AMINO ACID CATABOLISM IN LIVER: GLYCINE AND GLUTAMINE

HARRY STEPHEN EWART

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AMINO ACID CATABOLISM IN LIVER: GLYCINE AND GLUTAMINE

by

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Department of Biochemistry
Memorial University of Newfoundland

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ABSTRACT

The liver removes substantial quantities of glycine and glutamine from the circulation in animals ingesting high-protein diets. Glycine and glutamine, however, do not accumulate in liver tissue during a protein meal, and in fact, their hepatic concentrations may actually decrease. Thus, there appears to be an activation of hepatic glycine and glutamine catabolism at this time. Glycine and glutamine catabolism are initiated within mitochondria via the glycine cleavage system and glutaminase, respectively. Rapid activation of these enzymes has been demonstrated in intact mitochondria from glucagon-injected rats. However, it was unknown whether similar activations occur as part of an hepatic physiological response to protein intake. In this thesis, glycine and glutamine catabolism have been extensively studied in intact mitochondria from rats fed on a high-protein diet or given a single high-protein meal.

It was discovered that intact liver mitochondria from rats fed on a high-protein diet for six days catabolise glycine and glutamine at enhanced rates compared to mitochondria from rats fed a normal-protein diet. Glycine and glutamine catabolism were also stimulated in normal-protein-fed rats if they ingested a single high-protein meal for 2 hours before being killed. Thus, flux through the glycine cleavage system and through glutaminase is able to respond rapidly, according to the protein intake experienced

during an individual meal. Investigations using whole cells or isolated, intact liver from such animals allow similar conclusions. Thus it is proposed that activation of the glycine cleavage system and glutaminase is a normal hepatic response in animals ingesting a high-protein meal.

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Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
H-protein	lipoic acid-containing aminomethyl transferase; component of the glycine cleavage system
HEPES	N-2-hydroxyethylpiperazine -N'-2-ethanesulphonic acid
L-protein	lipoamide dehydrogenase; component of the glycine cleavage system
MOPS	3-[N-morpholino]propane sulponic acid
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
OAA	oxaloacetate
P-protein	glycine decarboxylase; component of the glycine cleavage system
Pi	inorganic phosphate
PEP	phosphoenolpyruvate
2PG	2-phosphoglycerate
T-protein	^{5}N, ^{10}N-methylene-tetrahydrofolate synthesising protein; component of the glycine cleavage system
Tris	Tris[hydroxymethyl]aminomethane

Chapter 1

Glycine and glutamine metabolism

Introduction

The disposal of excess amino acids after ingestion of a protein meal is primarily an hepatic function (Jungas et al., 1992). The response of the liver, in part, will depend on the previous dietary history of the animal. For example, in animals fed on a diet high in protein, the capacity of the liver to metabolise amino acids is greatly enhanced, an adaptation which involves increases in the activities of the amino acid-catabolising enzymes, usually through increases in their concentrations. Amino acid metabolism in liver also shows an ability to respond to the quite variable amounts of amino acids that might be ingested during individual meals. Two mechanisms have been described whereby such an immediate response can occur. One mechanism involves enzyme induction. Krebs (1972) has reviewed the hepatic enzymes which respond in this way. They are typically cytoplasmic proteins with short half-lifes. Thus enzymes such as tyrosine aminotransferase (Watanabe et al., 1968), tryptophan pyrollase (Feigelson and Greengard, 1962), and histidase (Sahib and Krishna Marti, 1969) are induced, often hundreds-of-fold, following a meal and subsequently decrease to near zero. The second mechanism involves regulation by covalent modification, where enzymes such as phenylalanine hydroxylase (Fisher and Pogson, 1984) and branched-chain α-ketoacid dehydrogenase (Harris et al., 1985) are modulated by

phosphorylation/dephosphorylation of enzyme subunits.

There are, however, a number of amino acids whose hepatic catabolism following a meal is not understood. The work in this thesis focuses on the hepatic disposal of two amino acids, glycine and glutamine, following a protein meal. Large amounts of these amino acids are taken up by the liver of animals ingesting a high-protein meal (Yamamoto et al., 1974). Yet the liver concentration of glycine, and sometimes of glutamine, is lowered at this time, suggesting a stimulation of intra-hepatic metabolism. Hepatic glycine and glutamine catabolism are initiated in mitochondria by the glycine cleavage system and glutaminase, respectively. Neither of these enzymes appears to be rapidly induced or modified by covalent modification. In this chapter the glycine cleavage system and glutaminase are reviewed and their role in hepatic metabolism discussed.

Glycine

Glycine is structurally the simplest of amino acids, yet its metabolism is complex. Glycine can be synthesised in a number of ways by cells, so under normal conditions it is a nonessential amino acid. However, dietary glycine may be required for growth of pre-term infants (Jackson et al., 1981), and because of its high content in collagen, glycine has been suggested to become conditionally indispensable in wound healing and repletion of tissue after depletion (Yu et

al., 1985). In addition to being incorporated into proteins, glycine is involved in a number of synthetic reactions (**Fig. 1.1**). First, glycine is glucogenic. This involves conversion to serine. The serine may also be converted to phospholipid, either directly as phosphatidylserine, or following conversion to ethanolamine. Cleavage of glycine results in its α-carbon being passed to tetrahydrofolate to give methylene-tetrahydrofolate, making glycine an important source of one-carbon units. In haemopoietic cells, glycine condenses with succinyl-CoA to give δ-aminolevulinic acid, which is the committed step in heme synthesis. Glycine carbon is incorporated at positions 4 and 5 of the purine ring, while its nitrogen is incorporated at the number 7 position. In kidney, glycine and arginine are used to form guanidoacetic acid which will be converted to creatine. Glycine is also a participant in bile acid conjugation. Finally glycine is involved in sarcosine, glutathione, and hippurate synthesis. Apart from these processes, glycine is also an inhibitory neurotransmitter in spinal cord and brain.

Blood and liver glycine concentrations

The concentration of glycine in rat plasma is usually reported in the range of 100-400 nmoles per mL (Yamamoto et al., 1974; Rémésy et al., 1978; Eriksson et al., 1989). As

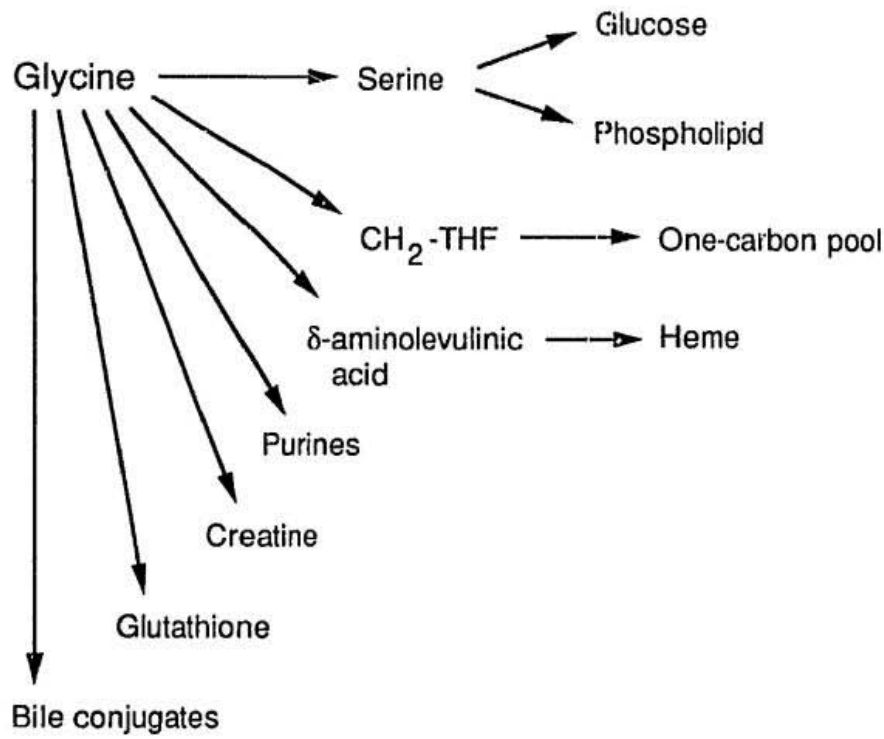


Fig. 1.1. Metabolic fates of glycine and serine

would be expected, the level of circulating glycine depends on the nutritional circumstance of the animal and on the level of protein in the diet. The relationship is, however, an inverse one. Rats fed high-protein diets exhibit lower levels of glycine in plasma than rats fed low or adequate protein diets (Ishikawa, 1976; Rémésy et al., 1978; Peters and Harper, 1985; Fafournoux et al., 1990). In fact, glycine levels in arterial blood are actually decreased following a protein meal (Yamamoto et al., 1974; Rémésy et al., 1978; Fafournoux et al., 1990). In the studies of Yamamoto et al. (1974) rats were meal-fed on a 30% casein diet such that they ate their entire daily food intake in two hours. While the concentration of other amino acids in the plasma of these rats following the meal was equal to or much higher than those in fasted rats, glycine was lowered to about half the level seen in fasted rats and was depressed further after four hours. This effect was evident whether glycine was measured in plasma or whole blood (Ishikawa, 1976). These findings, and those of Rémésy et al. (1978) and Fafournoux et al. (1990) where rats were fed ad libitum, show that during the absorptive period, despite an increase in portal glycine concentration, arterial levels are paradoxically decreased. In agreement with these findings is the reported diurnal rhythm of glycine in rat plasma (Eriksson et al., 1989). Most amino acids in blood are lowest in concentration prior to the onset of darkness and increase in the plasma during the dark

cycle, the period of active feeding. Glycine is the only amino acid whose level peaks just prior to darkness and is then subsequently lowered as the dark cycle commences.

The decrease in circulating glycine in fed rats is the result of a greatly increased hepatic uptake of glycine at this time. Ishikawa (1976) used the portal-hepatic vein difference to calculate the hepatic extraction of amino acids in rats fed 50% casein diets. The hepatic uptake of glycine was found to be 72.5%, the highest for all amino acids. Fafournoux et al. (1990) report a similar level of hepatic uptake in rats fed a 60% casein diet measured 4-5 hours after the beginning of feeding, compared with a value of about 25% in animals fed a 15% casein diet. In contrast, non-hepatic tissues in fed rats do not extract large amounts of glycine (Ishikawa, 1976).

Despite the increased uptake of glycine by the liver of high-protein-fed rats, the level of glycine in the liver tissue of these animals is decreased (Ishikawa, 1976; Peret et al., 1981; Rémésy et al., 1983). In rats adapted for 21 days to 5, 13, and 50% casein diets, the liver glycine levels were 4830, 2080, and 580 nanomoles per gram of tissue respectively (Rémésy et al., 1983). However, it is clear that the depression of liver glycine concentration occurs rather rapidly. Peret et al. (1981) switched rats fed a 10% casein diet to a 70% casein diet which resulted in a dramatic reduction in the liver glycine concentration, from 2204 to

964 nanomoles per gram tissue, after the first day of high-protein feeding.

Together, these findings suggest that hepatic glycine catabolism is stimulated in animals fed diets high in protein.

Routes of glycine catabolism

Early studies on the metabolism of glycine in rat liver showed that glycine could be converted to serine (Arnstein and Neuberger, 1953), a process that involved the incorporation of the α-carbon of glycine as the ß-carbon of serine (Nakada et al., 1955). It was thought, however, that this pathway involved glyoxylate as an intermediate, which could be detected in homogenates incubated with glycine. The scheme proposed transamination of glycine with pyruvate or α-ketoglutarate to give glyoxylate. Glyoxylate carbon could then be released as CO_2 and formate, with the formate being converted to serine (Nakada and Sund, 1958). Glyoxylate can also be formed from glycine via the action of the D-amino acid oxidase, but the high Km of the enzyme for glycine suggests that this would make a minor contribution (Niems and Hellerman, 1962). Conversion of glycine to glyoxylate is now thought to represent only a minor component in overall glycine catabolism.

The bulk of glycine catabolism in vertebrates is now known to occur by way of the hepatic glycine cleavage system

(Yoshida and Kikuchi, 1972, 1973). The overall reaction catalysed by the glycine cleavage system is:

$$\text{Glycine} + \text{THF} + NAD^+ \rightarrow CO_2 + NH_3 + \text{methylene-THF} + NADH + H^+$$

The methylene-THF can react with a second molecule of glycine to form serine and regenerate THF, a reaction catalysed by serine hydroxymethyltransferase. This scheme has been termed the "glycine cycle" (Snell, 1984) and is illustrated in **Fig. 1.2.**

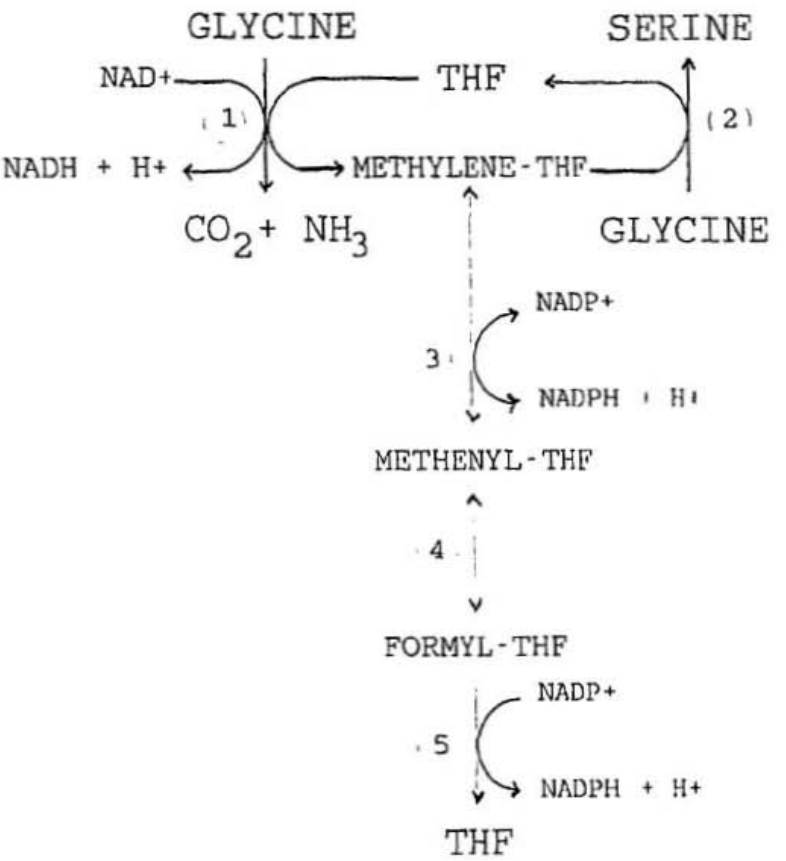
The glycine cleavage reaction

Bacteria

The glycine cleavage reaction was first described by Sagers and Gunsalus in 1961 in cell-free extracts from the anaerobic bacterium Diplococcus glycinophilus. In this organism glycine is used in a fermentive process to form acetate. It was shown that extracts of D. glycinophilus catalysed a tetrahydrofolate- and pyridoxal phosphate-dependent cleavage of glycine to one-carbon units. In this process carbon-1 of glycine was released as CO_2 and carbon-2 was transferred to tetrahydrofolate to yield a hydroxymethyl derivative that was in equilibrium with methylene-THF. Evidence was also reported for the subsequent involvement of the carbon-1 in serine formation. The GCS was soon found to occur in other bacteria including Escherichia coli (Pitts

Fig. 1.2. Glycine catabolism by the glycine cleavage system and subsequent metabolism of the resulting methylene-tetrahydrofolate

1. Glycine cleavage system
2. Serine hydroxymethyltransferase
3. Methylene tetrahydrofolate dehydrogenase
4. Methylene tetrahydrofolate cyclohydrolase
5. Formyl tetrahydrofolate oxidoreductase


GLYCINE
SERINE
NAD+
THF
(1)
(2)
NADH + H+
METHYLENE-THF
CO2+ NH3
GLYCINE
NADP+
3
NADPH + H+
METHENYL-THF
4
FORMYL-THF
NADP+
5
NADPH + H+
THF

and Crosbie, 1962) and the aerobic soil bacterium, Arthrobacter globiformis (Jones and Bridgeland, 1966). In each case glyoxylate could not be detected as an intermediate.

Birds

At about the same time, Richert et al. (1962) reported that the metabolism of glycine in avian liver occurred in a similar fashion. Liver homogenates of pigeon, duck, and chicken were shown to release the carbon-1 of glycine as CO_2. Carbon-2 reacted with another molecule of glycine to form serine. It was further found that the rates of CO_2 release were decreased in liver homogenates from ducks that had been fed diets deficient in vitamin B-6, niacin, or folic acid. Restoration of activity could be achieved in these homogenates by addition of the appropriate cofactors - pyridoxal-5-phosphate, NAD^+, or THF, respectively. This suggested the direct participation of these cofactors in the reaction.

Rats

The first indication that rat liver possessed the enzymes for glycine cleavage came from findings that rat liver mitochondria (Kawasaki et al. 1966) or extracts solubilised from such mitochondria (Sato et al., 1969a) could synthesize two molecules of glycine from one molecule each of

serine, bicarbonate, and ammonia. This represented the reverse of glycine cleavage and occurred preferentially in the absence of oxygen. It was further shown that methylene-THF was an intermediate which combined with ammonia and CO_2 to give glycine (Motokawa and Kikuchi, 1969a).

Sato et al. (1969b) were the first to report the reaction in the physiological direction of glycine cleavage. Under aerobic conditions, extracts from acetone-dried mitochondria catalysed the cleavage of glycine to methylene-THF, ammonia, and CO_2. Glycine cleavage was stimulated by NAD^+ which acted as a hydrogen acceptor in the reaction. THF was required. In the absence of THF, however, an exchange of the carboxyl group of glycine with carbon-14 labelled bicarbonate could be demonstrated. This activity is a partial reaction in glycine cleavage and had previously been demonstrated as a characteristic of the bacterial glycine cleavage system (Klein and Sagers, 1966).

Other vertebrates

A comparative study of glycine catabolism subsequently showed that the glycine cleavage system occurs in the livers of a variety of vertebrate species (Yoshida and Kikuchi, 1972). In mammals the glycine cleavage system was shown to occur in pig, cow, goat, sheep, dog, rabbit, guinea pig, and human liver. Activities were also reported for fish, reptiles, and amphibians. Birds and reptiles exhibit the

highest activities of vertebrates. Rat liver possesses the highest mammalian activities.

Tissue distribution

Yoshida and Kikuchi (1973) measured glycine cleavage activity in several tissues of rat and chicken. For both species the liver exhibited by far the greatest activity. But kidney, testis and brain showed moderate activity in both species. Other tissues tested including lung, small intestine, and skeletal muscle had low or neglegible activities.

In tissues expressing glycine cleavage activity, the reaction appears to represent the major route of glycine catabolism (Yoshida and Kikuchi, 1972; 1973). In kidney where there is a high D-amino oxidase activity it was possible that some of the glycine was degraded to glyoxylate. However, while kidney mitochondria or homogenate catalysed the production of CO_2 from 1-^{14}C- glycine, the soluble fraction which contains the oxidase did not (Yoshida and Kikuchi, 1973).

Mitochondria

The glycine cleavage system is confined to the mitochondria of all tissues so far tested. The system is bound to the inner membrane (Motokawa and Kikuchi, 1971; Hiraga et al., 1972). The enzyme was not released from the

membrane upon digitonin treatment or during freezing and thawing cycles, but it was found to be solubilised from the membrane by treatment with the detergent lubrol (Motokawa and Kikuchi, 1971). By comparison with the effects of such treatment on other membrane proteins it was concluded that the glycine cleavage system was loosely bound to the inner mitochondrial membrane. It was subsequently reported that this association with the inner membrane was probably as an enzyme complex (Hiraga et al., 1972).

The components of the glycine cleavage system

Components of the glycine cleavage system have been isolated from a number of bacterial (Klein and Sagers, 1966; Kochi and Kikuchi, 1969, Freudenberg and Andreesen, 1989), plant (Walker and Oliver, 1986), and animal sources (Kikuchi and Hiraga, 1982). The glycine cleavage system consists of four proteins which are as follows: 1). a pyridoxal phosphate - dependent glycine decarboxylase; 2). a lipoic acid-containing aminomethyl transferase; 3). an ^{5}N, ^{10}N-methylene-tetrahydrofolate synthesising protein; and 4). a flavin-containing lipoamide dehydrogenase. In animals and plants these proteins are referred to as P-, H-, T-, and L-proteins, respectively. In bacteria they are referred to as P_1, P_2, P_3, and P_4.

Measurement of activity and purification

The complex has not been isolated intact. The strategy employed in purification procedures has been to separate the individual proteins and assay activity by recombining fractions. Two activities have been used to monitor purification steps. Glycine cleavage system activity requires the presence of all four of the enzyme proteins. Activity may be determined by quantifying the release of $^{14}CO_2$ from fractions incubated in the presence of 1-^{14}C-glycine. The activity can also be assayed by measuring [^{14}C]-formaldehyde formation from 2-^{14}C-glycine (Motokawa and Kikuchi, 1974). Alternatively, the activity can be measured in the direction of glycine synthesis. This is done under anaerobic conditions in the presence of methylene-THF, ammonia and [^{14}C]-bicarbonate and involves assessing the incorporation of $^{14}CO_2$ into acid-stable products. A second activity is due to the glycine-bicarbonate exchange reaction which is a partial reaction in the cleavage of glycine (Sato et al., 1969b). The activity of P- or H-protein can be monitored by measuring the exchange of the carboxyl group of glycine with [^{14}C]-bicarbonate. Thus P-protein can be assayed in the presence of added H-protein and vice versa. An interesting feature of the glycine cleavage system is that individual proteins of the enzyme complex in one species are interchangeable with the corresponding components from another, over a wide variety of

species. For example P-protein from A. globiformis will catalyse the exchange reaction in the presence of H-protein from chicken (Fujiwara et al., 1979), rat (Hayasaka et al., 1980), and human (Motokawa et al., 1977) liver. This fact has been used to advantage in purification procedures where the components isolated from one species can be used in the assay for activity in fractions prepared from other species.

Proteins of the glycine cleavage system

P-protein has been purified to homogeneity from liver mitochondria of rat (Hayasaka et al., 1980) and chicken (Hiraga and Kikuchi, 1980a). In each case the P-protein is a homodimer with a relative molecular weight of about 210 000. It is a pyridoxal-phosphate-dependent enzyme, responsible for the decarboxylation of the carboxyl carbon of glycine and the transfer of the aminomethyl remnant to the H-protein. Unlike the decarboxylase components of the dehydrogenase complexes for pyruvate, α-ketoglutarate, and the branched-chain keto-acids, P-protein is virtually inactive by itself. For activity, the presence of H-protein is required (Motokawa and Kikuchi, 1972). H-protein can be replaced by lipoic acid, but this substitution results in far less activity (Kochi and Kikuchi, 1976).

The structures of the vertebrate P-proteins that have been studied appear very similar. A rabbit anti-rat P-protein antibody crossreacted with P-proteins from carp, frog, snake,

chicken, cow, and human tissue (Hayasaka et al., 1980). Recently, the sequences of chicken and human P-protein have been deduced following isolation of cDNA clones (Kume et al., 1991). These proteins were 84% identical and most amino acid substitutions were conservative (Kume et al., 1991).

H-protein is a small, acidic , heat stable protein (Motokawa and Kikuchi, 1969b) that contains lipoic acid as a prosthetic group. H-protein appears to play an analogous role to the lipoate-containing acyltransferases (E_2 subunits) of the pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and branched-chain α-ketoacid dehydrogenase complexes (Fujiwara et al., 1979). It functions as a carrier of the aminomethyl intermediate between the active sites of P-protein and T-protein. The primary structure of H-protein from chicken has been determined by direct sequencing of the purified protein (Fujiwara et al., 1986), and for cow (Fujiwara et al., 1991) and human (Fujiwara et al., 1991; Koyata and Hiraga, 1991) P-proteins by sequencing their cDNA clones. These proteins were very similar. The human H-protein was 97% and 86% identical to the bovine and chicken proteins, respectively (Fujiwara et al., 1991). The site of attachment of lipoic acid to these H-proteins was found to be lysine 59. Their molecular masses, calculated from their sequences, are each about 14 000 when the lipoic acid group is included.

The functional glycine decarboxylase is an enzyme

complex consisting of P- and H-proteins (Hiraga and Kikuchi, 1980a). Very low decarboxylase activity is exhibited by P-protein alone. P-protein activity, as measured by the bicarbonate-glycine exchange reaction, was increased more than 100 000-fold by the addition of H-protein (Hiraga and Kikuchi, 1980b). These researchers also showed that when a mixture of H- and P-proteins were subject to gel filtration a considerable amount of H-protein eluted with P-protein. The existence of these two proteins as a complex was also demonstrated using sucrose gradient centrifugation. In each procedure, the ratio of H-protein to P-protein was 2:1. Because P-protein is a homodimer, it was suggested that one molecule of H-protein binds to each P-protein subunit.

T-protein is a tetrahydrofolate-dependent enzyme which catalyses the degradation of the H-protein-bound intermediate to ammonia and methylene-THF. In this, it catalyses a unique reaction which involves C-S and C-N bond cleavage. T-protein has been purified from the liver of rat (Motokawa and Kikuchi, 1974), chicken (Okamura-Ikeda et al., 1982), and cow (Okamura-Ikeda et al., 1991). The molecular weights of these proteins were 33 000, 37 000, and 38 000, respectively. Isoelectric-focusing showed that the chicken protein was basic with a pI of 9.8. The primary structure of bovine T-protein has been determined from its cDNA (Okamura-Ikeda et al., 1991). The calculated molecular weight of the mature

protein was 40 534, in agreement with the apparent molecular weight of the purified protein.

L-protein is considered to be lipoamide dehydrogenase, a flavin-containing protein (Kikuchi, 1973). Lipoamide dehydrogenase is a homodimer with a subunit molecular weight of about 55 000 (Carothers et al., 1989). As a component of the glycine cleavage system it functions to reoxidise lipoic acid back to the disulfide form through the transfer of reducing equivalents to NAD^+. Kochi et al. (1986) suggested that the lipoamide dehydrogenase of the glycine cleavage system was the same protein as the E_3 component used by other mitochondrial enzyme complexes. This was based on the inhibition of the glycine cleavage system by pyruvate, α-ketoglutarate, and branched-chain α-keto acids, an effect proposed to be caused by provision of reducing equivalents to the glycine cleavage system , through a shared common E_3. This view was challenged by Carothers et al. (1987) who reported two immunologically distinct forms of lipoamide dehydrogenase, one of which appeared to be associated with the glycine cleavage system. The possibility of lipoamide dehydrogenase isoenzymes is also supported by the finding that humans with a genetic defect in E_3 do not exhibit hyperglycinemia (Carothers et al., 1989). Hakozaki and Honda (1990) showed that rat liver lipoamide dehydrogenase was separated into three forms using ion-exchange chromatography.

In reconstitution experiments, however, the glycine cleavage system did not exhibit a particular specificity toward any of the three forms. Moreover, the three forms could not be distiguished immunologically and were revealed as a single band on SDS-polyacrylamide gel electrophoresis. Thus, it is still unclear whether L-protein of the glycine cleavage complex is identical to the E_3 protein.

Mechanism

A scheme for the mechanism of the glycine cleavage reaction is outlined in **Fig. 1.3**. The overall glycine cleavage reaction may be divided into three separate partial reactions. The first event, the decarboxylation of glycine, occurs via a sequential mechanism in which glycine and H-protein bind to P-protein before the release of any product (Fujiwara and Motokawa, 1983). Glycine forms a Schiff base with the carbonyl group of pyridoxal phosphate bound to P-protein (Hiraga and Kikuchi, 1980b). H-protein, which may be regarded as a co-substrate, binds to P-protein at a separate site (Fujiwara and Motokawa, 1983). Binding of H-protein and glycine to P-protein occurs randomly as does the release of products (Fujiwara and Motokawa, 1983). Decarboxylation results in the release of CO_2 and not bicarbonate (Fujiwara and Motokawa, 1983) and the generation of an H-protein intermediate. In the reaction, the lipoic acid prosthetic group of H-protein is reduced and one of the sulphydryl

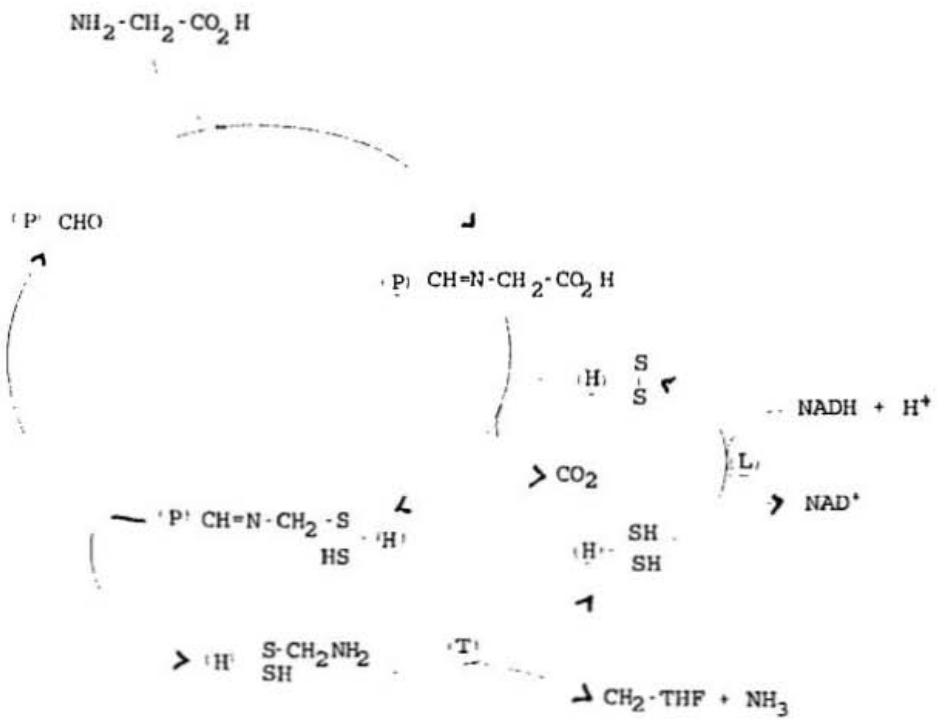


Fig. 1.3. Scheme for the overall reaction of the glycine cleavage system.

groups binds the intermediate, which is now recognised to be methylamine (Hiraga and Kikuchi, 1980a; Fujiwara et al., 1984). Methylamine is then degraded to methylene-THF and ammonia in the reaction catalysed by T-protein. In this second partial reaction, ammonia release appears to preceed formation of methylene-THF (Fujiwara et al., 1984). THF is not an absolute requirement for this reaction but greatly stimulates the rate of catalysis, presumably through removal of formaldehyde from the T-protein active site (Fujiwara et al., 1984). The third partial reaction is the reoxidation of the reduced lipoic acid of H-protein to its disulfide form. This step is catalysed by L-protein and involves the transfer of reducing equivalents to NAD^+.

Fate of glycine cleavage system products

Flux through the glycine cleavage system produces CO_2 and NH_3 which can participate in intramitochondrial metabolism. For example, both of these products are precursors of carbamylphosphate, and therefore, may enter the urea cycle. The fate of the second carbon, as methylene-THF, may be entry into the one-carbon pool, complete oxidation to CO_2, or conversion to serine. The synthesis of purines, apart from requiring intact glycine as a precusor, also involves the incorporation of carbon from methenyl- and formyl-THF, both of which may be derived from methylene-THF (see **Fig. 1.2**). In uricotelic animals such as birds and snakes,

complete oxidation to CO_2 does not appear to occur because of extremely low activity of formyl tetrahydrofolate oxidoreductase (Yoshida and Kikuchi, 1972). In these animals an important physiological role of the glycine cleavage system is the provision of the methylene carbon for uric acid synthesis.

In rats, oxidation of methylene-THF to CO_2 does occur, which will regenerate THF. But this appears to occur only to a minor extent. Hampson et al. (1983) reported that formation of $^{14}CO_2$ from 2-^{14}C-glycine in rat liver mitochondria is very low compared to flux through the glycine cleavage system as measured by $^{14}CO_2$ release from 1-^{14}C-glycine. Similar observations were made with rat kidney slices (Rowsell and Al-Naama, 1982). In rats the primary fate of the α-carbon of glycine is incorporation into serine as outlined in **Fig. 1.2**. This is evident from experiments in vitro where glycine disappearance was observed to be twice the serine and ammonia formation. The coupled flux through the glycine cleavage system and serine hydroxymethyl transferase will thus result in the following net reaction:

$$2\ \text{Glycine} + H_2O + NAD^+ \rightarrow \text{Serine} + NH_3 + CO_2 + NADH + H^+$$

Conversion to serine is the dominant route for gluconeogenesis from glycine which will now be discussed.

Gluconeogenesis from glycine

In dogs treated with phlorizin the reabsorption of glucose by renal tubules is compromised. In such animals, ingestion of glycine results in an increase in the appearance of glucose in urine, and on this basis, glycine was classified as a glucogenic amino acid (see Fruton and Simmonds, 1959). In fasted normal and diabetic rats, addition of U-^{14}C-glycine to arterial blood leads to the appearance of radioactivity in plasma glucose (Hetenyi et al., 1988). Glycine, however, is a poor precursor of glucose in rat hepatocytes (Beliveau and Freedland, 1982; Rémésy et al., 1983), except when these are isolated from animals adapted to high-protein diets (Rémésy et al., 1983). Glycine is efficiently converted to glucose in cat hepatocytes (Beliveau and Freedland, 1982).

The conversion of glycine to aminoacetone and subsequently to pyruvate is one potential glucogenic route, but this does not appear to be important in mammals (Bender, 1985). This glucogenicity of glycine , instead, derives from its conversion to serine (Snell, 1984). As illustrated in **Fig. 1.4** gluconeogenesis from serine may proceed either through cytosolic serine dehydratase (EC 4.2.1.13) which yields pyruvate, or via mitochondrial serine aminotransferase (EC 2.6.1.51), which converts serine to hydroxypyruvate. The

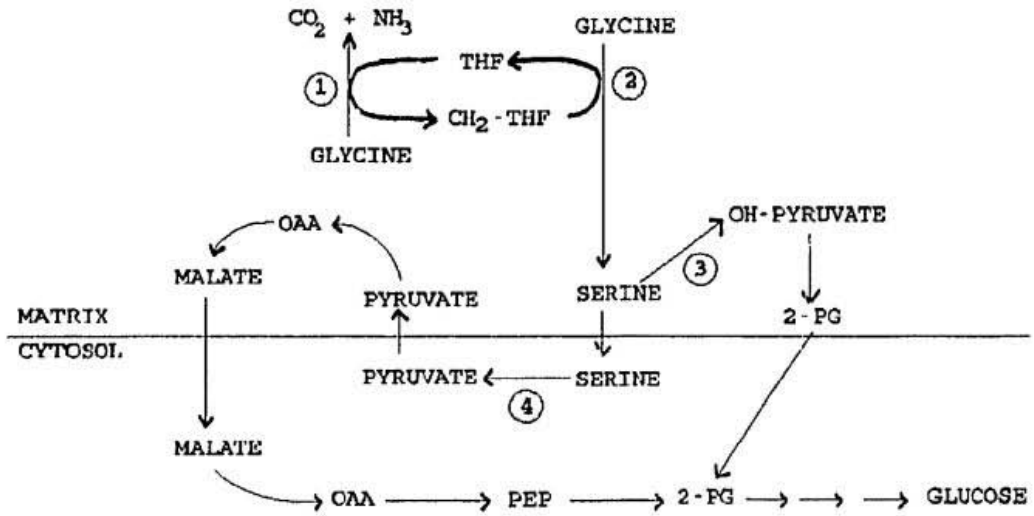


Fig. 1.4. Gluconeogensis from glycine and serine

1. Glycine cleavage system
2. Serine hydroxymethyltransferase
3. Serine aminotransferase
4. Serine dehydratase

relative importance of these two pathways varies with species and their diet. Transamination is the major pathway for gluconeogenesis from serine in carnivores (Rowsell et al., 1979) and in humans (Snell, 1986), but in rats the deamination pathway appears to predominate (Bhatia et al., 1975). But even in rats, flux through the transaminase may be significant. For example, use of inhibitors of phosphoenolpyruvate carboxykinase, and therefore of serine dehydratase-mediated gluconeo-genesis, suggests that upwards of 30% of the serine flux may proceed via serine aminotransferase (Snell, 1984). It has also been noted that, in rats fed high-protein diets, gluconeogenesis from serine (and glycine) may proceed primarily through this pathway (Oda et al., 1982; Rémésy et al., 1983).

Defects in glycine metabolism

The first description of clinical symptoms of hyperglycinemia were reported in 1961 (Nyhan et al., 1961; Childs et al., 1961). Glycine levels were 10 and 40 times the normal level in plasma and urine, respectively. The patient suffered from lethargy, seizures, prolonged episodes of vomiting and ketoacidosis. At the time it was referred to as idiopathic hyperglycinemia. It was later reported that hyperglycinemia represents a group of disorders in amino acid metabolism which could be divided into two distinct diseases, ketotic and nonketotic hyperglycinemia

(Baumgartner, 1969). Ketotic hyperglycinemia accompanies defects in branched-chain keto-acid metabolism (Nyhan, 1978). Non-ketotic hyperglycinemia results from a primary defect in the glycine cleavage system.

Nonketotic hyperglycinemia is an autosomal recessive disorder characterised by high levels of glycine in plasma and cerebrospinal fluid in the absence of excess organic acids (Nyhan, 1989). Clinically nonketotic hyperglycinemia is a serious disease with no effective therapy and a high neonatal mortality rate. Those surviving are severely mentally retarded and suffer from intractable seizures. Most nonketotic hyperglycinemic patients have a defect in P-protein (Tada and Hayasaka, 1987; Kure et al, 1991, 1992a). The disorder can also arise through a defect in H-protein or T-protein (Hiraga et al., 1991), but there has been no report of the disease arising from a defect in lipoamide dehydrogenase. Nonketotic hyperglycinemia is a relatively rare disorder in many countries occurring in 1 in 250,000 births (Nyhan, 1989). The incidence is very high in the population of Northern Finland where the occurence is 1 in every 12,000 births (von Wendt et al., 1979). The reason for this has been traced to a defect in the gene coding for P-protein, which was evident in 70% of the Finnish patients tested (Kure et al., 1992a). The lesion was revcaled as a single G to T substitution which resulted in amino acid alteration in the decarboxylase from Ser 564 to Ile 564.

The enzymatic diagnosis of nonketotic hypergly-cinemia until recently required liver biopsy. Kure et al (1992b) have recently demonstrated that the GCS is expressed in peripheral B lymphocytes when they are transformed by Epstein-Barr virus. This method was shown to be a reliable means of assessing GCS activity in patients, obviating the need for liver biopsy. The severe consequences of a genetic defect in the glycine cleavage system speaks to the need for an understanding of the metabolic regulation of hepatic glycine catabolism.

Regulation of the Glycine Cleavage System

Branched-chain amino acids

While the components and reaction mechanism of the glycine cleavage system have been established, its metabolic regulation is poorly understood. O'Brien (1978) and Kochi et al. (1986) reported inhibition of the glycine cleavage system by branched-chain α-keto acids. These researchers incubated mitochondria with branched-chain α-keto acids at concentrations of 2 and 5 mM , respectively, and observed a 40-50% inhibition of glycine decarboxylation. The locus of this inhibition was reported to be the lipoamide dehydrogenase. However, the physiological concentration of branched-chain α-keto acids are in the micromolar range

(Schauder, 1984), so this mechanism, if operative, should only be so under pathological conditions.

Oxidation-reduction in mitochondrial pyridine nucleotides

It has also been proposed that the glycine cleavage system could be regulated by changes in the oxidation-reduction states of mitochondrial pyridine nucleotides (Hampson et al., 1983; 1984). In perfused liver, conditions such as infusion of ß-hydroxybutyrate or octanoate, which lead to reduction in the mitochondrial NADH redox couple, inhibit glycine decarboxylation, whereas acetoacetate infusion stimulates glycine decarboxylation (Hampson et al., 1984). When intact rat liver mitochondria were incubated under state IV conditions in the presence of an oxidisable substrate, flux through the glycine cleavage system was strongly inhibited (Hampson et al., 1983). Glycine decarboxylation was also inhibited by respiratory chain inhibitors such as rotenone. Conversely, glycine decarboxylation was stimulated in state III over state IV and was maximally stimulated by the addition of an uncoupler. Thus the glycine cleavage system was shown to be stimulated in mitochondria under oxidising conditions, and inhibited under reducing conditions. It was further shown, by direct measurement of mitochondrial pyridine nucleotides, that flux through the glycine cleavage system was correlated with changes in both the NAD(H) and NADP(H) redox couples.

Effects of hormones

Addition of glucagon (or cAMP) to freshly isolated hepatocytes stimulates flux through the glycine cleavage system in a dose dependent manner, with maximum stimulation occurring at about 10^{-7} M glucagon (Jois et al., 1989). The glycine cleavage system is also stimulated in hepatocytes isolated from glucagon injected rats (Jois et al., 1990a). In isolated perfused liver, glucagon, catecholamines, phenylephrine, and vasopressin stimulate glycine oxidation two- to three-fold (Brosnan et al., 1990; Jois et al., 1990b). Thus hormones known to act through cyclic-AMP as well as those known to act through calcium are able to stimulate hepatic glycine catabolism. The stimulation of the glycine cleavage system appears to be fairly stable since the effect persists in mitochondria isolated from rats injected with glucagon (Jois et al., 1989). In these experiments, isolation of mitochondria was performed without any precaution to preserve phosphorylation state. The effect of glucagon on the glycine cleavage system is, therefore, to be contrasted with its effects on cytoplasmic enzymes such as glycogen phosphorylase which are typically labile and reversible upon removal of hormone.

High-protein feeding

As discussed earlier, the liver extracts large amounts

of glycine from the circulation in rats fed high-protein diets, and yet, in this situation the liver concentration of glycine decreases. This suggests that there is a stimulation of hepatic glycine catabolism in these animals. Regulation of the glycine cleavage system by branched-chain amino acids or by changes in mitochondrial redox state do not explain these findings. Feeding a diet high in protein raises both the circulating (Schauder,1984) and liver (Fafournoux et al., 1990) levels of branched-chain amino acids and also leads to a lower mitochondrial NAD^+/NADH ratio in liver (Peret et al, 1981) and hence, by the mechanisms discussed above, would be expected to inhibit glycine catabolism. That this does not occur suggests that these mechanisms are physiologically ineffective and that other regulatory mechanisms must exist. Regulation by glucagon, on the other hand, is a distinct possibility, since ingestion of a high-protein diet is known to increase the circulating concentrations of this hormone (Robinson et al., 1981). In the studies where mitochondria were isolated from glucagon-injected rats (Jois et al., 1989), a supraphysiological concentration of hormone was used to elicit a response. Stimulation of the glycine cleavage system could be demonstrated following a single injection of glucagon made 25 minutes prior to isolation of mitochondria. Whether these findings have a physiological counterpart, in the situation of rats fed a high-protein meal, where an increase in circulating glucagon (in vivo) is known to occur

(Robinson et al., 1981), is a focus of this thesis. In **Chapter 3** this possibility is examined by determining whether flux through the glycine cleavage system is stimulated in mitochondria isolated from rats fed high-protein diets or meals.

Hormone effects on mitochondrial metabolism

The effect of hormone treatment on mitochondrial metabolism has been extensively studied and has been the subject of a number of reviews (Halestrap, 1986; 1989; Denton and McCormack, 1990). Many of the effects are persistent in that they are retained by mitochondria isolated from hormone-treated animals. For a comprehensive list of mitochondrial parameters stimulated in isolated mitochondria from hormone-treated animals the reader is referred to Halestrap (1989). In addition to the glycine cleavage system, these include stimulation of pyruvate metabolism (Adam and Haynes , 1969), succinate oxidation (Yamazaki, 1975), citrulline synthesis and flux through glutaminase (Lacey et al., 1981). It is possible that many of these effects which persist following glucagon treatment, may also be evident in mitochondria isolated from rats fed a high-protein diet. Thus the hypothesis that the glycine cleavage system will be stimulated in mitochondria isolated from rats fed high-protein diets or meals was extended to include glutaminase

(**Chapter 4**). This enzyme, which is responsible for the bulk of glutamine metabolism in liver (Horowitz and Knox, 1968), will now be discussed.

Glutamine

Glutamine is a quantitatively more important amino acid than glycine, being the most abundant amino acid in blood (0.5 - 0.6 mM), and reaching high concentrations in tissue (> 6 mM). Under normal circumstances, glutamine is a nonessential amino acid because of the occurrence of glutamine synthetase in many tissues. Glutamine is, however regarded as a conditionally essential amino acid during catabolic illnesses, and is, therefore, sometimes provided in parenteral fluids. Glutamine plays a prominent role as a transport molecule for ammonia in the circulation, protecting the brain from ammonia toxicity. Glutamine nitrogen is donated in a number of biosynthetic reactions including formation of purines, pyrimidines, glucosamine, and NAD^+. Glutamine is also a precursor of neurotransmitters, glutamate and γ-aminobutyric acid. A number of cell-types including intestinal cells, lymphocytes, reticulocytes, and tumour cells use glutamine as a repiratory fuel. In liver, glutamine is a precursor of glucose and urea. Alanine derived from glutamine in the gut is also converted to glucose in the liver. In kidney, glutamine plays an important role in acid-base homeostasis, as a major source of ammonia for excretion

during acidosis.

The first observations on hepatic glutamine metabolism were made by Krebs in 1935. He found that liver homogenates were able to synthesis glutamine from ammonium glutamate in an energy requiring process. Liver preparations also contained a "glutamine-splitting" activity which converted glutamine to ammonium glutamate. A description of the metabolic role for both glutamine synthesising and degrading enzymes with in liver tissue, however, has come only recently with an understanding of the unique regulatory feature of liver glutaminase in being activated by ammonia (product activation) and the discovery that glutaminase and glutamine synthetase are located in different hepatocyte populations (Häussinger, 1989).

Phosphate-activated glutaminase

The catabolism of glutamine in tissues is carried out in large part by the activity of glutaminase (L-glutamine amidohydrolase; E.C. 3.5.1.2) which catalyses the following irreversible reaction:

$$\text{Glutamine} + H_2O \rightarrow \text{Glutamate} + NH_4^+$$

The enzyme is sometimes referred to as phosphate-dependent or phosphate-activated glutaminase because its activity is accelerated in the presence of phosphate (Carter and Greenstein, 1947; Errera and Greenstein, 1949; Errera, 1949).

Other ions such as sulphate and arsenate also activate glutaminase, but are far less effective.

There are two isozymes of glutaminase, kidney-type and liver-type. This was first noted by Krebs (1935) who observed that glutaminase activity in liver differed from that of kidney and other tissues in pH optimum and in not being inhibited by glutamate. Horowitz and Knox (1968) have studied the kinetic characteristics and tissue distribution of the two isoenzymes. The liver-type enzyme is found only in adult liver and possibly lung. In contrast, the kidney-type is widely distributed in tissues. In addition to the relatively high activities found in kidney, brain, and small intestine, lower activities of the kidney-type glutaminase are also present in heart, lung, fetal liver, and hepatoma tissue. Kidney-type glutaminase has also recently been reported in skin (Keast et al., 1989).

Curthoys et al. (1976) reported that the liver- and kidney-type enzymes could be distinguished immunologically. Antiserum prepared against rat kidney glutaminase did not cross-react with the liver enzyme, but did cross-react with the enzyme in brain and small intestine supporting the contention that the enzymes in kidney, brain and small intestine are similar. However, Shapiro et al. (1987) found that antibodies raised to the kidney enzyme did react with a 58 kDa peptide from rat liver extract which they suggest constitutes the liver enzyme. Smith and Watford (1988) showed

that antibodies to liver glutaminase react, although weakly, with glutaminase of kidney, brain, and intestine. A cDNA clone for liver glutaminase has been isolated and sequenced (Smith and Watford, 1990). Comparison with a previously determined cDNA sequence for kidney-type (brain) glutaminase (Banner et al., 1988) uncovered a region of 123 amino acids showing 80% identity (Smith and Watford, 1990). Thus, while kidney-type and liver-type glutaminase are different proteins, they share common epitopes which may be the result of a common evolutionary origin.

Other activities

There have been at least two glutaminase-like activities described which are not catalysed by the above enzymes. Carter and Greenstein (1947) reported a type of glutaminase activity which was active only in the presence of pyruvate and other α-keto acids. It has since been shown by Meister (1953) that this activity is due to the sequential action of 2 cytosolic enzymes: glutamine-oxo-acid aminotransferase (E.C. 2.6.1.15) and ω-amidase (E.C. 3.3.1.3). The reactions involved are

glutamine + α-ketoglutarate ↔ α-ketoglutaramate + glutamate

and

α-ketoglutaramate + H_2O → α-ketoglutarate + NH_4^+

Giving the net reaction

$$\text{glutamine} + H_2O \rightarrow \text{glutamate} + NH_4^+$$

Although the transaminase works in both directions, glutamine degradation is favoured because ω-amidase rapidly converts α-ketoglutaramate to α-ketoglutarate (Cooper and Meister, 1977). These enzymes are widely distributed in animal tissues, and because the glutamine aminotransferase can utilise the α-ketoacids of tyrosine and methionine, the reaction sequence is regarded as a salvage pathway for essential amino acid carbon and sulfur (see Cooper, 1988). Thus, Wu and Thompson (1990) reported that skeletal muscle incubated *in vitro* produce tyrosine from glutamine and p-hydroxyphenylpyruvate, while Häussinger *et al*. (1985a) reported the transamination of α-keto-γ-methiobutyrate or p-hydroxyphenylpyruvate with glutamine in perfused liver. In each situation, however, high concentrations of the α-keto acids had to be used, suggesting that this is a minor pathway for glutamine utilisation *in vivo*.

A second type of glutaminase activity, independent of phosphate, but activated by maleate occurs in extracts from liver, kidney, and brain (Katunuma *et al*., 1968). This activity has been referred to as maleate-activated or phosphate-independent glutaminase. The activity has been identified as a partial reaction of γ-glutamyltranspeptidase (Curthoys and Kuhlenschmidt, 1975; Tate and Meister, 1975). γ-glutamyltranspeptidase (E.C. 2.3.2.2) plays a key role in

glutathione metabolism. The enzyme can use glutamine only at a rate of 1-2% of the rate at which it uses glutathione (Tate and Meister, 1975), but this is increased markedly in the presence of maleate (Curthoys and Kuhlenschmidt, 1975) or hippurate (Thompson and Meister, 1980). In kidney during chronic acidosis, glutamine utilisation by γ-glutamyl-transpeptidase has been suggested to contribute to renal ammoniagenesis (Welbourne and Dass, 1981a). This was consistent with reports that in acidotic rats both hippurate concentration (Welbourne and Dass, 1981b) and kidney γ-glutamyltranspeptidase activity (McFarlane Anderson and Alleyne, 1977) are increased. On the other hand, when γ-glutamyltranspeptidase is inhibited by acivicin, no effect on ammonia excretion from acidotic rats is observed, arguing against a glutaminase-type function for the enzyme *in vivo* (Shapiro and Curthoys, 1981).

Purification of liver glutaminase

Initial attempts to purify liver glutaminase met with only partial success. Huang and Knox (1976) achieved a 15-fold increase in the specific activity of glutaminase from rat liver using ammonium sulphate precipitation and Sepharose chromatography. Patel and McGivan (1984), using ion-exchange chromatography, obtained a rat liver preparation enriched 60-fold in glutaminase specific activity, but attempts to remove glutamate dehydrogenase, the major contaminant, were

unsuccessful. These procedures were hampered by the instability of the enzyme in homogenates and dilute solutions. This problem does not appear to have been encountered during isolation of the kidney-type glutaminase, which was first purified to homogeneity from pig kidney by Kvamme and collegues in 1970, and subsequently from pig brain (Svenneby, 1970) and rat tissues (Curthoys et al., 1976; Haser et al., 1985). It should also be noted that these purifications were aided by the property of the kidney-type enzyme to aggregate in phosphate-borate buffer and to disaggregate in Tris buffer, a trait not possessed by the liver enzyme (Huang and Knox, 1976).

Rat liver glutaminase has now been purified to near homogeneity by two different laboratories (Heini et al., 1987; Smith and Watford, 1988). The problem of enzyme instability was largely overcome by addition of protease inhibitors in the buffer solutions. The basic approach involved ammonium sulphate fractionation as well as ion exhange and hydroxyapatite chromatography. Similar results were obtained in both laboratories. The relative molecular mass of the protein was found to be about 170 000, while the molecular mass of the subunits, determined by sodium dodecyl sulphate/ polyacrylamide gel electrophoresis, was 57 000 to 58 000. Heini et al. (1987) suggested that glutaminase is, therefore, composed of three identical subunits. Purified glutaminase exhibited an apparent Km for glutamine of 17 mM

and a pH optimum between 7.8 and 8.2 (Smith and Watford, 1988). The K_a for phosphate could not be determined because dialysis against Tris buffer to remove the anion resulted in irreversible loss of activity. The enzyme was not inhibited by glutamate, nor was the purified glutaminase affected by maleate, an activator of γ-glutamyl transpeptidase. A small (10%) activation of glutaminase, however, was detected in the presence of N-acetylglutamate, at concentrations of 2-5 mM.

Association of glutaminase with the inner mitochondrial membrane

In early studies, Errera (1949) showed that phosphate-activated glutaminase was bound to insoluble liver particles that were precipitated during centrifugation. Guha (1962) later showed that this activity was associated with mitochondria. When mitochondria are treated with digitonin, glutaminase is found in the inner membrane + matrix fraction, and can be released into solution by sonication or treatment with detergents (Kalra and Brosnan, 1973). However, after the gentler treatment of freezing and thawing of liver mitochondria, more than 90% of the activity of glutaminase was found to be associated with the membrane fraction, while matrix enzymes were released (McGivan et al., 1980). These results were taken to indicate that liver glutaminase is loosely associated with the inner mitochondrial membrane.

The association of glutaminase with the inner

mitochondrial membrane appears to have important kinetic consequences. McGivan et al. (1980) studied membrane-bound and soluble forms of liver glutaminase. In membrane preparations, glutaminase exhibited an apparent Km for glutamine of about 6 mM, but this value increased to 21 mM when the enzyme was released to solution by sonication. The glutamine dependence curve was sigmoidal for the solubilised enzyme. McGivan et al. (1985) showed that the addition of mitochondrial membranes to partially purified glutaminase reduced this sigmoidicity and resulted in a Km for glutamine closer to that for the membrane-bound preparations. Other results, with intact mitochondria, showed that under isotonic conditions, the affinity of glutaminase for glutamine was sigmoidal but became more hyperbolic if osmolarity was decreased (McGivan et al., 1985). Together, these results suggested to these workers that liver glutaminase could be regulated in situ by reversible association with the inner mitochondrial membrane.

Properties of glutaminase

Liver glutaminase shows positive cooperative binding of glutamine (Snodgrass and Lund, 1984; Szweda and Atkinson, 1989). Activation of glutaminase often involves an increase in the enzyme's affinity for glutamine. A number of substances that activate the enzyme will now be discussed.

The stimulation of liver glutaminase by ammonia was

initially shown in isolated mitochondria (Charles, 1968) and later in perfused liver (Häussinger et al., 1975) and isolated hepatocytes (Joseph and McGivan, 1978a). The stimulation of glutaminase by ammonia is also evident in disrupted mitochondria suggesting that its effect is a direct one, and not the result of transport effects or other events requiring an intact membrane (McGivan et al., 1980; McGivan and Bradford, 1983a). At cytosolic pH, glutaminase was virtually inactive in the absence of ammonia and, therefore, this molecule is regarded as an obligatory activator Verhoeven et al. (1983). There is, however, no agreement on whether ammonium (NH_4^+) or ammonia (NH_3) is the active species (see Szweda and Atkinson, 1990a).

Bicarbonate stimulates flux through glutaminase in intact mitochondria (Joseph and McGivan, 1978b), in disrupted mitochondria (McGivan et al., 1980), and in hepatocytes (Baverel and Lund, 1979). The effect is not large and there is some indication that it depends on the presence of ammonia (Verhoeven, et al., 1983). Bicarbonate does not stimulate flux through glutaminase in perfused liver (Häussinger et al., 1980) and it has no effect on the partially purified enzyme (Patel and McGivan, 1984).

Inorganic phosphate stimulates flux through glutaminase in intact mitochondria (Lacey et. al., 1981)and in disrupted mitochondria (McGivan et al., 1980). The activity of partially purified glutaminase has been shown to be

absolutely dependent on phosphate (McGivan et al, 1985) and complete removal of phosphate results in irreversible loss of activity (Smith and Watford, 1988). It has been reported that in disrupted mitochondria 70% of the glutaminase activity is independent of added phosphate (McGivan and Bradford, 1983b). It would appear, however, that this is only true when the incubations are carried out with optimal concentrations of glutamine and ammonia and at alkaline pH (Szweda and Atkinson, 1989). Under conditions that simulate physiological conditions glutaminase was found to be strongly affected by phosphate.

The pH sensitivity of liver glutaminase is opposite to that diplayed by the kidney enzyme, in being inhibited by acidosis and stimulated by alkalosis. Flux through glutaminase in perfused liver is very dependent on changes in perfusate pH. For example, Lueck and Miller (1970) observed a 50% reduction in glutamine catabolism when perfusate pH was lowered from 7.45 to 7.15. Increases in pH result in a stimulation of glutaminase flux (Häussinger et al., 1980). Thus, glutaminase responds to changes of pH in the physiological range. Affect of pH on glutaminase has also been noted in isolated hepatocytes (Kashiwagura et al., 1985) and mitochondria (Verhoeven et al., 1983), and the effect has been extensively studied in frozen-thawed mitochondria by Szweda and Atkinson (1989). In frozen-thawed mitochondria glutaminase activity increased 8-fold as pH was raised from

7.1 to 7.7. This was accompanied by a decrease in the K_a for phosphate from 21 to 8.9 mM. Since flux through glutaminase became independent of pH when both phosphate and ammonia were present at saturating concentrations, but not when only one of these effectors was saturating, it was concluded that increases in pH stimulate glutaminase by affecting the phosphate and the ammonia sensitivity of the enzyme.

A number of other substances have been reported to affect glutaminase activity. These include leucine, ATP, N-acetylglutamate, and magnesium ion. Leucine stimulates flux through glutaminase in isolated hepatocytes (Baverel and Lund, 1979), but does not stimulate the partially purified enzyme (Patel and McGivan, 1984). Since leucine is known to inhibit ornithine transcarbamylase, its action is believed to be secondary to an accumulation of ammonia (see McGivan et al., 1988). ATP activates glutaminase in disrupted mitochondria by increasing the phosphate sensitivity of the enzyme (McGivan et al., 1980). The effect appears to be additive to those of other effectors (ammonia and bicarbonate). Flux through glutaminase is stimulated in intact mitochondria under conditions that favour production of N-acetylglutamate and it has been proposed that this compound is responsible (Meijer and Verhoeven, 1986). However no effect of N-acetylglutamate is evident in disrupted mitochondria (Joseph and McGivan, 1978b) while its effect on the purified enzyme is small (Smith and Watford, 1988). The

information on the effects of magnesium ion on glutaminase are somewhat equivocal. Treatment of intact mitochondria with EDTA stimulates flux through glutaminase, an effect that can be reversed by addition of low concentrations of magnesium (Joseph et al., 1981a). Magnesium affected the stimulation of glutaminase during hypotonic incubation, and the activity of the solubilised enzyme during reconstitution with mitochondrial membranes (McGivan et al., 1985). It was suggested that magnesium ion interferes with the association of glutaminase with the inner membrane. Recently, however, Szweda and Atkinson (1990) have reported that magnesium ion stimulates glutaminase in frozen-thawed membrane preparations. The effect was dependent on pH, being most effective near 7.4. The effect was mediated by an increase in the affinity of glutaminase for phosphate.

Hepatocyte heterogeneity in glutamine metabolism

The concept of hepatocyte heterogeneity in metabolic function is well accepted and has been the subject of a number of reviews (Häussinger, 1989; 1990; Jungermann and Katz, 1982; 1989). Hepatocytes are described as being either periportal or perivenous. Periportal hepatocytes are those nearest the sinusoidal inflow, and therefore, receive portal blood rich in oxygen, nutrients, and hormones. Perivenous hepatocytes are nearer the sinusoidal outflow, and so, must contend with blood of a lower oxygen, nutrient, and hormone

content, but which is somewhat enriched by substances released from periportal cells. The size of the periportal and perivenous compartments is not clear cut, and it has been pointed out by Häussinger (1990), that the distinction will depend on the pathway under consideration. In terms of metabolic zonation, it is now generally accepted that amino acid degradation, ureagenesis, and gluconeogenesis occur in the periportal zone, while glycolysis, ketogenesis, and xenobiotic metabolism are perivenous events (Jungermann and Katz, 1989). Hepatocyte heterogeneity in ammonia and glutamine metabolism is especially pronounced. This has been shown in metabolic studies involving the isolated, perfused liver (Häussinger, 1983), in experiments with hepatocyte preparations enriched in either periportal or perivenous cells (Watford and Smith, 1990), and by immunohistochemical localisation of liver enzymes (Gebhardt and Mecke, 1983; Saheki et al., 1983). Glutamine synthetase is confined to a small population of cells (about 7%) surrounding the terminal hepatic venule (Gebhardt and Mecke, 1983), while glutaminase (Watford and Smith, 1990) and the urea cycle enzymes (Saheki et al.,1983; Gaasbeek-Janzen et al., 1984) are found in a much larger periportal region.

Metabolic significance

The physiological significance of glutamine degradation and ureagenesis occurring "upstream" of glutamine synthesis

was illustrated in the metabolic studies of Häussinger (1983). In these studies comparison was made between metabolic fluxes during antegrade and retrograde perfusion. When rat liver was perfused in the normal, antegrade direction, added ammonia was mainly converted to urea. In the absence of added ammonia, that ammonia arising endogenously was shown to be converted to glutamine. In each situation, the effluent ammonia concentration was low. If, on the otherhand, the perfusion was in the retrograde direction, added ammonia was primarily converted to glutamine, while ammonia arising endogenously was washed out. Effluent ammonia concentration during retrograde perfusion was, therefore, relatively high. These differences in metabolic outputs with perfusion direction disappeared when methionine sulfoximine, a transition state analog inhibitor of glutamine synthetase, was present in the perfusate.

From these findings, it was proposed that glutamine and urea synthesis together constitute an efficient ammonia detoxification system (**Fig. 1.5**). Ammonia in sinusoidal blood will first come in contact with cells capable of urea synthesis. NH_3 and not NH_4^+ is regarded as the substrate for carbamylphosphate synthase I (Cohen et al., 1985). The Km of carbamylphosphate synthase I for NH_3 is about 13 μM in intact mitochondria, which is somewhat higher than the estimated NH_3 concentration in normal liver (Cohen et al., 1985).

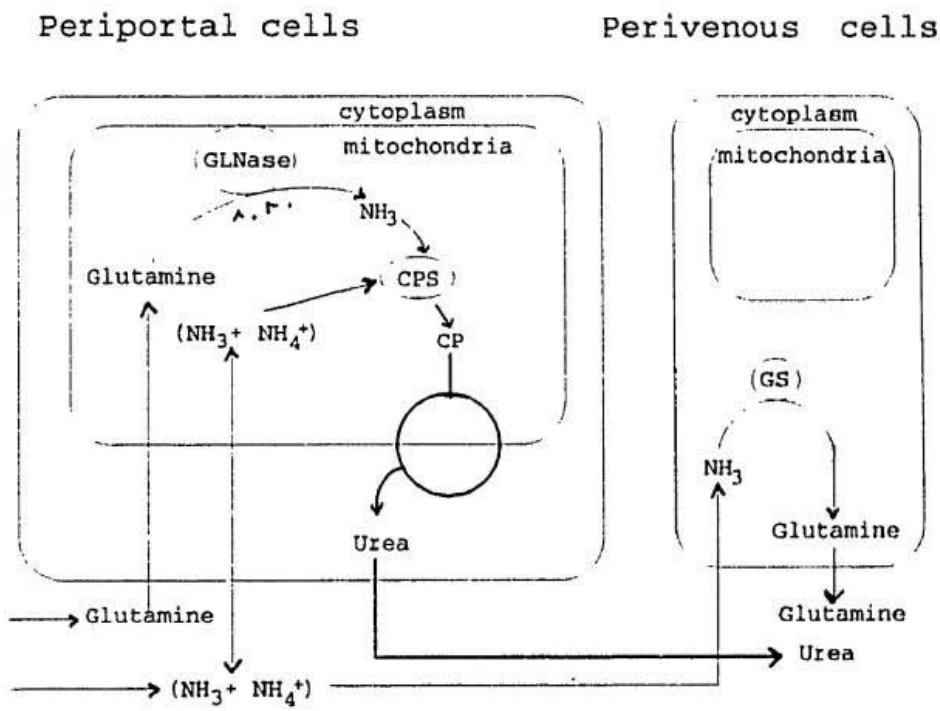


Fig. 1.5. Hepatocyte heterogeneity in glutamine metabolism and its involvement in urea synthesis and ammonia detoxification (modified from Häussinger, 1989).

Therefore, it is expected that carbamylphosphate (and urea) synthesis will be stimulated as NH_3 is elevated (Cohen et al., 1985). The stimulation by ammonia of glutaminase flux may also be an important influence on urea synthesis. First, it has been reported that carbamylphosphate synthase I has preferential access to the ammonia generated by glutaminase (Meijer, 1985). Second, it has been suggested that the stimulation of glutaminase by ammonia is a signal of the availability of ammonia for urea synthesis and the need for glutamate for the synthesis of N-acetylglutamate, the obligatory activator of carbamyl-phosphate synthase I (Szweda and Atkinson, 1990a). If ammonia is not converted to urea in periportal cells it may be scavenged by glutamine synthetase in perivenous cells (Häussinger, 1989). Thus, hepatic detoxification of ammonia involves a co-ordination of glutamine metabolism and urea cycle activity.

Transport of glutamine across the plasma and mitochondrial membranes

Glutamine entry into liver was shown to be mediated by a different transport protein than that responsible for alanine entry (Joseph et al., 1978). This activity, termed System N, is sodium-dependent and is specific for glutamine, asparagine, and histidine (Kilberg et al., 1980). System N has not been found in other body tissues, although a somewhat analogous system, N^m, exists in muscle (Christensen, 1990). A

100 kDa protein responsible for system N has recently been partially purified (Tamarappoo et al., 1992). Although System N is responsible for the bulk of glutamine removal by liver, some uptake occurs via System A, which is also sodium-dependent, and by System L, a sodium-independent transporter (Fafournoux et al., 1983; Low et al., 1991). System L, however, is involved primarily in glutamine efflux (Fafournoux et al., 1983; Burger et al., 1989), and consistent with this role, its activity is greater in perivenous cells than in periportal cells (Burger et al., 1989).

System N, unlike System A, is slow to undergo adaptive change (Christensen, 1990). Glutamine transport by system N is not stimulated by glucagon or insulin during several hours of exposure (Kilberg et al., 1980), but induction does occur with prolonged exposure of cell culture to hormones (Gebhardt and Kleeman, 1987). System N was not stimulated in hepatocytes from rats starved for 48 hours (Hayes and McGivan, 1982), but stimulation was demonstrated in liver membrane vesicles prepared from rats starved for 60 hours (Low et al., 1992). Glutamine transport by system N is also induced in animals fed high-protein diets (Rémésy et al., 1988; Fafournoux et al., 1990). System N has been shown to be stimulated in perfused liver by increases in pH (Lenzen et al., 1987). Inhibition of glutamine transport has been demonstrated in the presence of physiological concentrations

of histidine which competes for uptake (Häussinger et al., 1985b). Glutamine transport is therefore suggested to be a potential site of regulation of hepatic glutamine metabolism.

The movement of glutamine into liver mitochondria is mediated by a specific transport protein which can be inhibited by the sulphydryl blocking reagent meraslyl (Joseph and Meijer, 1981). Uptake of glutamine by liver mitochondria appears to be an electroneutral event which is driven by the mitochondrial pH/cytosolic pH gradient (Soboll et al., 1991). Initial rates of transport are rapid (Joseph and McGivan, 1978b) and far exceed maximal rates of glutaminase flux (Kovacevic and Bajin, 1982). Transport activity is reported to maintain a mitochondrial/cytosolic gradient for glutamine of approximately 3 (Häussinger et al., 1985b). Very high concentrations of matrix glutamine have been reported for liver perfused with a physiological glutamine concentration (0.6 mM) at alkaline pH (Lenzen et al., 1987). In these experiments, mitochondrial glutamine concentration was reported to increase from about 15 mM to 50 mM when perfusate pH was raised from 7.3 to 7.7. These values, however, were determined following freeze-clamping and fractionation of liver in nonaqueous solvents, a procedure which is likely to lead to organellar membrane damage and redistribution of glutamine.

The possibility that transport of glutamine either across the plasma membrane, or into the mitochondrial matrix

could be regulatory for its metabolism has been examined using the concept of control strength (Pogson et al. 1990; Low et al., 1990). In these studies, L-glutamate-γ-hydrazide was used to inhibit glutaminase in hepatocytes and in mitochondria. This compound did not have an effect on glutamine transport processes at the concentrations used (Low et al., 1990). It was found that the degree of inhibition of glutamine metabolism in cells and in mitochondria at a given concentration of L-glutamate-γ-hydrazide was essentially the same. This suggested that regulation of glutamine catabolism could be exerted solely within the mitochondria (ie. glutaminase). Glutaminase was, therefore, assigned a flux control coefficient equal to 1.0 (Pogson et al., 1990). However, when the hepatocyte experiments were done in the presence of histidine, to compete with glutamine for uptake by System N, flux through glutaminase was inhibited. Conversely, incubations done in the presence of tryptophan, which inhibits glutamine efflux via System L, increased flux through glutaminase. The flux control coefficients in these situations were 0.31 and -0.30 for System N and System L, respectively. The transport of glutamine across the plasma membrane can, therefore, exert a regulatory influence on cellular glutamine metabolism.

Regulation of glutamine metabolism by portal ammonia and hormones

The stimulation of glutaminase is usually associated with production of urea and glucose. Glutamine at 10 mM is readily metabolised to glucose and urea in perfused liver (saheki and Katunuma, 1975) and in isolated hepatocytes (Krebs et al., 1976). However, at physiological concentrations glutamine is poorly metabolised unless effectors such as ammonia (Lund and Watford, 1975) or glucagon (Joseph and McGivan, 1978a) are present. The result of blood-borne effectors on hepatic glutamine metabolism will now be discussed.

The portal glutamine and ammonia concentrations are in the vicinity of 0.5-0.6 and 0.2-0.3 mM, respectively (Lund and Watford, 1976). When these concentrations are perfused, flux through glutaminase is stimulated as monitored by release of $^{14}CO_2$ from 1-^{14}C-glutamine (Häussinger, 1983) or U-^{14}C-glutamine (Häussinger and Sies, 1979) and urea production is increased. In isolated hepatocytes, ammonia (< 1 mM) stimulates the conversion of glutamine to urea, glucose and glutamate (Joseph and McGivan,1978a). The half-maximal effect of NH_4Cl occurred at 0.15 mM, and thus, concentrations found in the portal blood are effective in stimulating glutamine utilisation.

The gut releases more ammonium into the portal

circulation when presented with a glutamine load (Buttrose et al., 1987). In this situation hepatic uptake of glutamine was increased and there was a stimulation of glutaminase flux. This occurred if ammonium chloride was infused directly into the portal vein, suggesting that hepatic uptake of glutamine under these conditions is driven by the activation of intracellular glutamine metabolism (Buttrose et al., 1987). In the postabsorptive rat the splanchnic bed, gut and liver, is responsible for about 70% of glutamine removal from the circulation, with the gut accounting for approximately two-thirds of this uptake (Welbourne et al, 1986). The kidney accounts for much of the remainder. In metabolic acidosis the kidney becomes the primary consumer of glutamine (Schrock and Goldstein, 1981; Phromphetcharat et al., 1981; for review see Welbourne, 1987). Renal glutaminase activity is stimulated, lowering arterial glutamine concentration, and thereby reducing the glutamine load to the splanchnic bed (Welbourne et al., 1986). The gut still effectively extracts glutamine, but its product release shifts from alanine to ammonium and glutamate. This has the effect of stimulating hepatic glutamine metabolism, through the activating effect of ammonia on glutaminase flux and by providing substrate for glutamine synthetase. The latter process predominates since net glutamine release is observed (Welbourne, 1986; Welbourne et al., 1986). These changes in liver glutamine metabolism are important in providing glutamine for renal ammoniagenesis

and bicarbonate production during acidosis (Welbourne and Phromphetcharat, 1984).

A number of hormones stimulate glutamine metabolism. Flux through glutaminase is activated in mitochondria isolated from rats injected with glucagon (Lacey et al., 1981). Glucagon (or cyclic AMP) stimulates production of glucose and urea in hepatocytes (Joseph and McGivan,1978a; Ochs, 1984; Kashiwagura et al., 1985). Flux through glutaminase is also stimulated in mitochondria isolated from these cells, or cells treated with other hormones such as catecholamines (Corvera and Garcia-Sainz, 1983). Catecholamines, vasopressin, and angiotensin II are all effective in increasing glutamine utilisation in hepatocytes (Joseph et al., 1981b; Ochs, 1984; Vincent et al., 1989).

The stimulation of gluconeogenesis from glutamine by glucagon is accompanied by a decrease in the intracellular concentration of glutamine which is consistent with an activation of glutaminase (Joseph and McGivan, 1978a). It has been shown, however, that stimulation of glutamine utilisation by glucogenic hormones also involves actions of these hormones on enzymes later in the gluconeogenic sequence (Ochs, 1984; Kashiwagura et al., 1985). Hormonal stimulation of gluconeogenesis is associated with a decrease in α-ketoglutarate (Verhoeven et al., 1985; Häussinger and Sies, 1984; Staddon and McGivan, 1984). This is thought to be due to the activation of α-ketoglutarate dehydrogenase by calcium

(McCormack, 1985). It has recently been reported that α-ketoglutarate inhibits phosphoenolpyruvate carboxykinase, and that this occurs at concentrations of α-ketoglutarate expected in the cell during basal conditions (Titheradge et al., 1992). It is therefore proposed that hormones stimulate gluconeogenesis by removing the inhibition of phosphoenolpyruvate carboxykinase by α-ketoglutarate. Thus, the effect of hormones on glutamine metabolism is the result of activation of mitochondrial processes, flux through glutaminase and α-ketoglutarate dehydrogenase.

Problem of investigation

The liver is an important site for the catabolism of glycine and glutamine. From the discussions so far, it is clear that the metabolic regulation of these processes is poorly understood. The principal observations in this regard are that the hepatic glycine cleavage system and glutaminase are activated in rats following a massive injection of glucagon. Injection of glucagon is a non-physiological event, and it is unclear under what conditions in vivo these enzymes are activated. The focus of this thesis is a study of hepatic glycine and glutamine metabolism in animals fed on high-protein diets or meals, which are physiological situations associated with increased levels of blood glucagon.

Chapter 2

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 150-200 g were purchased from Charles River Limited (Montréal, P.Q.). Animals were housed 2 or 3 in a cage and had free access to a standard Purina pellet diet and tap water. They were kept in a room with a 12:12 light:dark cycle. Lights were off from 8pm to 8am (normal light cycle) or from 8am to 8pm (reversed light cycle). Animals were housed under these conditions for at least 5 days before being placed on a purified diet.

Purified Diets

The normal-protein diet (15% casein) and the high-protein diet (60% casein) used throughout these studies are modified AIN-76 diets (Bieri *et al.*, 1977). Their composition is detailed in **Table 2.1**. These diets are isocaloric. In the high-protein diet, sucrose and corn starch were reduced in favour of the casein. In each diet the ratio of sucrose to corn starch was the same. In one set of experiments (**Chapter 4**) additional protein diets were used where lactalbumin replaced the casein as the protein.

Feeding of rats

Diet protocol

Animals were fed either the high-protein or normal-protein diet *ad libitum* for periods up to 6 days. Food was

Table 2.1. Composition of the 15% and 60% casein diets

	15% casein diet	60% casein diet
	(g/kg diet)	
Casein	148.5	598.5
Sucrose	533.0	192.0
Corn starch	170.0	61.0
Corn oil	50.0	50.0
Alphacel	50.0	50.0
Mineral mix (AIN 76)[1]	35.0	35.0
Vitamin mix (AIN 76A)[1]	10.0	10.0
Choline bitartrate	2.0	2.0
L-methionine	1.5	1.5

[1] The composition of the mineral mix and vitamin mix are given in Bieri *et al.* (1977).

provided in powder-form in a metal feeder suspended from the edge of the cage. Animals rapidly adjusted to the diets (**Table 2.2**). Rats fed the high-protein diet tended to lose about 3 g body weight on the first day of the diet but this was followed by normal weight gain on subsequent days. In a few instances, where weight gain did not occur, the animal was removed from the study.

It was important to measure blood and liver amino acids in animals fed on these diets. This was to ensure that the decrease in circulating and hepatic glycine concentrations, observed in animals fed on high-protein diets (see **Chapter 1**), was occurring in our studies. This is reported here as a preliminary experiment. Arterial blood samples were taken at 9 am (see below for the methods used). **Table 2.3** details the blood amino acids in rats fed the protein diets. As expected glycine levels were depressed in animals fed the high-protein diet (141 nmoles/ml versus 205 nmoles/ml). However, the levels of other amino acids were also depressed, notably serine, glutamine, alanine, tyrosine, and hyroxyproline. Surprisingly, the levels of many amino acids were quite similar in these animals despite the difference in protein content of the diets. Only the branched-chain amino acids, proline, and arginine were elevated in the blood of high-protein-fed animals. These findings agree with those of Peret et al. (1981) who reported a substantial adaptation of hepatic amino acid metabolism in rats following the first day

Table 2.2. Change in body weight of rats fed the 15% and 60% casein diets. Results are mean ± S.D. of four rats for each group. These rats were used to determine the blood and liver amino acids levels presented in **Table 2.3** and **2.4**. Day 1 denotes the initial weight.

Diet	Body weight (g)				Net gain (g)
	Day 1	Day 2	Day 4	Day 6	
15% casein	213.5 ± 2.2	217.3 ± 3.0	225.5 ± 5.0	237.0 ± 7.1	23.5
60% casein	214.8 ± 3.2	211.5 ± 2.5	220.5 ± 1.6	238.3 ± 3.1	23.5

Table 2.3. Amino acid levels in arterial blood from rats fed 15% and 60% casein diets. The rats were fed the respective diets for 6 days. The results are the mean ± S.E.M. of samples taken from four rats. * significantly different from value in 15% casein group (student t-test; $p < 0.05$).

	Casein (15%)	Casein (60%)
	(nanomoles/milliliter of blood)	
Cysteic acid	19.59 ± 2.0	17.75 ± 1.1
Taurine	392.64 ± 24.9	462.44 ± 25.6
Hydroxyproline	47.18 ± 5.9	15.45 ± 3.1*
Threonine	405.87 ± 74.9	347.51 ± 23.2
Serine	340.26 ± 29.2	235.29 ± 18.4*
Asparagine	80.45 ± 8.0	88.68 ± 4.4
Glutamic acid	251.72 ± 19.6	245.18 ± 24.9
Glutamine	664.91 ± 33.8	460.26 ± 9.7 *
Proline	460.71 ± 50.0	765.13 ± 63.2*
Glycine	205.12 ± 15.3	141.41 ± 11.9*
Alanine	732.27 ± 47.5	559.88 ± 29.5*
Citrulline	75.97 ± 7.2	85.76 ± 6.9
Amino-n-butyric	34.06 ± 31.6	23.68 ± 6.8
Valine	254.51 ± 20.0	768.58 ± 50.1*
Cystine	45.86 ± 3.0	48.46 ± 5.2
Methionine	85.12 ± 5.4	75.75 ± 5.9
Isoleucine	101.27 ± 6.7	264.79 ± 19.8*
Leucine	150.03 ± 11.0	418.10 ± 29.7*
Tyrosine	191.59 ± 7.1	129.55 ± 5.5*
Phenylalanine	69.62 ± 2.8	73.37 ± 3.4
Tryptophan	96.85 ± 34.9	83.79 ± 17.8
Hydroxylysine	0.21 ± 0.2	0.51 ± 0.5
Ornithine	44.44 ± 5.5	58.25 ± 1.5
Lysine	786.68 ± 60.4	963.38 ± 61.5
Histidine	71.52 ± 5.3	61.53 ± 12.0
Arginine	159.18 ± 9.6	220.14 ± 12.5*

of high-protein-feeding.

In liver tissue of these animals (**Table 2.4**), the branched-chain amino acids, citrulline, and amino-n-butyric acid were increased by high-protein-feeding while the hepatic levels of most other amino acids did not differ from those in rats fed the 15% casein diet. This was with two exceptions. First, glycine was only 517 nmole/g liver in rats fed the high-protein diet compared to 1445 nmoles/g liver in rats fed the 15% casein diet. The other exception was glutamine whose level was decreased by 43% in liver of animals fed the high-protein diet.

Protocol to examine the effect of ingestin a diet for a single day

In the studies on glycine metabolism the effect of changing the protein content of the diet for one day was examined (**Chapter 3**). In these experiments, rats were fed the high-protein or normal-protein diet for 5 days followed by one day of feeding (19 h) on the alternate diet (ie. normal-protein-fed rats were switched to the high-protein diet and vice versa). This was done by placing the rat in a clean cage with the new diet at 2 pm and animals were killed the following day at 9 am (normal light cycle). Some animals were fed on the same diet for the entire six days (controls).

Table 2.4. Liver amino acids from rats fed 15% and 60% casein diets. The rats were fed the respective diets for 6 days. The results are the mean ± S.E.M. of liver samples taken from four rats. * significantly different from value in 15% casein group (student t-test; $p < 0.05$).

	Casein (15%)	Casein (60%)
	(nanomoles/gram liver tissue)	
Cysteic acid	118.34 ± 17.7	99.15 ± 9.9
Taurine	7004.54 ± 2894.2	11042.60 ± 3637.8
Hydroxyproline	47.06 ± 8.8	57.55 ± 8.6
Threonine	437.76 ± 51.1	424.84 ± 20.4
Serine	658.96 ± 208.9	295.22 ± 33.7
Asparagine	71.57 ± 11.2	85.24 ± 1.6
Glutamic acid	1295.36 ± 224.4	1193.60 ± 45.4
Glutamine	5968.96 ± 498.4	3431.29 ± 30.0*
Proline	230.19 ± 31.2	489.66 ± 113.7
Glycine	1445.44 ± 279.3	517.79 ± 21.2*
Alanine	2461.65 ± 186.6	2405.26 ± 285.3
Citrulline	81.63 ± 21.7	159.02 ± 6.4*
Amino-n-butyric	20.26 ± 5.3	71.21 ± 10.5*
Valine	230.74 ± 51.1	739.61 ± 60.2*
Cystine	79.72 ± 14.9	84.31 ± 9.1
Methionine	78.68 ± 14.9	93.65 ± 8.5
Isoleucine	106.02 ± 23.0	277.27 ± 16.1*
Leucine	179.57 ± 52.0	415.54 ± 25.5*
Tyrosine	72.59 ± 2.0	67.80 ± 4.1
Phenylalanine	46.25 ± 2.2	63.75 ± 2.8
Tryptophan	20.78 ± 5.7	11.91 ± 4.1
Hydroxylysine	0.00 ± 0.0	0.00 ± 0.0
Ornithine	113.01 ± 23.3	111.41 ± 10.7
Lysine	932.63 ± 211.1	1164.62 ± 149.0
Histidine	669.41 ± 34.0	583.71 ± 20.4
Arginine	42.47 ± 7.8	76.18 ± 10.4

Protocol to examine the effect of feeding a single meal

A similar protocol was used to investigate the effect of a single meal on glycine and glutamine catabolism. In these experiments, rats were fed the normal-protein or high-protein diet for 3 to 4 days. When they were to be used, rats were put in clean cages and were deprived of food for 6-7 hours, but had free access to tap water. This short period of food deprivation occurred during the light period when the animals would not normally be actively feeding. This was found to be necessary to ensure that the rats were truly postabsorptive and that they ate promptly when they were given a meal. Meals were provided at the start of the dark period. Meals consisted of either the diet to which the rats had been accustomed during the previous 3-4 days of feeding, or the alternate diet. In other words, rats fed the normal-protein diet for 3-4 days were given either a high-protein or normal-protein meal while animals fed the high-protein diet were given either a high-protein or normal-protein meal. Some animals did not receive meals and were sacrificed at this time (controls). Rats were killed 2 or 4 hours following the start of a meal.

Injection of rats with glucagon

In experiments involving glucagon, rats maintained on the standard pellet diet were injected (intraperitoneally)

with 0.1 mg glucagon/100 g body weight dissolved in 0.9% saline/0.05% bovine serum albumin. Control rats were injected with vehicle. Rats were sacrificed 20-30 minutes after injection.

Blood and Liver sampling

Rats were anaesthetised with an intraperitoneal injection of Nembutal (sodium pentobarbitol) at a dose of 6.5 mg/100g body weight . About 1 ml of blood was removed from the abdominal aorta into a heparinised syringe. For the measurement of liver amino acids, a piece of liver tissue was freeze-clamped using metal tongs cooled in liquid nitrogen. This was done immediately after taking the arterial blood sample.

Blood and liver amino acids

About 0.2 mL of blood was added to a pre-weighed Eppendorf tube containing 0.2 mL of 10% sulfosalicylic acid and 50 µL amino-ethyl-cysteine (125mM; internal standard). The tube was then weighed. The contents were vortexed vigorously and the tube was placed on ice for 30 minutes. 0.20 mL lithium citrate buffer (0.2 N) was then added and the sample was centrifuged at 12 000 x g for 2 min. On a few occasions where the supernatant was coloured, a second 2 min. centrifugation was performed to clear it. The supernatant was adjusted to pH 2.2 using lithium hydroxide (3 N).

Liver samples were ground to powder in liquid nitrogen and stored at -70°C. In preparation for amino acid analysis 1 g of frozen tissue powder was added to a cold tared tube containing 4 mL of 6% perchloric acid and 75 µL of internal standard. The sample was homogenised using a motor-driven teflon pestle which just fitted the centrifuge tube. The samples were then centrifuged at 12 000 x g for 10 min. The supernatant was neutralised with KOH and placed on ice for 30 minutes. The potassium perchlorate was removed by centrifugation. The resulting supernatant was diluted 2:1 with lithium citrate buffer and HCl was used to adjust pH to 2.2.

Amino acids were measured on a Beckman model 121-M amino acid analyser as described by Lee (1974).

Isolation of Liver mitochondria

Rats were killed by cervical dislocation. The liver was homogenised in a medium modified from Hampson et al. (1983) using a hand-held teflon homogeniser. The isolation medium contained mannitol, 225 mM; sucrose, 75 mM; Ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), 1 mM; N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 5 mM; pH 7.4. The homogenate was centrifuged for 10 min. at 600 x g and 4°C in a Beckman centrifuge. The resulting supernatant was centrifuged for 10 min. at 8200 x g to pellet the mitochondria. The mitochondrial pellet was

resuspended in homogenisation medium and centrifuged again at 8200 x g for 10 min. This washing step was repeated twice. The final pellet was resuspended in the isolation medium to give a concentration of 50-70 mg/ml mitochondrial protein as determined by the biuret test using bovine serum albumin (BSA) as standard (Gornall et al., 1949). The yield of mitochondria was similar for high protein- and normal-protein-fed rats. The mitochondria were acceptable if they displayed a respiratory control ratio greater than 4 with 2-oxoglutarate as substrate.

Respiratory control ratio and succinate oxidation

Oxygen uptake was measured using a Clark-type electrode. The respiration medium contained KCl, 140 mM; KH_2PO_4, 4 mM; $MgCl_2$, 2.5 mM; ethylenediamine tetraacetic acid (EDTA), 1 mM; HEPES, 5 mM; BSA, 1 mg/ml; pH 7.4. The medium was equilibrated with respect to temperature (30°C) and air saturation. Rates of succinate oxidation (**Chapter 4**) were determined at ADP and succinate concentrations of 0.5 mM and 5 mM respectively. The mitochondrial concentration was 1-2 mg of mitochondrial protein in a chamber volume of 1.76 mL.

Preparation of mitochondrial membranes

Mitochondrial membranes were prepared according to Szweda and Atkinson (1989). A stock solution of mitochondria was diluted 1:1 with distilled water and subjected to three

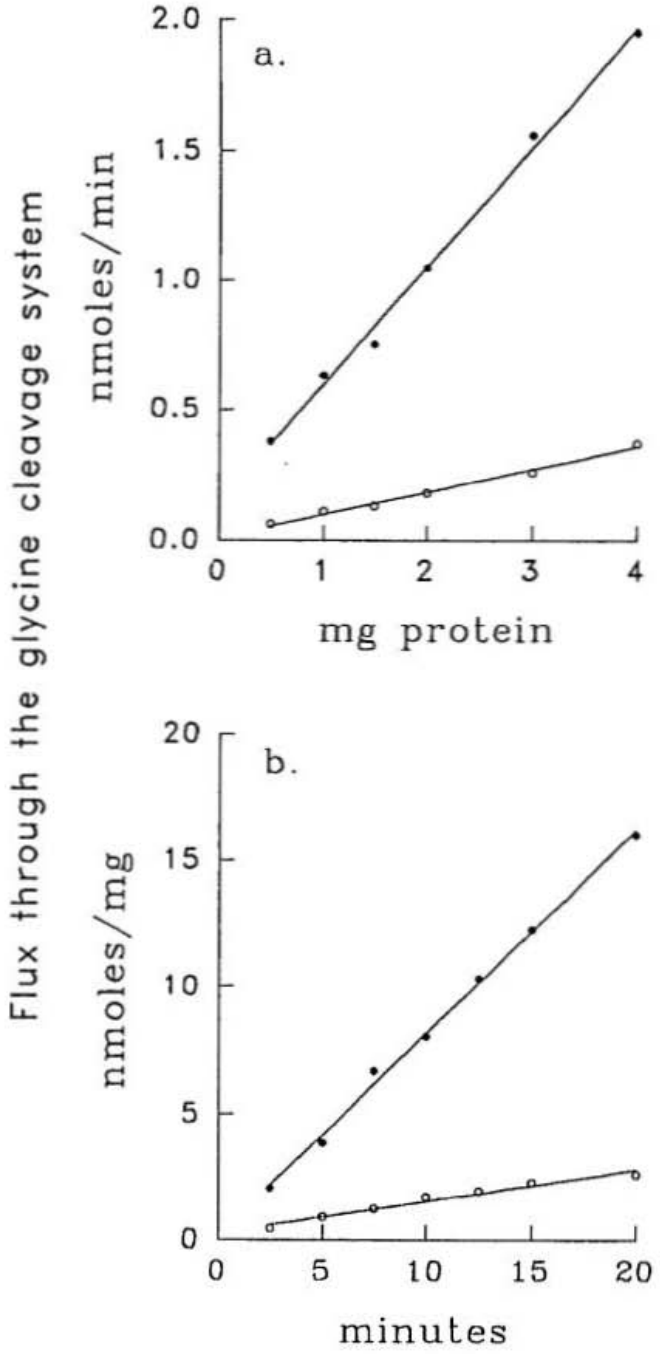
cycles of freezing in liquid nitrogen and thawing in water (37°C). The suspension was then diluted 5-fold with isolation medium and the membranes were sedimented by centrifugation for 20 min. at 40 000 x g.

Mitochondrial Incubations

Flux through the glycine cleavage system

Flux through the glycine cleavage system was measured by quantifying the release of $^{14}CO_2$ by intact mitochondria incubated with 1-^{14}C-glycine. The standard incubation medium (KCl-based medium) consisted of KCl, 125 mM; NaCl, 5 mM; $MgCl_2$, 2.5 mM; K_2HPO_4, 2.5 mM; 3-[N-morpholino]propane sulponic acid (MOPS), 10 mM; Tris[hydroxymethyl]aminomethane hydrochloride, 7 mM; EGTA, 1 mM; 1-^{14}C-glycine, 5 mM (300-500 dpm/nmol); ADP, 1 mM; pH 7.4. The flux rate was linear for up to 20 minutes and for up to 4 mg/ml mitochondrial protein (**Fig. 2.1**). Incubations were routinely carried out at 30°C for 10 to 15 minutes at a final protein concentration of 1 mg/ml. The flask was sealed with a rubber stopper equipped with a centerwell. Injection of 0.3 ml of 30% $HClO_4$ into the flask terminated the incubation and released the CO_2 from solution. CO_2 was trapped in filter paper immersed in 0.3 ml NCS tissue solubiliser (Amersham Canada Limited, Oakville, Ontario) contained within the centerwell. CO_2 was collected for 1 hour after which time the centerwells were transferred to scintillation vials containing Omnifluor. Radioactivity

Fig. 2.1. Flux through the glycine cleavage system in rat liver mitochondria as a function of protein concentration (0-4 mg mitochondrial protein, 13 min.) and time (0-20 min. with 1 mg protein). Glycine concentration was 5 mM. Mitochondria were isolated from rats fed a high-protein (●) and normal-protein (O) diet for 6 days. results are the average of 2 individual experiments.


Flux through the glycine cleavage system
a.
nmoles/min
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
mg protein
b.
nmoles/mg
20
15
10
5
0
0
5
10
15
20
minutes

was determined using an LKB 1214 scintillation counter which uses radium-226 source as an external standard. In some experiments we used a simplified incubation medium which consisted of mannitol, 225 mM; sucrose, 75 mM; HEPES, 5 mM, pH 7.4; EGTA, 1 mM and 1-^{14}C-glycine, 5mM. To this medium KH_2PO_4, 2.5 mM or ADP, 1 mM were added as required. This medium was utilised when the requirement for phosphate and ADP in the stimulation of flux through the glycine cleavage system was examined (**Chapter 3**).

The fate of the number 2 carbon of glycine (CO_2 or serine) was investigated by monitoring the release of $^{14}CO_2$ from mitochondria incubated in the presence of 2-^{14}C-glycine and by determining serine production (**Chapter 3**). Mitochondria were incubated in the KCl-based medium under the conditions described for measuring flux through the glycine cleavage system but 2-^{14}C-glycine was present in place of 1-^{14}C-glycine. The reaction was stopped by addition of 0.3 ml of 30% sulfosalicylic acid. Following collection of $^{14}CO_2$, the acidified samples were first centrifuged for 4 min. in an Eppendorf microcentrifuge (12 000 x g) to remove protein and then an aliquot of the supernatant was adjusted to a pH of 2.2 with lithium hydroxide. Serine concentration of these samples was measured on a Beckman model 121-M amino acid analyser as described by Lee (1974).

Flux through the glycine cleavage system is sensitive to submicromolar concentrations of free calcium (Jois et al., 1990b). In experiments where glycine cleavage system flux was measured in the presence of various concentrations of free calcium (**Chapter 3**), EGTA was maintained at 1 mM in the incubation medium, while different amounts of $CaCl_2$ were present. This was done essentially as described by Denton et al. (1978). Stock solutions of EGTA and EGTA plus $CaCl_2$ were combined in differing ratios. Because the EGTA concentration was the same in each stock solution, only $CaCl_2$ concentration was altered (from 0 to 0.95 mM) in order to achieve the desired level of free calcium. Free calcium was calculated from $CaCl_2$:EGTA ratios using the computer programme EQCAL (Biosoft, Milltown NJ 08850, USA) in which the equilibrium composition is determined by a free energy minimisation method (Eriksson, 1979). The stability constants reported by Fabiato and Fabiato (1979), adjusted for temperature, were used in the calculation.

Flux through glutaminase

Flux through glutaminase was measured in intact mitochondria as described by Lacey et al. (1981). Mitochondria (2-3 mg) were incubated at 30°C for 10 minutes in a final volume of 1 mL which contained KCl, 100 mM; glutamine, 20 mM; KH_2PO_4, 10 mM; succinate, 5 mM; rotenone, 5 µg/ml; EGTA, 1mM; Tris-HCl, 20 mM; pH 7.4. There was a lag

in the formation of glutamate during the first 2.5 minutes of incubation after which time the reaction became linear (**Fig. 2.2**). Flux was linear with protein up to 3 mg/ml mitochondrial protein. Incubations were stopped with 0.3 mL perchloric acid (7%). The deproteinised extracts were neutralised and assayed for glutamate enzymically according to Bernt and Bergmeyer (1974). The change in glutamate concentration during the 10 minute incubation was used to calculate flux through glutaminase.

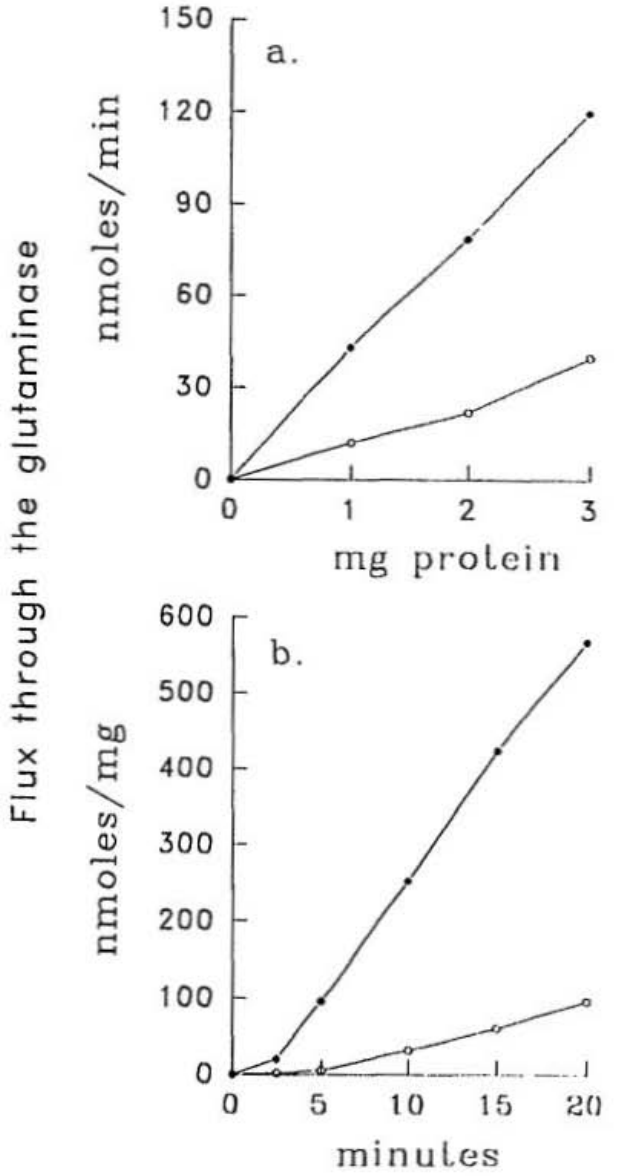
Citrulline synthesis

Citrulline synthesis was determined in intact mitochondria (3-4 mg) incubated for 4 minutes at 30°C in a medium which contained KCl, 80 mM; L-ornithine, 20 mM; KH_2PO_4, 10 mM; NH_4Cl, 5mM; $KHCO_3$, 10 mM; succinate, 10 mM; Tris-HCl, 20 mM; pH 7.4 (Lacey et al., 1981). Incubations were stopped by adding 0.3 mL of perchloric acid (7%). The samples were analysed for citrulline (Herzfeld and Raper, 1976).

Glutaminase activity

Glutaminase activity was determined by incubating mitochondrial membranes (1 mg/ml) for 10 minutes at 37°C in a medium that contained mannitol, 300 mM; glutamine, 20 mM; NH_4C , 0.7 mM; HEPES, 20 mM; pH 7.4; and various levels of KH_2PO_4 (Szweda and Atkinson, 1989). Reaction rates were

Fig. 2.2. Flux through glutaminase in rat liver mitochondria as a function of protein concentration (0-3 mg mitochondrial protein, 10 min.) and time (0-20 min. with 2 mg protein). Glutamine and phosphate concentration were 20 mM and 10 mM, respectively. a. Mitochondria were isolated from rats fed on the high- (●) or normal-protein (O) diet for 3-4 days. Results are from one experiment. b. Rats were fed on the normal-protein diet for 3-4 days and then given a meal of the high-protein (●) or normal-protein (O) diet for 2 hours. Results are the average of 2 individual experiments.

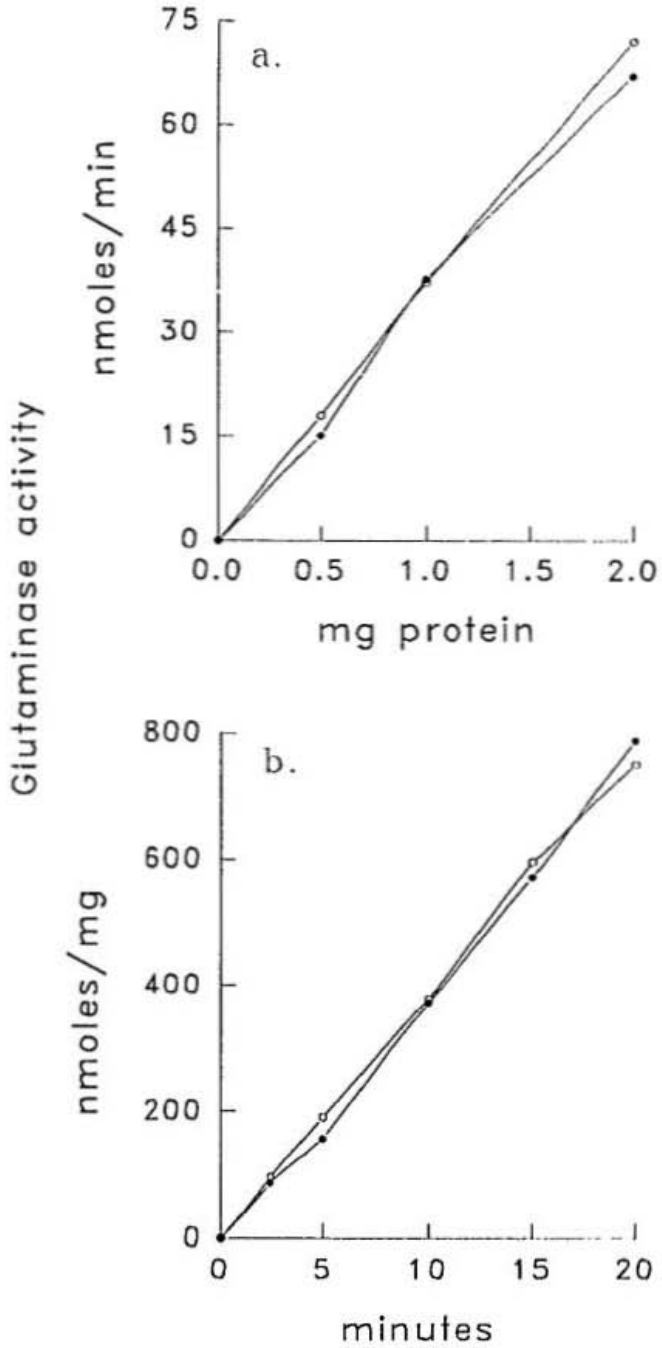

Flux through the glutaminase
a.
nmoles/min
150
120
90
60
30
0
0
1
2
3
mg protein
b.
nmoles/mg
600
500
400
300
200
100
0
0
5
10
15
20
minutes

linear for up to 20 minutes and up to 2 mg/ml membrane protein (**Fig. 2.3**). In the standard assay 1 mg/ml protein was incubated for 10 minutes. Incubations were stopped with 0.3 mL perchloric acid (7%). The deproteinised extracts were neutralised and assayed for glutamate enzymically according to Bernt and Bergmeyer (1974).

Measurement of mitochondrial matrix volume

Methods for measuring mitochondrial matrix volume have been reviewed by Halestrap (1989). We used the distribution of $^{3}H_2O$ and ^{14}C-sucrose to determine the total water and extramatrix spaces, respectively. Mitochondrial matrix volume is the difference between these two spaces. An inherent problem is that this is the difference between two large numbers. Most often matrix volume will be only 10-15% of the total water space (Halestrap, 1986). Thus small errors in measuring either space will result in a large error in the matrix volume determination. The procedure of determining matrix volume using $^{3}H_2O$ and ^{14}C-sucrose involves a short incubation of a mitochondrial suspension with these substances, and subsequent separation of the mitochondria from solution. In the experiments on glycine accumulation, removal of mitochondria from solution was done by simply pelleting the mitochondria by centrifugation (see next section). In our hands, this technique gave values for the matrix volume that were somewhat higher than those reported

Fig. 2.3. Glutaminase activity in mitochondrial membranes from rat liver as a function of protein concentration (0-2 mg membrane protein, 10 min.) and time (0-20 min. with 2 mg protein). Glutamine concentration was 20 mM. Intact mitochondria were isolated from rats fed a normal-protein diet and given a high-protein (●) or normal-protein (O) meal for 2 hours.


Glutaminase activity
a.
nmoles/min
75
60
45
30
15
0
0.0
0.5
1.0
1.5
2.0
mg protein
b.
nmoles/mg
800
600
400
200
0
0
5
10
15
20
minutes

in the literature. For subsequent experiments, we therefore decided to separate the mitochondria from solution by centrifugation through an oil layer (**Fig. 2.4**). An advantage of this procedure is a more complete removal of extramatrix water from the pellet (Halestrap, 1989). Silicone oil is commonly used for such procedures. However it has been observed by Cohen et al. (1987) that total water space, sucrose space, and matrix volume determined by this method varied inversely with the amount of protein used for the determination. The reason for this effect is not known. For this reason we investigated the possibility of using a different oil, bromododecane, for our purposes (**Fig. 2.5**). The total water space and the sucrose space tended to be higher when using bromododecane than when silicone oil was used. However, the matrix volumes determined using the two oils were quite similar, especially when 2 or 3 mg of mitochondrial protein were loaded. Thus bromododecane is at least as effective for use in measuring matrix volume as silicone oil. Since bromododecane is much easier to work with than silicone oil, because of its lower viscosity, we therefore used this oil in subsequent experiments (**Chapter 3 and Chapter 4**). The procedure is described below.

Intact mitochondria were pre-incubated in the KCl-based medium used to measure flux through the glycine cleavage system (**Chapter 3**) or in that used to measured glutaminase flux (**Chapter 4**) in a final volume of 1.25 ml and at a

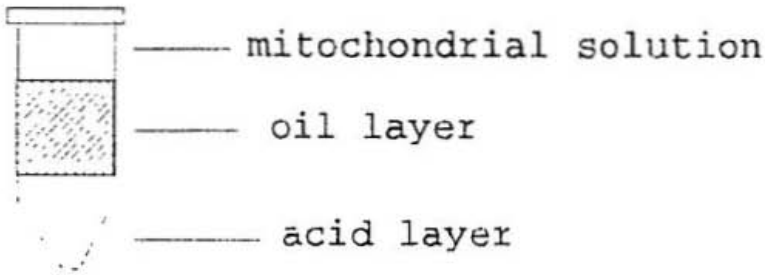
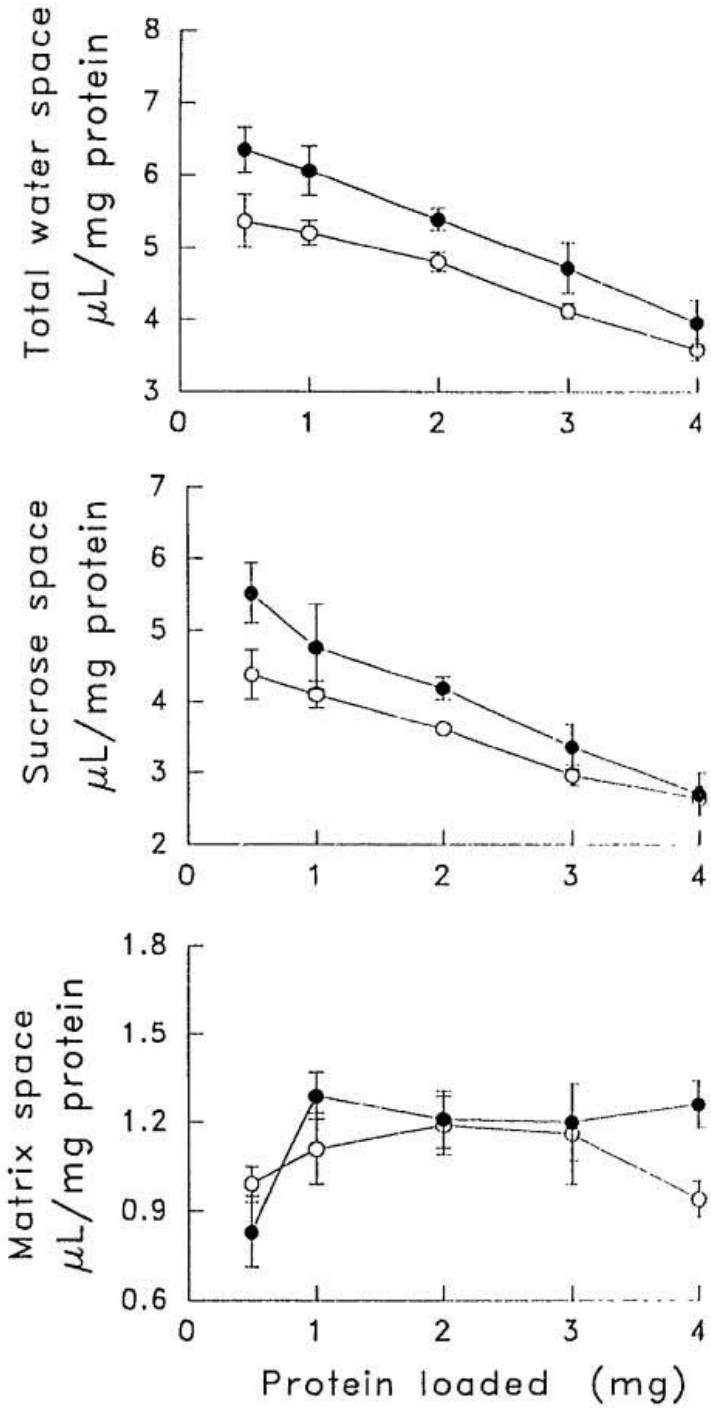


Fig. 2.4. Centrifugation of mitochondria through oil. 100 µL perchloric acid (30%) is placed in an Eppendorf tube and overlaid with 0.7 ml oil (eg. silicone oil; density equal to 1.026). The mitochondrial suspension is pipetted on top of the oil and the tube is immediately centrifuged, separating the mitochondria from their suspending solution, and pelleting them in the acid.

Fig. 2.5. Total water space, a., sucrose space, b., and mitochondrial matrix space, c., determined by centrifugal-filtration through bromododecane oil (●) or silicone oil (O). The results are the mean ± S.E.M. of 4 and 5 separate experiments , respectively. The procedure was as outlined in the text except mitochondria were pre-incubated at concentrations of 1, 2, 4, 6, and 8 mg/ml in order to load the desired amount as a 0.5 ml aliquot on top of the oil. The density of each oil was 1.026.


Total water space
μL/mg protein
8
7
6
5
4
3
0
1
2
3
4
Sucrose space
μL/mg protein
7
6
5
4
3
2
0
1
2
3
4
Matrix space
μL/mg protein
1.8
1.5
1.2
0.9
0.6
0
1
2
3
4
Protein loaded (mg)

protein concentration of 4 mg/ml. After 6 minutes 15 µl of $^{3}H_2O$ (20 µCi/ml) and 15 µl of ^{14}C-sucrose (10 µCi/ml) were added. An aliquot of 0.5 ml was immediately transferred to an Eppendorf centrifuge tube (1.5 mL) which contained 100 µl of 15% perchloric acid overlaid by 0.7 ml of the oil mixture (bromododecane and dodecane, 1:0.05 v:v). Mitochondria were separated from the medium through the oil layer into the acid layer by centrifugation in an Eppendorf centrifuge (10,000 x g for 1 min.). Following centrifugation the top layer was removed with a Pasteur pipette. Residual radioactivity was removed from the sides of the tube and the top of the oil layer by flushing with a gentle stream of tap water for 1 minute. This procedure also removed part of the oil layer, but left the acid layer undisturbed. A Pasteur pipette was then used to remove the remaining oil except for a small layer which formed a meniscus with the acid layer. This small amount of oil did not affect the counting of the sample. The pellet was resuspended in the acid layer before being transfered to a scintillation vial containing scintillation fluid. Complete transfer was assured through 2 x 0.25 mL washes with distilled water. Vials were quickly capped to prevent loss of $^{3}H_2O$ through evaporation. The samples were counted for ^{3}H and ^{14}C using ScintiVerse E (FisherScientific, Ontario, Canada) scintillation fluid in a LKB 1214 scintillation counter. Corrections were made for both ^{3}H overspill into the ^{14}C channel and ^{14}C overspill into the ^{3}H

channel. Samples were counted after 1 or 2 days to allow chemiluminescence to subside. Samples were counted for three successive cycles and averaged. The mitochondrial matrix space was calculated as the difference between the $^{3}H_2O$ space and ^{14}C sucrose space.

During the centrifugal-filtration of the mitochondria through the oil layer, not all of the protein that was loaded reaches the acid layer. Therefore values for matrix volume (μL/mg mitochondrial protein) based on the total protein loaded will be an underestimate (Pande, per. comm.). The protein sedimented by centrifugation of mitochondria was determined in a parallel series of experiments in which the mitochondrial suspension (0.5 ml) was taken from the same incubation flasks. In this case, Eppendorf tubes used for separating mitochondria contained 1.25 M KCl in place of perchloric acid. This change was necessary because the pellet formed from centrifuging mitochondria into perchloric acid was difficult to dissolve. Following centrifugation, the top layer was removed as described above. The oil layer and part of the KCl layer were removed by aspiration. Since the oil that remained adhering to the sides of the tube was found to interfere with the biuret protein assay, it was necessary to remove this oil. This was done by filling the tube to overflowing with a continuous stream of 1.25 M KCl. This caused the oil to float to the surface where it was removed by padding the surface with a Kimwipe. The pellet was then

treated with 0.2 mL deoxycholic acid for 2 or 3 hours or overnight at 4°C. The protein in the sample was then measured using the biuret procedure with BSA as standard. A reagent blank contained 50 µL 1.25 M KCl.

Glycine accumulation

Glycine transport was measured in intact mitochondria in the presence of 13 µM rotenone to inhibit glycine oxidation. Mitochondria were pre-incubated, with shaking, at a concentration of 2 mg/ml for 6 minutes at 30°C in the standard incubation medium used for measuring glycine cleavage system flux. 5 mM 1-^{14}C-glycine (specific activity = 400 dpm/nmol) was added and samples were taken at 15 and 45 seconds and immediately separated from the medium by centrifugation in an Eppendorf centrifuge (12 000 x g for 1 min.). The movement of glycine into the mitochondria was quantified by determining the radioactivity associated with the pellet. In these experiments, parallel incubations were carried out with unlabelled glycine and $^{3}H_2O$ (20 µCi/ml) and U-^{14}C-sucrose (10 µCi/ml) to determine the matrix space and also to correct for glycine distributed in the extramatrix space.

Measurement of inorganic phosphate in the mitochondrial matrix

Intact mitochondria were incubated for 5 minutes in the

same medium used to determine flux through glutaminase. Mitochondria were incubated in a volume of 2 mL at a concentration of 4 mg/mL. Following incubation, three successive 0.5 mL aliquots of mitochondrial suspension were removed for determination of mitochondrial phosphate, protein, and volume. One aliquot was transferred to an Eppendorf tube containing 0.1 mL trichloroacetic acid (10%) overlaid with 0.7 mL bromododecane/dodecane (1:0.05 v:v). Mitochondria were separated from the incubation medium through the oil by centrifugation in an Eppendorf centrifuge (12 000 x g for 1 min.). The acid layer was analysed for inorganic phosphate according to Baginski et al. (1967). A second aliquot of mitochondrial solution was transfered to an Eppendorf tube in which the trichloroacetic acid was replaced with 0.1 mL of KCl (1.25 M). The amount of protein that was pelleted by centrifugation of mitochondria through the oil was determined in this tube as described previously (page 85). Prior to removal of the third aliquot, 30 µL of $^{3}H_2O$ (20 µCi/mL)/U-^{14}C-sucrose (10 µCi/mL) was added to the remaining 1 mL of mitochondrial solution. An aliquot was immediately transferred to an Eppendorf tube containing 0.1 mL of perchloric acid (7%) overlaid with 0.7 mL of the oil. This sample was used to determine mitochondrial matrix space as described on page 83. The calculation of matrix phosphate concentration required that we subtract the extramatrix phosphate in the pellet. Extramatrix phosphate was calculated

from the sucrose space and the inorganic phosphate concentration of the supernatant.

Preparation of Hepatocytes

Hepatocytes were isolated by the collagenase perfusion method as described by Krebs et al. (1974) except hyaluronidase was omitted from the perfusate. Collagenase A (Clostridium histolyticum) was perfused at a concentration of 30 mg/ml. Cell viability was greater than 95% as determined by trypan blue (0.02%) exclusion.

Hepatocyte incubations

Flux through the glycine cleavage system

Hepatocytes (3-5 mg dry weight) were incubated in a total volume of 1 ml Krebs-Henseleit medium at 37°C for 30 minutes. The flask was gassed with O_2/CO_2 (95:5) for 20 s following the addition of the hepatocytes. Glucagon (10^{-5} M), dissolved in 10 mM HCl, was added to the incubations to give a final concentration of 10^{-7} M. An equivalent amount of 10 mM HCl was added to control incubations. At the end of the incubation the flask was sealed with a rubber stopper equipped with a centerwell and 0.4 ml of 30% $HClO_4$ was injected into the flask. This terminated the incubation and released the CO_2 from solution which was collected as described.

Flux through glutaminase

This was measured by determining the liberation of $^{14}CO_2$ from 1-^{14}C-glutamine. Hepatocytes were incubated as described above except 1-^{14}C-glutamine (1 mM) replaced glycine and ammonium chloride was present where indicated.

Before this technique could be used it had to be validated. Two potential problems had to be addressed. First, flux through glutaminase will produce 1-^{14}C-glutamate. $^{14}CO_2$ release occurs in the Krebs cycle after glutamate is converted to α-ketoglutarate. Thus, there are two steps between glutaminase and the actual release of $^{14}CO_2$ and isotope dilution is possible. Second, a suspension of isolated hepatocytes contains both periportal and perivenous hepatocytes, and therefore, both glutaminase and glutamine synthetase are present. As a result there is a potential for some 1-^{14}C-glutamate to be converted back to glutamine without release of $^{14}CO_2$. To test the validity of using $^{14}CO_2$ release from 1-^{14}C-glutamine to determine glutaminase flux, we compared it with glutamine disappearance, determined chemically. Methionine sulfoximine was used to inhibit glutamine synthetase (**Table 2.5**). In these experiments, hepatocytes (about 35 mg dry weight/ml) were incubated in a final volume of 3 ml Krebs-Henseleit medium containing NH_4Cl (2 mM). Glucagon (10^{-7} M) and methionine sulfoximine (1 mM) were present where indicated. The reaction was started by

Table 2.5. Flux through glutaminase in rat hepatocytes determined by measuring $^{14}CO_2$ release from 1-^{14}C-glutamine and by the chemical disappearance of glutamine.[1] Values are Mean ± SEM with the number of experiments indicated in parentheses. Rates are nmoles/mg dry wt./min. A negative sign indicates a net formation of glutamine. Details are given in the text. Methionine sulfoximine (MSO; 1 mM) and glucagon (10^{-7} M) were present where indicated.

Additions	Glutamine disappearance	$^{14}CO_2$ release	Glutamate formation	Glutamine disappearnce / $^{14}CO_2$ release
none	-0.062 ± 0.09 (5)	0.19 ± 0.01 (5)	0.11 ± 0.01 (5)	-
MSO	0.18 ± 0.04 (5)	0.20 ± 0.01 (5)	0.15 ± 0.02 (5)	0.89
Glucagon	0.12 ± 0.05 (4)	0.35 ± 0.02 (4)	0.047 ± 0.01 (4)	0.34
Glucagon + MSO	0.25 ± 0.03 (3)	0.32 ± 0.01 (4)	0.068 ± 0.01 (4)	0.79

[1] Experiment was in collaboration with Stephen A. Squires and the data are also reported in his M.Sc. thesis.

addition of 1-^{14}C-glutamine (1 mM) and immediately 1 ml of hepatocyte solution was removed and acidified (zero time). The remaining 2 ml were incubated for 30 minutes. At this time a second 1 ml aliquot was removed from the flask and acidified. The remaining 1 ml in the flask was acidified and $^{14}CO_2$ was collected in 0.4 ml NCS tissue solubiliser as previously described. The zero time and 30 minute samples were neutralised and glutamine concentration was determined using glutaminase and glutamate dehydrogenase according to Lund (1974).

It is clear from our findings that measuring flux through glutaminase in hepatocytes, by determining glutamine disappearance, is confounded by the presence of glutamine synthetase in the incubations (**Table 2.5**). Only when glutaminase is stimulated by glucagon, or when glutamine synthetase is inhibited by methionine sulfoximine, is a net degradation of glutamine evident. Flux through glutaminase determined using release of $^{14}CO_2$ from 1-^{14}C-glutamine was higher than that determined chemically and it was independent of added methionine sulfoximine, suggesting that 1-^{14}C-glutamate is not converted back to glutamine, but enters the Krebs cycle. Indeed, $^{14}CO_2$ release matches very well the disappearance of glutamine determined chemically in incubations containing methionine sulfoximine. Thus, the method of collecting $^{14}CO_2$ from 1-^{14}C-glutamine is acceptable

for determining flux through glutaminase and methionine sulfoximine is not necessary for the assay. This is in agreement with experiments of Vincent et al. (1989).

Preparation of 1-^{14}C-glutamine

To prepare 1-^{14}C-glutamine from 1-^{14}C-glutamate, a 15 000 g supernatant from a rat liver homogenate (1 part tissue plus 3 parts 0.9% NaCl) was used as a crude preparation of glutamine synthetase. The incubation mixture was as described by Baverel and Lund (1979) and consisted of: NH_4Cl (15.6 mM); $MgCl_2$ (15.6 mM); ATP (7.8 mM); L-1-^{14}C-glutamate (5.6 mM); phosphocreatine (7.8 mM); creatine kinase (10 units/ml); enzyme preparation (0.2 ml/ml); Tris-HCl buffer (78 mM); pH 7.4. The mixture was incubated for 3 hours at 37^{o}C. The incubation was stopped by addition of 0.1 ml perchloric acid (10%). The pH of the supernatant of the deproteinised sample was adjusted to 7.0 using 50% (w/v) K_2CO_3.

Glutamine was separated from any 1-^{14}C-glutamate by column chromatography using Sephadex QAE (Häussinger et al., 1983). Glutamate is retained by the column, while glutamine passes through. Fractions containing radioactivity were pooled and lyophilised. Thin layer chromatography was then used to confirm the presence of 1-^{14}C glutamine. In all instances, more than 98% of the radioactivity in the spot applied to the plate was recovered by scraping the glutamine

spot.

Measurement of flux through the glycine cleavage system in perfused liver

Single pass perfusion of livers was carried out for 30 minutes as described by Sies (1978). The perfusate was Krebs-Henseleit medium containing lactate (2.1 mM), pyruvate (0.3 mM), and glycine (0.3 mM; 1-^{14}C-glycine 20 dpm/nmol). Glycine decarboxylation rate was determined by measuring the $^{14}CO_2$ content of the perfusate leaving the liver. Perfusate was collected every 5 min. for 30 s under mineral oil for this purpose. The perfusate was also analysed for CO_2, O_2, and pH using a Ciba Corning 238 pH/blood gas analyser. Following the perfusion the entire liver was removed and dried to constant weight in an oven set to 50°C.

Perfusate samples were removed from below the oil using a 21G 1.5" needle. 5 ml was injected into a sealed 25 ml flask containing 0.4 ml HCl (1 N). $^{14}CO_2$ was collected in a centerwell containing 0.4 ml NCS tissue solubiliser as previously described. Duplicate or triplicate determinations were done for each sample.

In some experiments maintenance of liver function during the perfusion was assessed by measuring the levels of tissue adenine nucleotides. The tissue specific activity of 1-^{14}C-glycine was also determined at this time. In these

experiments approximately 2 g of liver tissue was removed from a lobe at 10 min. and immediately freeze-clamped using aluminum tongs cooled in liquid nitrogen. The rest of the lobe was immediately ligated with surgical thread. This proved effective in preventing leakage of perfusate during the balance of the perfusion. At 30 min. a second 2 g sample of tissue was freeze-clamped and the perfusion was terminated.

Measurement of adenine nucleotides and glycine specific activity

The frozen liver was powdered, extracted in perchloric acid, centrifuged, and neutralized as described on page 70. Adenine nucleotides were determined as decribed by Hems and Brosnan (1970). For determination of the specific radioactivity of glycine it was necessary both to measure the chemical content of glycine and the radioactivity associated with it. Chemical glycine was determined on a Beckman model 121-M amino acid analyser (Lee, 1974). A parallel run on the amino acid analyser was performed with a second aliquot of sample, but without ninhydrin, and 1 ml fractions were collected. Radioactivity associated with the glycine peak was pooled and counted.

Chemicals

Diet components were obtained from ICN (Cleveland, Ohio)

except for the cornstarch and L-methionine which were purchased from Sigma Chemical Co., Ltd. (St. Louis, Mo.) and the corn oil (Mazola) which was purchased from Best Foods Canada, Inc. (Etobicoke, Ont.). 1-^{14}C-glycine, 2-^{14}C-glycine, 1-^{14}C-glutamate, $^{3}H_2O$, U-^{14}C-sucrose and Omnifluor were obtained from Dupont-New England Nuclear (Mississauga, Ont.). The toluene and Scintiverse used were from Fisher Scientific (Nepean, Ont.). Enzymes, glucagon, bromododecane, dodecane, and bovine serum albumin (prepared from fraction V; essentially fatty acid-free) were from Sigma Chemical Co., Ltd. (St. Louis, Mo.). Silicone oil was from Dow Corning (William F. Nye, Inc., New Bedford, Ma. USA). QAE Sephadex (A-25) was obtained from Pharmacia (Canada) Inc., Baie d'Urfe, P.Q. Collagenase A was from Boehringer-Mannheim Canada (Laval, P.Q.). Heparin (sodium injection USP) was purchased from Allen and Hanburys (Glaxo Canada, Ltd., Montréal, P.Q.). Other reagents were of analytical grade.

Treatment of Data

Experimental results were reported as means ± standard error. In **Chapter 3** data were compared using the Wilcoxon-Mann-Whitney test with the exception of those presented in **Table 3.1** which were analysed using a 3-factor analysis of variance with comparisons made using the Tukey multiple comparisons test. In this case the flux through the glycine cleavage system was compared in the various incubation media

using liver mitochondria from rats fed the high- or normal-protein diet for 2 or 6 days. In the other chapters comparisons were made using a Student t-test. In all situations a probability, $p < 0.05$, was regarded as indicating statistical significance.

The data reported for **Fig. 4.3** and **4.4** were plotted using the computer programme Graphpad (GraphPAD Software, San Diego, California, USA) where the curve of best fit (sigmoidal curve in **Fig. 4.3**, rectangular hyperbola in **Fig. 4.4**) is arrived at through an iterative process employing an algorithm which determines the parameters that minimize the sum of squares of differences between the dependent variable in the equations and the observations. For **Fig. 4.3**, the data were transformed to a linear plot Log ($v_o/V_{max} - v_o$) versus Log [S] (Hill equation), in order to estimate the K_m and V_{max} values for the data. For **Fig. 4.4**, the k_a and V_{max} values were estimated using a Lineweaver-Burk plot of the data. In each case, the analysis was performed for each individual experiment within a data set.

Chapter 3

Glycine catabolism in mitochondria from rats fed high-protein diets and meals

Synopsis

Glycine catabolism was studied in intact rat liver mitochondria by measuring the release of $^{14}CO_2$ from 1-^{14}C-glycine. Mitochondria from rats fed a high-protein diet for 6 days showed an enhanced ability to catabolise glycine compared to those from rats fed a normal-protein diet. Glycine catabolism was also stimulated in normal-protein-fed rats if they ingested a single high-protein meal for two hours prior to sacrifice, thus illustrating the rapid response of the glycine cleavage system to protein intake. The stimulation of glycine catabolism in rats fed a high-protein diet or meal was not evident if the mitochondria were incubated in the absence of inorganic phosphate (omitting ADP had no effect on the rate). Mitochondria from high-protein- and normal-protein-fed rats did not differ in their ability to accumulate glycine, a process which occurred far too rapidly to impose a limit on the rate of flux through the glycine cleavage system. Glycine cleavage system flux is sensitive to sub-micromolar concentrations of free calcium. In the presence of optimal free calcium (0.25 μM), glycine cleavage system flux was high, but similar in mitochondria from rats fed the different diets. The stimulation of glycine catabolism by high protein-feeding was not associated with a change in mitochondrial matrix volume. Furthermore, mitochondria from rats fed a high-protein meal maintained an

enhanced ability to catabolise glycine compared to those from rats fed a normal-protein meal when incubated in hypotonic solutions of very low osmolarity.

Introduction

The changes in glycine levels in blood and liver tissue in animals fed a high-protein diet suggest that activation of hepatic glycine catabolism occurs at this time (**Chapter 2**). However few studies have assessed glycine catabolism in the livers of such animals. Matsuda *et al*. (1973) reported that glycine decarboxylation was enhanced in liver slices from chickens and rats fed on high-protein diets, while Petzke *et al*. (1986) reported that hepatocytes from rats fed high-protein diets had increased glycine cleavage activity. Both of these studies involved adapting animals to the high-protein diets, and did not examine possible short-term effects that may occur following an individual meal. Thus the physiological regulation of hepatic glycine metabolism is not well understood.

As discussed in detail in **Chapter 1**, the glycine cleavage system can be inhibited by branched-chain a-keto acids (O'Brien, 1978) or by conditions which lead to a more reduced state of the pyridine nucleotide redox couples (Hampson *et al*., 1983). However a high-protein diet or meal will raise hepatic levels of branched-chain amino acids

(Fafournoux et al., 1990) and also leads to a lower mitochondrial $NAD^+/NADH$ ratio in liver (Peret et al, 1981), and thus, glycine catabolism would be expected to be inhibited. That this does not occur suggests that these mechanisms are physiologically ineffective.

Prior to the work described in this thesis, it was discovered that glycine catabolism in isolated hepatoyctes is stimulated by glucagon (Jois et al., 1989). The stimulatory effect of glucagon on glycine catabolism is also evident in intact, fully functioning, mitochondria isolated from rats that had been injected with the hormone 25 minutes prior to sacrifice. Since ingestion of a high-protein meal leads to an increase in the concentration of glucagon in the circulation (Robinson et al., 1981), it is possible that this hormone may be an important signal for the hepatic removal of glycine at this time. Therefore, it was hypothesised that the hepatic glycine cleavage system would be activated in animals fed a high protein diet or meal and that this would be evident in mitochondria isolated from these animals.

Objectives

The main objectives of this present study were (1) to determine flux through the glycine cleavage system in intact mitochondria from rats fed on high- or normal-protein diets and (2) to investigate the possible short-term effect of high-protein-feeding by determining whether the glycine

cleavage system is stimulated immediately after a high-protein meal, and if so, whether this is a function of the previous dietary history of the animal.

Protocols

In these studies rats were kept under the normal light cycle. Rats were fed the 15% or 60% casein diet for 2 to 6 days depending on the experiment. Rats were usually sacrificed between 8 and 9 am. The procedure for investigating the effects of an individual meal was described in **Chapter 2**. The times of sacrifice in these experiments were 8pm (controls), 10pm (2 hr meal), and 12 midnight (4 hr meal).

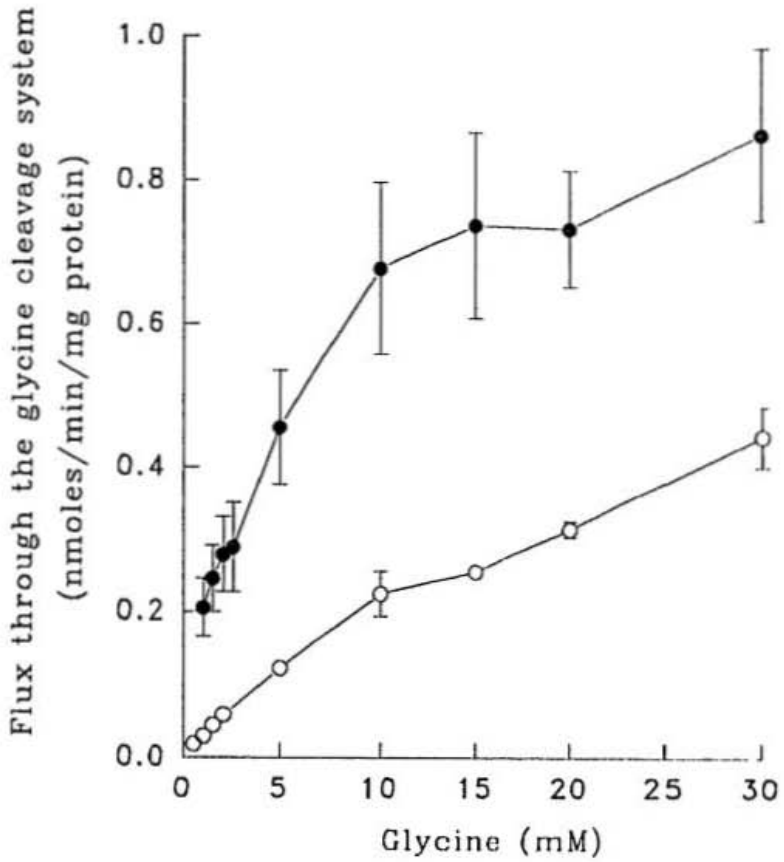
Results

Stimulation of glycine catabolism by a high protein diet or meal

Flux through the glycine cleavage system was stimulated in intact mitochondria from rats fed a high-protein diet for six days compared to rates in mitochondria from rats fed a normal-protein diet (**Fig. 3.1**). The stimulation was especially pronounced at the lower, physiological concentrations of glycine where it was about six-fold compared to two-fold at very high concentrations.

The stimulation of flux through the glycine cleavage

Fig. 3.1. Glycine decarboxylation in mitochondria from high protein- and normal protein-fed rats. Mitochondria were isolated at 9am and incubated in the KCl-based medium described in the Materials and Methods. Results are means ± S.E.M. for four separate experiments. Glycine decarboxylation in mitochondria from high protein-fed rats (●) was significantly higher than that in mitochondria from normal protein-fed rats (O) at each of the glycine concentrations tested (Wilcoxon-Mann-Whitney test, $p<0.05$).


Flux through the glycine cleavage system
(nmoles/min/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
Glycine (mM)

system did not gradually develop over the six day period of feeding the high-protein diet. This is evident from the results presented in **Table 3.1** which show that rats fed for just two days on the high-protein diet exhibited a stimulated rate of glycine catabolism, which was similar to that obtained in mitochondria from rats fed for six days on the diet, provided inorganic phosphate was present in the medium. This was shown using the standard KCl-based medium which contained phosphate, as well as in parallel incubations using the isolation medium. The effect of added phosphate on the glycine cleavage system flux and the use of the isolation medium as a simplified incubation medium are discussed in the next section.

This finding lead to an investigation of the effect of feeding the high-protein diet to rats for a single day (**Table 3.2**). In this experiment rats were fed for five days on the high-protein or normal-protein diet, but were then switched to the alternate diet for one day. What was remarkable was that this single day of feeding on the high-protein diet was sufficient to stimulate the glycine cleavage system. The glycine cleavage system rate that resulted was indistinguishable from that observed following six days of feeding on the high-protein diet. These data also showed that a single day of feeding on the normal-protein diet resulted in low rates of glycine catabolism in rats otherwise fed the high-protein diet. Thus, the glycine cleavage system responds

Table 3.1. Flux through the glycine cleavage system in mitochondria isolated from high-protein and normal-protein-fed rats. Mitochondria were incubated in the KCl-based medium as outlined in the Methods. Parallel incubations were carried out in the isolation medium and the isolation medium + 2.5 mM potassium phosphate. ADP (1 mM) was present where indicated. The results are the mean ± S.E.M. of four separate experiments. *significantly different from rates in mitochondria isolated from normal-protein-fed rats, incubated in the same medium (Tukey multiple comparisons test, $p < 0.05$).

Diet	Days on diet		Rate (nmoles/min.mg mitochondrial protein)		
			KCl medium	Isolation medium	Isolation medium with K_2HPO_4
HP	6	ADP	0.47 ± 0.05*	0.10 ± 0.02	0.62 ± 0.17*
		-	0.57 ± 0.05*	0.11 ± 0.02	0.46 ± 0.08*
	2	ADP	0.53 ± 0.11*	0.11 ± 0.02	0.58 ± 0.10*
		-	0.63 ± 0.15*	0.13 ± 0.02	0.46 ± 0.06*
NP	6	ADP	0.09 ± 0.003	0.07 ± 0.01	0.11 ± 0.01
		-	0.07 ± 0.01	0.07 ± 0.01	0.09 ± 0.01
	2	ADP	0.15 ± 0.02	0.09 ± .01	0.19 ± 0.03
		-	0.13 ± 0.03	0.07 ± 0.01	0.14 ± 0.02

Table 3.2. Flux through the glycine cleavage system in mitochondria from rats f-i for a single day on the high-protein and normal-protein diets. Rats were given the alternate diet at 2:30 pm the day before sacrifice (dark 8pm-8am). Mitochondria were incubated in the KCl-based medium as outlined in the Methods. Other incubations were carried out in the isolation medium and the isolation medium + 2.5 mM potassium phosphate. ADP (1 mM) was present where indicated. The results are the mean ± S.E.M. of four separate experiments. *significantly different from rates in mitochondria isolated from rats fed the high-protein diet one day, incubated in the same medium (Wilcoxon-Mann-Whitney test, $p < 0.05$).

Diet (5 days)	Diet (1 day)		Rate (nmoles/min.mg mitochondrial protein) KCl medium	Isolation medium	Isolation medium with K_2HPO_4
HP	NP	ADP	0.09 ± 0.03*	0.07 ± 0.01	0.11 ± 0.03*
		-	0.09 ± 0.03*	0.07 ± 0.02	0.09 ± 0.03*
NP	HP	ADP	0.41 ± 0.1	0.14 ± 0.02	0.46 ± 0.03
		-	0.61 ± 0.07	0.13 ± 0.02	0.53 ± 0.04

rapidly to dietary input. The question was how rapidly. Could the glycine cleavage system be activated following a single meal?

To answer this question a protocol was set up to assess glycine metabolism following a single, 2 or 4 hour, high-protein meal. In liver mitochondria from rats previously fed the normal-protein diet, ingestion of a high-protein meal resulted in a dramatic increase in flux through the glycine cleavage system (**Table 3.3**). Following the two hour high-protein meal, the flux through the glycine cleavage system in these rats was 0.67 ± 0.2 nmoles/min/mg protein compared to a value of 0.08 ± 0.01 nmoles/min.mg protein in mitochondria from rats not given a meal (first column of data in **Table 3.3**). The four hour high-protein meal resulted in an even greater stimulation (1.02 ± 0.27 nmoles/min.mg protein). Ingestion of a normal-protein meal did not significantly alter flux through the glycine cleavage system. However, in rats fed the high-protein diet, ingestion of a normal-protein meal lead to a decrease in glycine catabolism to low basal rate (0.10 ± 0.01 and 0.08 ± 0.02 nmoles/min.mg protein at 2 and 4 hours respectively). Thus the flux through the glycine cleavage system in liver mitochondria is rapidly and dramatically modulated by the protein content of a single meal.

Table 3.3. Flux through the glycine cleavage system in mitochondria from rats fed high-protein and normal-protein meals. Rats fed the high-protein or normal-protein diet for 2-3 days were deprived of food for six hours and then given a high-protein or normal-protein meal as specified. Meals began at the start of the dark period and rats were killed 2 or 4 hours later. Controls, those not receiving a meal, were killed at the start of the feeding period. Mitochondria were incubated in the KCl-based medium as outlined in the Methods. Other incubations were carried out in the isolation medium and the isolation medium + 2.5 mM potassium phosphate. ADP (1 mM) was present in each incubation. The results are the mean ± S.E.M. with the number of experiments in parentheses. * significantly different from the control; † significantly different from those that received a meal that matched the diet they had been accustomed to (Wilcoxon-Mann-Whitney test, $p < 0.05$).

Diet	Meal	Rate (nmoles/min.mg mitochondrial protein) KCL medium	KCl medium no K_2HPO_4	Mannitol medium	Mannitol medium with K_2HPO_4
HP	---	0.22 ± 0.05 (5)	---	0.12 ± 0.03 (5)	0.37 ± 0.06 (4)
HP	HP; 2 hr	0.38 ± 0.06 (3)	0.09 ± 0.005 (3)	0.16 ± 0.04 (3)	0.42 ± 0.07 (3)
HP	NP; 2 hr	0.10 ± 0.009*† (4)	0.09 ± 0.002 (3)	0.11 ± 0.01 (4)	0.17 ± 0.02*† (4)
HP	HP; 4 hr	0.43 ± 0.05 (3)	0.09 ± 0.01 (3)	0.16 ± 0.009 (3)	0.52 ± 0.07 (3)
HP	NP; 4 hr	0.08 ± 0.02*† (5)	---	0.10 ± 0.01 (5)	0.21 ± 0.02 (5)
NP	---	0.08 ± 0.01 (5)	---	0.11 ± 0.005 (5)	0.20 ± 0.02 (5)
NP	NP; 2 hr	0.13 ± 0.03* (3)	0.10 ± 0.01 (3)	0.14 ± 0.05 (3)	0.21 ± 0.07 (3)
NP	HP; 2 hr	0.67 ± 0.2*† (4)	0.13 ± 0.05 (3)	0.22 ± 0.05 (4)	0.90 ± 0.2** (4)
NP	NP; 4 hr	0.14 ± 0.02 (3)	0.09 ± C.008 (3)	0.09 ± 002 (3)	0.19 ± 0.03 (3)
NP	HP; 4 hr	1.02 ± 0.3** (5)	---	0.35 ± 0.1** (5)	1.9 ± 0.4** (5)

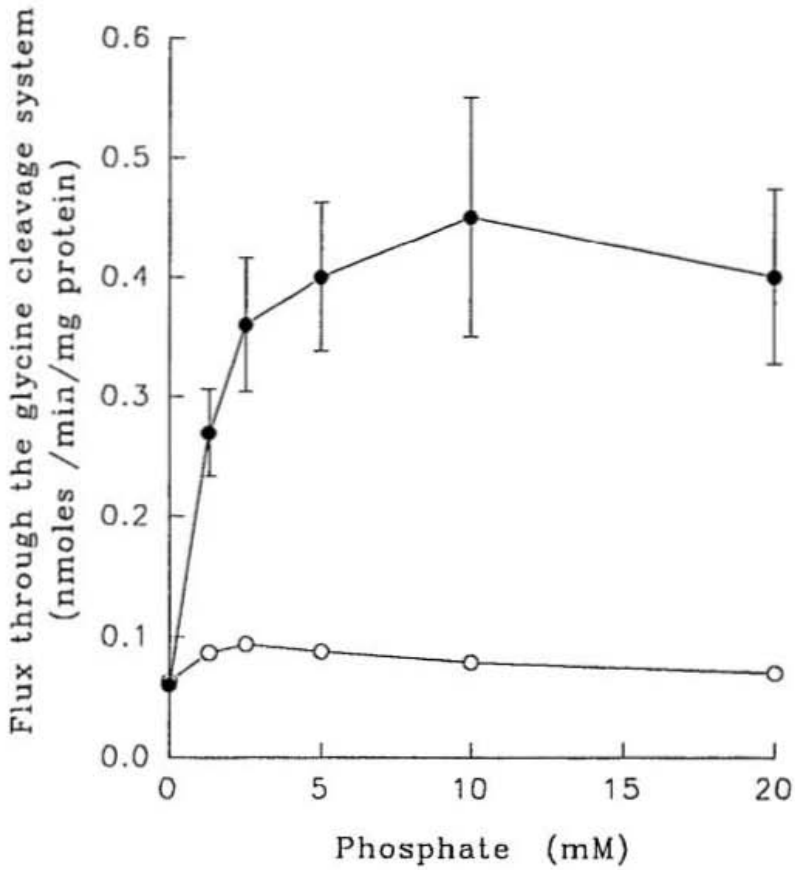
Dependence on inorganic phosphate

Previous work in our laboratory on the stimulation of the glycine cleavage system by glucagon-injection had revealed that the effect was dependent on the presence of inorganic phosphate in the incubation medium (Jois et al., 1992). Inorganic phosphate was also required for the stimulation of the glycine cleavage system brought about by high-protein feeding. This was determined using two different media. First, the effect was observed when phosphate was omitted from the KCl-based medium (**Table 3.3** and **Fig. 3.2**). As shown in **Fig. 3.2** the stimulation of glycine cleavage system by high-protein feeding was maximal when phosphate was about 2.5 mM. Phosphate had little effect on the rate obtained in mitochondria from rats fed normal-protein meals, even when present at 20 mM. Second, the effect could be demonstrated when mitochondria were incubated with the isolation medium (**Table 3.1 - 3.3**). The isolation medium was used as a simplified medium in order to help rule out the possibility that phosphate was interacting with other components of the medium. In this medium, as in the KCl-based medium, only low basal rates of glycine catabolism were apparent when phosphate was absent.

Stimulation of serine production from glycine

The glycine cleavage system releases the number 1 carbon

Fig. 3.2. Flux through the glycine cleavage system in rat liver mitochondria as a function of phosphate concentration. Rats were fed on a normal-protein diet for 4 days before being given a high-protein (●) or normal-protein (O) meal for two hours. Results are the mean ± S.E.M. of three separate experiments (high-protein meal) or the average of two experiments (normal-protein meal).


Flux through the glycine cleavage system
(nmoles /min/mg protein)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
Phosphate (mM)

of glycine as CO_2 and transfers the number 2 carbon to tetrahydrofolate to form methylene-tetrahydrofolate (see **Fig. 1.2**). The methylene carbon of methylene-tetrahydrofolate may either be oxidised (Hampson et al., 1983) or be transferred to a second glycine molecule to form serine via serine hydroxymethyltransferase. We therefore studied the fate of the second carbon of glycine by measuring the conversion of 2-^{14}C-glycine to $^{14}CO_2$ as well as measuring the quantity of serine produced (**Table 3.4**). In mitochondria from rats fed the high-protein diet serine is, quantitatively, the more important product, even under state 3 conditions where methylene-tetrahydrofolate oxidation is greatly enhanced . The rate of oxidation of methylene-tetrahydrofolate, as monitored by the release of $^{14}CO_2$ from 2-^{14}C-glycine, is somewhat increased by high-protein-feeding while serine production is greatly increased.

Effects of calcium

The glycine cleavage system is stimulated, in isolated mitochondria, in the presence of submicromolar concentrations of free calcium (Brosnan et al., 1990; Jois et al.,1990b). The effect of this ion on stimulation of flux through the glycine cleavage system in mitochondria from high-protein-fed rats therefore was investigated. Under the normal incubation conditions EGTA (1 mM) is present, and

Table 3.4. Serine production and the release of the one and two carbons of glycine as CO_2 by mitochondria from rats fed for 2 days on high-protein and normal-protein diets. Mitochondria were incubated (± 1 mM ADP) for 15 minutes in the KCl-based medium in the presence of 5 mM glycine labelled in the carbon-1 or carbon-2 position (300-500 dpm/nmol). The difference in serine levels in the incubation medium at t=0 min. and t=15 min. divided by the incubation time is serine production. Results are means ± S.E.M. for 4 separate experiments.

		nmoles/min.mg mitochondrial protein		
Diet		$^{14}CO_2$ from 1-^{14}C-glycine	$^{14}CO_2$ from 2-^{14}C-glycine	Serine production
High-protein	(-) ADP	0.49 ± 0.04	0.028 ± 0.004	0.56 ± 0.1
	(+) ADP	0.40 ± 0.06	0.14 ± 0.02	0.24 ± 0.08
Normal-protein	(-) ADP	0.057 ± 0.008	0.013 ± 0.003	0.071 ± 0.04
	(+) ADP	0.081 ± 0.01	0.092 ± 0.009	0.063 ± 0.02

thus, free calcium is negligible. In the present set of experiments, the desired free calcium concentrations were achieved by adding $CaCl_2$ (see **Chapter 2**). Calcium had no effect on glycine decarboxylation below about 0.1 µM (**Fig. 3.3a**), but a remarkable stimulation occurred over the range of 0.1 to 0.25 µM free calcium. When maximally stimulated (0.25 µM calcium), there was no longer any statistical difference between the flux through the glycine cleavage system in mitochondria from normal protein- and high protein-fed rats. Maximal flux was maintained at higher calcium concentrations provided ADP was present in the medium. Under state 4 conditions (no ADP), the flux was reduced above 0.3 µM calcium (**Fig. 3.3b**). Omission of ADP however, did not affect the degree of stimulation by free calcium below about 0.25µM. In a second set of experiments, free calcium was present at 0.25 µM, while glycine concentration was varied between 1 and 40 mM (**Fig. 3.4**). Under these conditions, where flux was maximally stimula'ed by calcium, there was no difference in the rate of glycine catabolism in mitochondria from high and normal protein-fed rats at any of the glycine concentrations tested.

Movement of glycine into mitochondria

The delivery of glycine to the glycine cleavage system is a potential site for metabolic regulation. Glycine transport was examined in intact mitochondria isolated from

Fig. 3.3. The sensitivity of glycine cleavage system flux to free calcium in the medium. Mitochondria were isolated from rats fed the high-protein (●) or normal-protein (O) diet for 2 days. Mitochondria were incubated in the KCl-based medium (5 mM glycine; 2.5 mM phosphate; 1 mM ADP) supplemented with $CaCl_2$:EGTA solutions of various ratios (see Materials and Methods). ADP was omitted from the incubations in b. Results are means ± S.E.M. for three separate experiments. One standard error bar has been omitted for clarity.

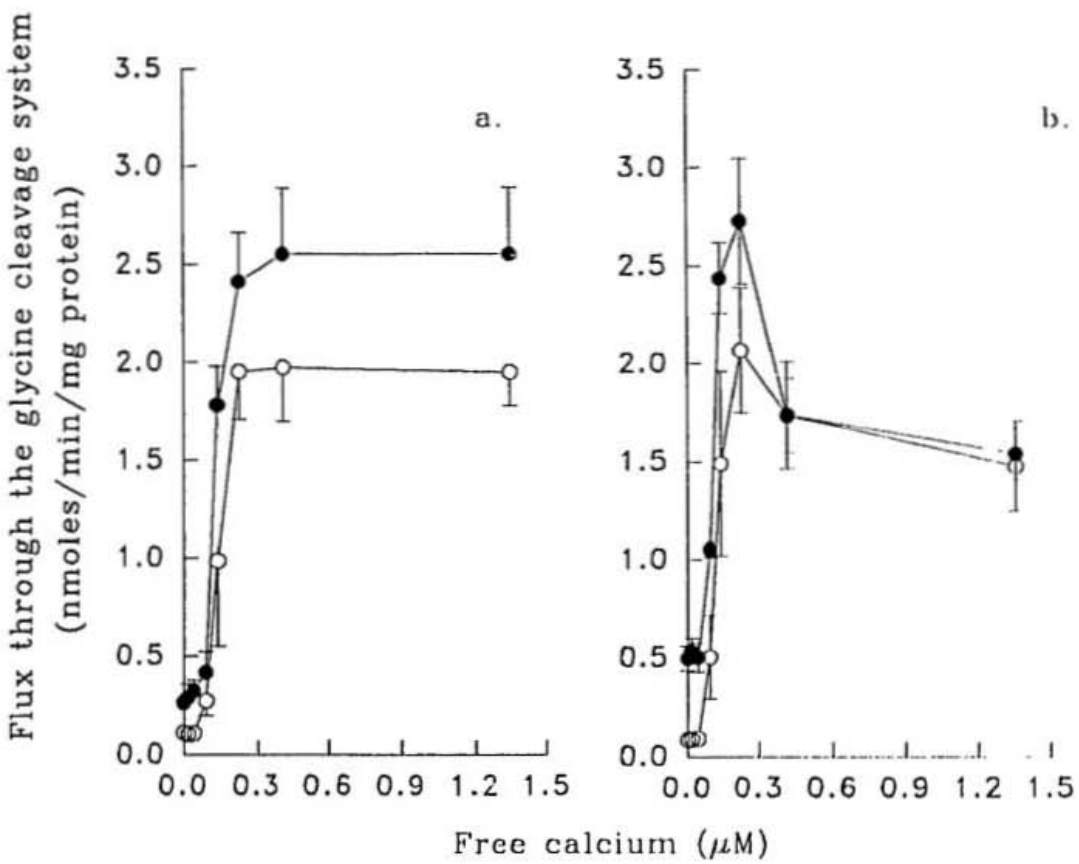
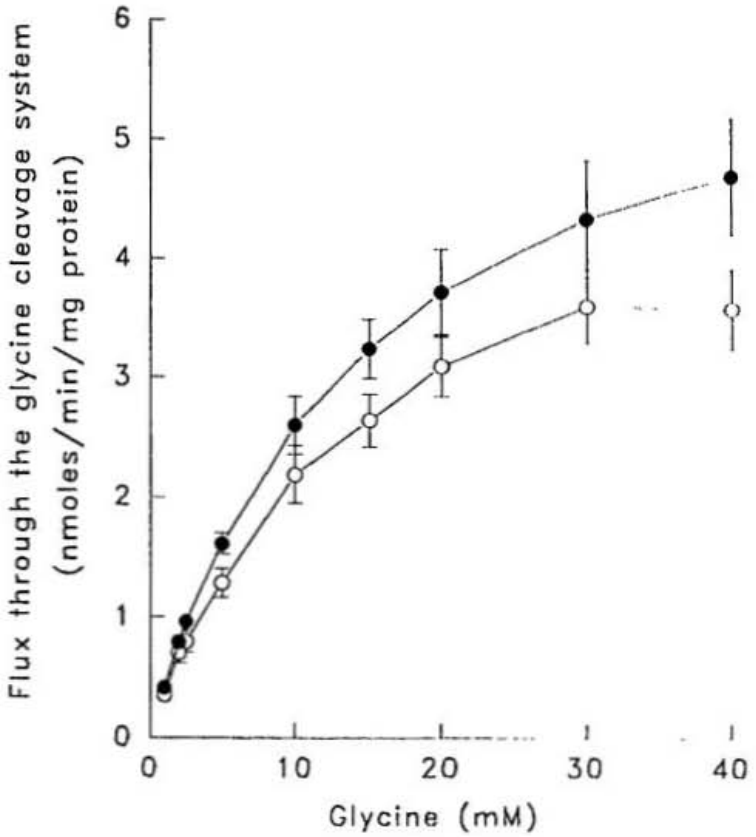

Flux through the glycine cleavage system
(nmoles/min/mg protein)
a.
b.
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0 0.3 0.6 0.9 1.2 1.5
Free calcium (μM)

Figure 3.4. Glycine decarboxylation in mitochondria from high protein- and normal protein-fed rats versus glycine concentration in the presence of 0.25 μM free calcium. Mitochondria were isolated from rats fed the high-protein (●) or normal-protein (O) diet for two days. Mitochondria were incubated in the KCl-based medium (5 mM glycine; 2.5 mM phosphate; 1 mM ADP) supplemented with $CaCl_2$ as described in the Materials and Methods. Results are means ± S.E.M. for four separate experiments. Glycine cleavage system flux in mitochondria from high protein-fed and normal protein-fed rats was not significantly different at any of the concentrations of glycine used (Wilcoxon-Mann-Whitney test, $p>0.05$).


Flux through the glycine cleavage system
(nmoles/min/mg protein)
0
1
2
3
4
5
6
0
10
20
30
40
Glycine (mM)

rats fed for 2 days on the diets (**Table 3.5**). Mitochondria from high protein- and normal protein-fed rats did not differ in their ability to accumulate glycine. Glycine accumulation was not inhibited by rotenone suggesting that movement was independent of respiration. Since the net accumulation of glycine was the same at 15 and 45 seconds, it is clear that transport was too rapid to be measured by these methods and that a rate based even on the 15 second data is an underestimate. This means that the initial rate of glycine transport was in excess of 32 nmoles/min.mg mitochondrial protein (8 nmoles/mg in 15 seconds multiplied by 4). Thus glycine delivery to the mitochondria is far too rapid to impose a limit even on the maximum rate of glycine decarboxylation, which we find to be about 2.5 nmoles/min.mg in the presence of 0.25 μM free calcium (**Fig. 3.4**).

Incubations in hypotonic media and measurement of mitochondrial matrix volume

A number of hormone actions on mitochondrial function can be mimicked by incubation in hypotonic media (Halestrap, 1989). Glycine cleavage system flux in intact mitochondria has recently been shown to be stimulated by hypotonicity (Brosnan et al., 1990; Halestrap et al., 1990). Hypotonic incubation results in a net movement of water into the mitochondrial matrix, and thus, matrix volume is increased. Halestrap has argued that an important part of the action of

Table 3.5. Glycine accumulation in intact mitochondria. Mitochondria from rats fed the high-protein or normal-protein diet for 2 days were preincubated for 6 minutes in the KCl-based medium with or without 13 μM rotenone. Glycine ($1-^{14}C$-glycine, 400 dpm/nmol) was added at a final concentration of 5 mM and the mitochondria were rapidly sedimented 15 or 45 seconds later. Accumulation of glycine was assessed by determining the radioactivity in the pellet with corrections for extra-matrix glycine as described in the Materials and Methods. Results are means ± S.E.M. for 6 separate experiments except for the 45 second determinations which are means of two experiments.

Diet	rotenone	Glycine accumulation (nmol/mg)	Matrix volume (µl/mg)	Matrix Glycine (mM)
High protein				
(15 s)	(-)	8.55 ± 1.14 (6)	2.73 ± 0.30 (5)	3.26 ± 0.44 (5)
	(+)	8.50 ± 0.90 (6)	2.34 ± 0.30 (5)	4.58 ± 0.78 (5)
(45 s)	(-)	10.32 (2)	-	-
	(+)	5.80 (2)	-	-
Normal protein				
(15 s)	(-)	7.28 ± 0.98 (6)	2.29 ± 0.51 (5)	4.62 ± 0.68 (5)
	(+)	8.04 ± 1.06 (6)	2.79 ± 0.37 (5)	3.11 ± 0.37 (5)
(45 s)	(-)	8.83 (2)	-	-
	(+)	8.13 (2)	-	-

glucagon and other glucogenic hormones involves an increase in mitochondrial matrix volume (see Halestrap , 1989). Accordingly, it was of importance to determine how hypotonicity would affect glycine catabolism in mitochondria from animals fed a high-protein meal. **Fig. 3.5** shows the effect of hypotonic incubation on flux through the glycine cleavage system from rats fed high-protein and normal-protein meals. We studied mitochondria from rats given a high protein or a normal protein meal, as well as from rats given a glucagon injection. Clearly hypotonic incubation had a marked stimulatory effect on glycine cleavage system flux in these mitochondria. However the findings suggest that this stimulation was of a different nature than the physiological stimulation brought about by feeding the high-protein meal. A stimulation by hypotonicity, for example, occurred in the absence of inorganic phosphate, although the effect was greater in the presence of this anion. It should also be noted that, at all levels of hypotonicity tested, the stimulatory effect of the high-protein meal was still evident, suggesting that the stimulation by high-protein feeding occurred through a different mechanism.

We repeated the above experiments, but used mitochondria from glucagon-injected and saline-injected rats (**Fig. 3.6**). Hypotonicity caused a large stimulation of glycine cleavage system flux in both preparations, an effect which did not require inorganic phosphate. Moreover, the effect of glucagon

Figure 3.5. The effect of osmolarity of the incubation medium on the rate of flux through the glycine cleavage system in mitochondria from rats fed a high-protein meal. Rats fed the normal-protein diet for 3 days were given a high protein (●,○) or normal protein (▲,△) meal for 2 hours. Solutions with different osmolarities were constructed by varying the concentration of KCl. Incubations were conducted in the presence (shaded symbols) or absence (open symbols) of 2.5 mM K_2HPO_4. Results are the means ± S.E.M. of 3 or 4 separate experiments. The negative error bars have been omitted for clarity. * significantly different from rates in mitochondria from rats fed the normal protein-meal.

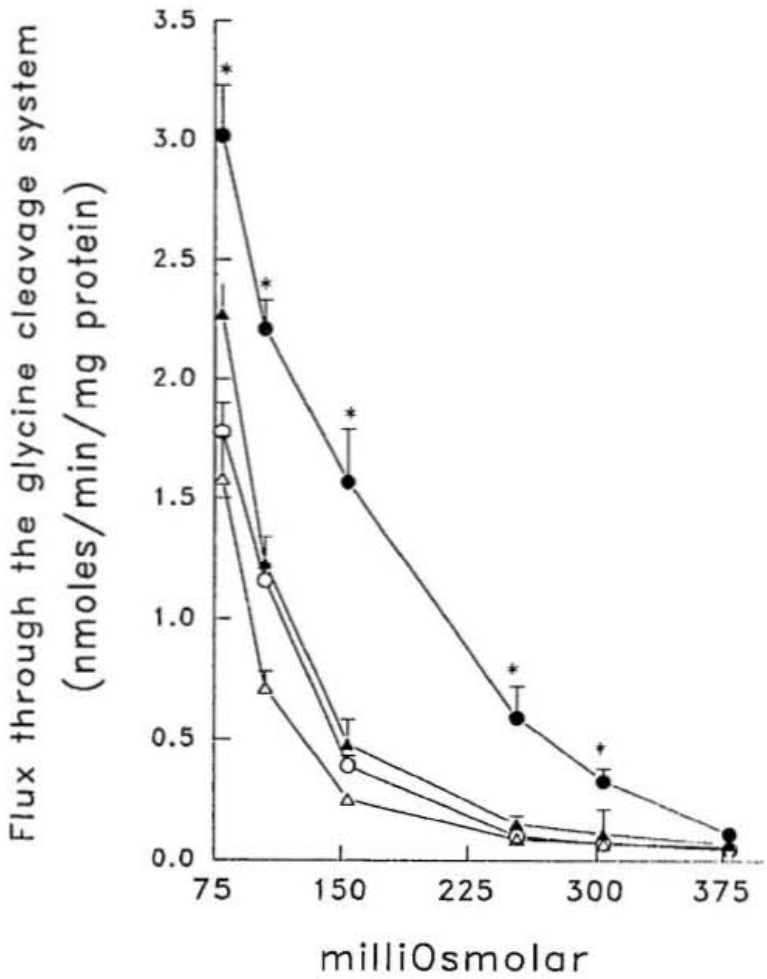
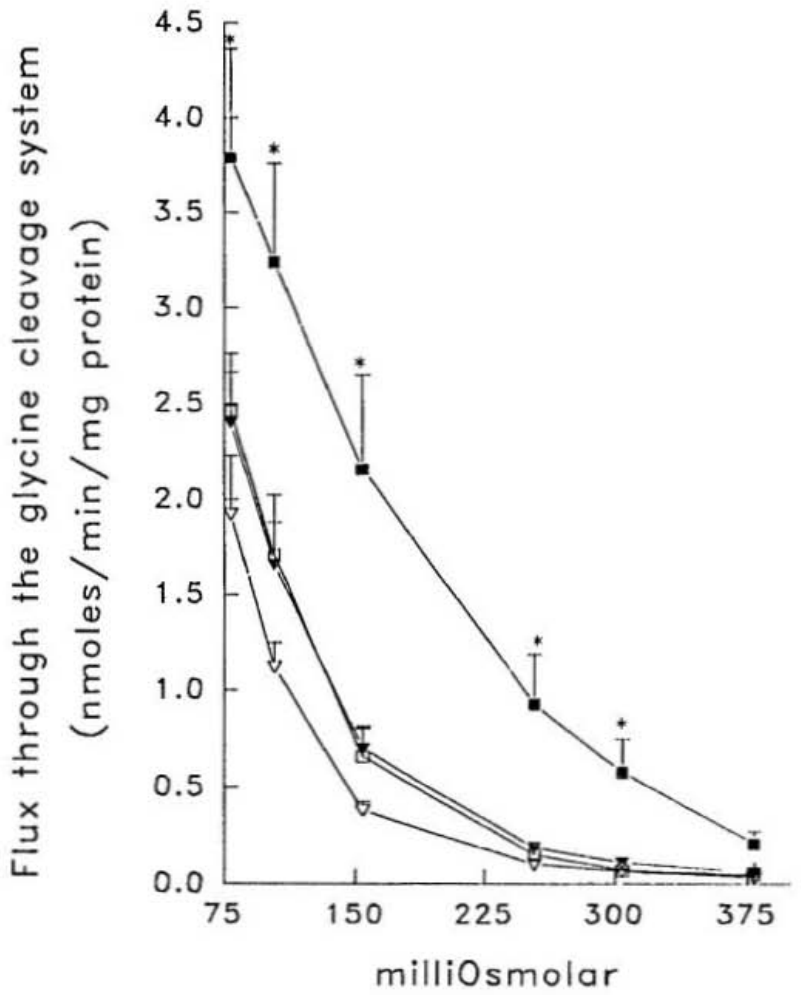

Flux through the glycine cleavage system
(nmoles/min/mg protein)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
75
150
225
300
375
milliOsmolar

Figure 3.6. The effect of osmolarity of the incubation medium on the rate of flux through the glycine cleavage system in mitochondria from rats injected with glucagon. Rats fed the standard chow diet were injected (ip) with 0.1 mg/100g bw. glucagon (■,□) or 0.9% saline/0.05% BSA (▼,▽). Solutions with different osmolarities were constructed by varying the concentration of KCl. Incubations were conducted in the presence (shaded symbols) or absence (open symbols) of 2.5 mM K_2HPO_4. Results are the means ± S.E.M. of 3 or 4 separate experiments. The negative error bars have been omitted for clarity. * significantly different from rates in mitochondria from rats injected with vehicle.


Flux through the glycine cleavage system
(nmoles/min/mg protein)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
75
150
225
300
375
milliOsmolar

was evident under hypotonic conditions. Thus the stimulation by glucagon-injection was similar to that produced by high-protein feeding, and appeared to be of a different nature than that produced by hypotonicity.

It was clearly important to determine the mitochondrial matrix volume in mitochondria from high protein- and normal protein-fed rats. **Table 3.6** shows that matrix volume was the same for mitochondria from rats given a high- or normal-protein meal. This was true even when phosphate was omitted from the incubations. Thus matrix volume remained unchanged despite large differences in the rate through the glycine cleavage system.

Discussion

The results of this investigation indicate that hepatic glycine catabolism is activated following a high protein meal. The effect elicited *in vivo* on the glycine cleavage system is both rapid and dramatic: the stimulation occurs within two hours from the start of a meal and involves a 4-to 6-fold enhancement of flux through the glycine cleavage system as measured in isolated mitochondria. In fact the protein content of the last meal is the single major determinant of the rate of glycine oxidation. Regardless of the rats' prior diet history, mitochondria from all rats fed the 15% casein meal oxidised glycine at a rate less than 0.15 nmoles/min.mg whereas the rate in mitochondria from animals

Table 3.6. Matrix volume and flux through the glycine cleavage system. Rats fed the normal protein diet for 3 days were given a high protein or normal protein meal for 2 hours. Matrix volume was determined by separating mitochondria from the incubation medium by centrifugal filtration through bromododecane oil as described in the Material and Methods. Results are the means ± S.E.M. of four separate experiments. K_2HPO_4 was present at a concentration of 2.5 mM where indicated.

Meal		Rate (nmoles/ min.mg protein)	Matrix volume (µl/ mg protein)
High protein	(−) K_2HPO_4	0.11 ± 0.02	1.23 ± 0.09
	(+) K_2HPO_4	0.45 ± 0.06	1.33 ± 0.09
Normal protein	(−) K_2HPO_4	0.09 ± 0.01	1.32 ± 0.07
	(+) K_2HPO_4	0.13 ± 0.02	1.29 ± 0.03

fed the 60% casein meal the rate was 0.38 or more (rates in KCL-based medium with phosphate).

The stimulation of flux through the glycine cleavage system by a high-protein diet or meal depended on the presence of inorganic phosphate in the incubation medium. The results reported here demonstrate that the requirement for phosphate was not attributable to a need for oxidative phophorylation since a stimulated rate was still evident when ADP was omitted from the incubations. These observations were also made with respect to the stimulation of flux through the glycine cleavage system following glucagon injection (Jois et al., 1992). Moreover, the stimulatory effect of glucagon was not apparent if phosphate was replaced with other permeant anions, such as thiocyanate or acetate, suggesting that the requirement for phosphate is quite specific (Jois et al., 1992). It should also be noted that omission of phosphate also prevents the stimulation by calcium (Jois et al., 1990b).

The glycine cleavage system was stimulated by concentrations of free calcium between 0.1 and 0.3 μM. It is known that the stimulation of the glycine cleavage system by calcium requires the ion to enter the mitochondria (Jois et al., 1990b). The maximal flux through the glycine cleavage system in the presence of calcium was about 2.5 nmoles/min/mg mitochondrial protein. This rate was markedly reduced at higher concentrations of calcium if ADP was not present in

the incubations. The reason for this is unknown. One possibility is that at calcium concentrations beyond 0.3 μM mitochondria become susceptible to calcium-induced matrix swelling and subsequent damage (Lê Quôc and Lê Quôc, 1988). These researchers studied the involvement of the ADP/ATP carrier in this process and report that the ability of calcium to modify inner mitochondrial membrane properties is determined by the orientation of the nucleotide binding site of the carrier. When ADP was present, the carrier was stabilised in a conformation that does not permit calcium-induced swelling to occur. Thus ADP may protect mitochondria from the harmful effects of calcium.

Halestrap and collegues have suggested that several of the effects of hormones and calcium on mitochondrial metabolism are mediated by an increase in mitochondrial matrix volume (see Halestrap, 1989). Changes in mitochondrial matrix volume do not appear to be involved in the regulation of the glycine cleavage system following a high-protein diet or meal. The stimulation of flux through the glycine cleavage system by hypo-osmolar conditions is accompanied by parallel changes in mitochondrial matrix volume (Brosnan et al., 1990; Halestrap et al., 1990), but it is shown here that this stimulation is somehow different from that brought about in vivo by feeding a high-protein meal. Indeed, direct measurement (under iso-osmotic conditions) showed that mitochondrial matrix volume was similar in mitochondria from

rats fed high-protein and normal-protein meals, and unlike flux through the glycine cleavage system, was independent of the presence of inorganic phosphate. We have also been unable to detect any change in mitochondrial matrix volume of mitochondria from glucagon-injected rats (Jois et al., 1992). These findings agree with those of Hamman and Haynes (1983) and Wingrove et al. (1984), but contrast with those of Halestrap and co-workers who report a 10-20% increase in mitochondrial matrix volume following glucagon treatment (see Armston et al., 1982). Such a discrepancy may be due to the difficulty in measuring small changes in matrix volume **(Chapter 2)**.

It has been shown in this chapter that the stimulation of glycine catabolism by high protein feeding has similar characteristics to that elicited by glucagon, and we consider it likely that the high circulating glucagon that is evident after a high protein meal (Robinson et al., 1981) may be an important signal in the stimulation of the glycine cleavage system at this time. The physiological significance of these findings is readily apparent. Ingestion of a high protein diet (or meal) necessarily involves ingestion of increased amounts of glycine. Since the body has no real capacity to store amino acids, other than the synthesis of functional proteins, excess amino acids must be catabolised. The pathway for the catabolism of glycine involves its conversion to serine in a fashion that consumes two molecules of glycine

with the formation of one molecule each of serine and CO_2. That this pathway is active is apparent from the increased serine production reported here. The fate of the serine may be direct oxidation to CO_2 or, more likely, conversion to glucose since gluconeogenesis is considered to be a normal prandial process after ingestion of protein (Jungas et al., 1992).

Chapter 4

Glutamine catabolism in liver mitochondria from rats fed on high-protein and normal-protein diets and meals

Synopsis

Hepatic glutaminase is rapidly activated in rats fed a single high-protein (60% casein) meal. Rats previously fed on a normal-protein (15% casein) diet for three to four days were given a high-protein meal for two hours. The high-protein meal increased the rate of flux through glutaminase in intact liver mitochondria nearly three-fold (20.6 ± 1.7 nmoles/min/mg protein versus 7.5 ± 2.9 nmoles/min/mg protein) at a Pi concentration of 10 mM. The activation of flux through glutaminase by a high-protein meal involved an increased sensitivity of glutaminase for Pi, an activator of the enzyme. We measured the concentration of Pi in the mitochondrial matrix and found that it did not differ in mitochondria from rats fed the high-protein and normal-protein meals, suggesting that the effect of the high-protein meal on the Pi sensitivity of glutaminase was not due to a change in the distribution of Pi across the inner mitochondrial membrane. Glutaminase activity, measured in mitochondrial membranes from freezed-thawed mitochondria, was unchanged by the high-protein meal.

Introduction

Hepatic glutaminase activity is induced in rats in response to chronic high-protein feeding (Matsuda et al., 1973; Watford et al., 1985). The more immediate, or acute

response of the enzyme to the challenge of a single high-protein meal has not been examined. However, glutaminase may respond in a similarly rapid fashion following a single high-protein meal as the glycine cleavage system reported in **Chapter 3.** Certainly, the short-term regulation of glutaminase by a variety of effectors and hormones, notably glucagon, supports the contention that glutaminase will be activated following a high-protein meal. It was, therefore, hypothesised that glutaminase would be activated in rats fed a single high-protein meal, and that this would be evident in mitochondria isolated from these animals. In these experiments, citrulline synthesis was also measured. The rate of citrulline synthesis is a function of the flux through carbamyl phosphate synthase I and ornithine transcarbamylase, and as such represents a measure of the flux through the mitochondrial portion of the urea cyle.

Protocol

In these experiments the rats were fed the 15% or 60% casein diets for 3-4 days before the effects of a single meal on glutamine catabolism were examined. In one experiment (**Fig 4.7**), glycine and glutamine metabolism were studied in mitochondria from rats fed 15% or 60% lactalbumin diets for six days.

Results

Stimulation of citrulline synthesis and flux through glutaminase by a high-protein meal

Flux through glutaminase was stimulated in intact mitochondria from rats fed a high-protein meal compared to rates in mitochondria from rats not given a meal or given a normal-protein meal (**Fig. 4.1**). The stimulation was evident both in rats which had been fed the high-protein diet or the normal-protein diet, thus illustrating the effectiveness of a single, two hour, high-protein meal in eliciting a stimulation of glutamine catabolism. Citrulline synthesis was also stimulated in these mitochondria (**Fig. 4.2**). The stimulation of citrulline synthesis by the high-protein meal, in absolute terms amounted to 31 nmoles/min.mg for rats previously fed either the normal-protein or high-protein diets. The degree of stimulation by the meal was greater in the mitochondria of rats fed the normal-protein diet than in those of rats fed the high-protein diet for three to four days (6.8-fold versus 2.7-fold). This was due to the higher premeal (control) rates of citrulline synthesis in the mitochondria of high-protein-fed rats compared to the rates in mitochondria of rats fed the normal-protein diet (18.68 nmoles/min.mg protein versus 3.43 nmoles/min.mg protein), an effect which presumably occurred because of induction of urea cycle enzymes in the rats fed the high-protein diet (Schimke,

Fig. 4.1. The effect of a single meal on flux through glutaminase in isolated rat liver mitochondria. Rats fed on the high-protein (a.) or normal-protein (b.) diet for 3-4 days, were given the HP (▮) or NP (□) meal for two hours. Controls (▨) did not receive a meal and were killed at the start of the feeding period. Results are means ± S.E.M. of four separate experiments. Results were compared using a Student t-test ($p<0.05$): * significantly different from the control (no meal); † significantly different from those that received a meal that matched the diet they had been accustomed to.

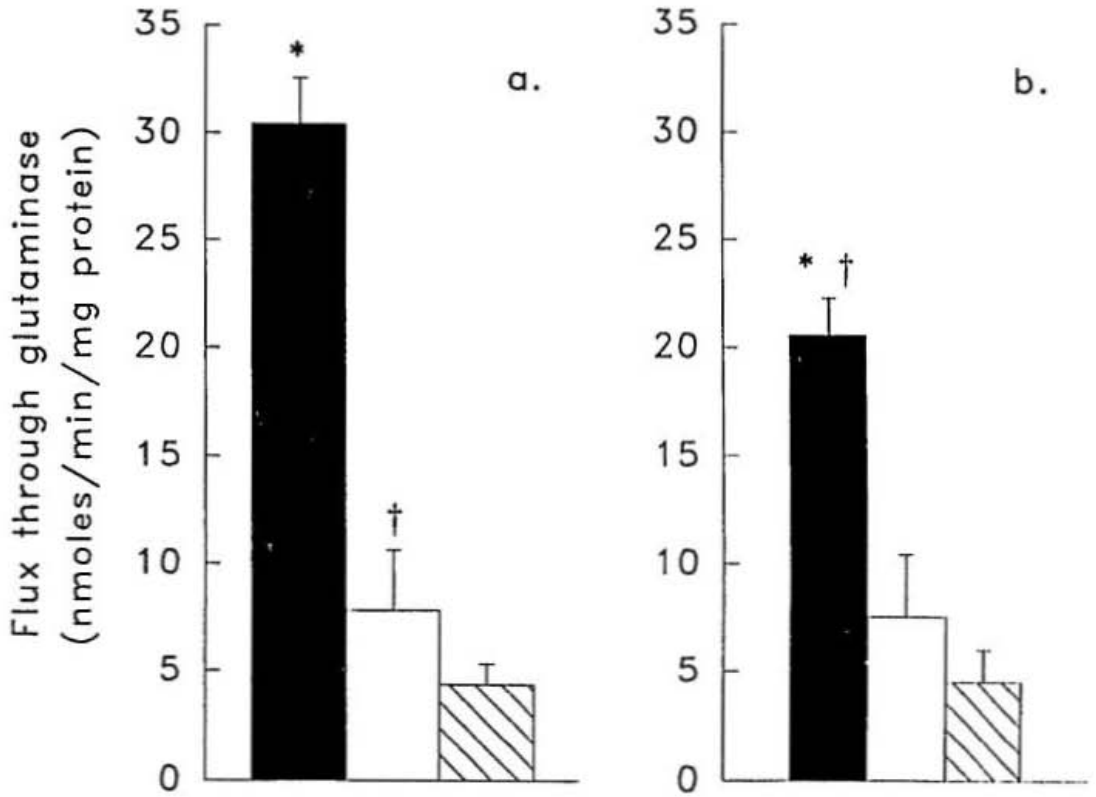
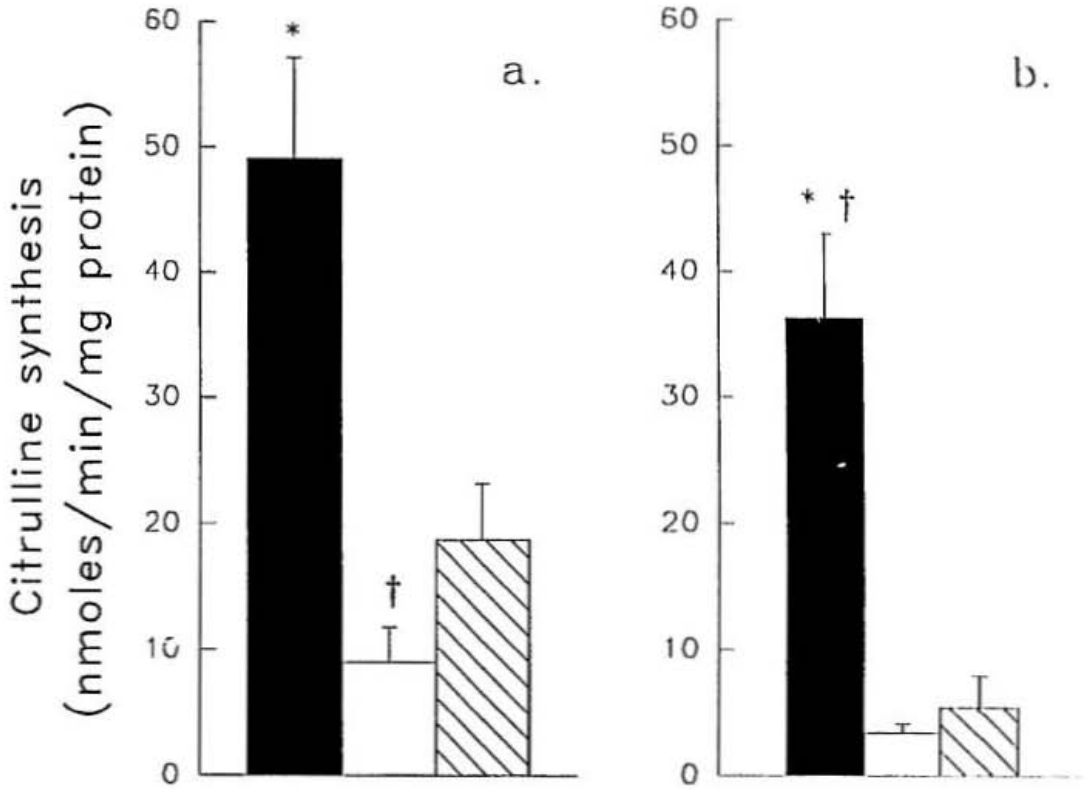

Flux through glutaminase
(nmoles/min/mg protein)
35
30
25
20
15
10
5
0
*
†
a.
b.

Fig. 4.2. The effect of a single meal on citrulline synthesis in isolated rat liver mitochondria. Rats fed on the high-protein (a.) or normal-protein (b.) diet for 3-4 days, were given the HP (■) or NP (□) meal for two hours. Controls (▨) did not receive a meal and were killed at the start of the feeding period. Results are means ± S.E.M. of three or four separate experiments. Results were compared using a Student t-test ($p<0.05$): * significantly different from the control(no meal); † significantly different from those that received a meal that matched the diet they had been accustomed to.


Citrulline synthesis
(nmoles/min/mg protein)
60
50
40
30
20
10
0
*
†
a.
b.

1962).

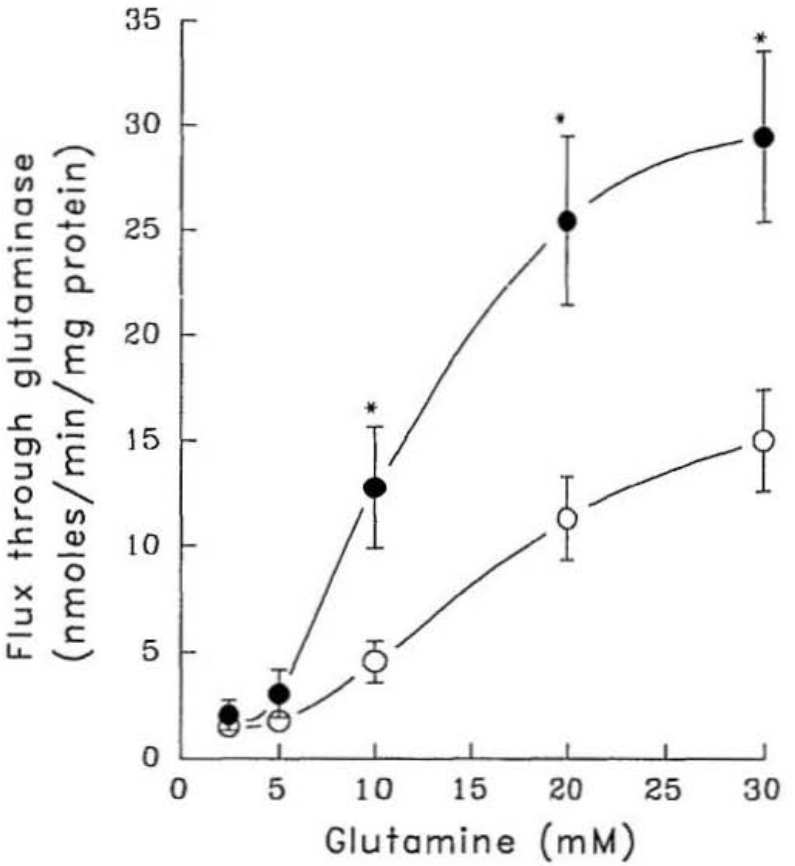
Sensitivity of flux through glutaminase to glutamine

Flux through glutaminase in isolated mitochondria showed a sigmoidal dependence on glutamine (**Fig. 4.3**). K_m and V_{max} values were calculated for these data as described in Chapter 2. In mitochondria from rats fed a high-protein meal, one-half maximal rate of flux through glutaminase was reached at a glutamine concentration of 12.4 ± 1.0 mM, compared to a value of 19.0 ± 1.0 mM observed for mitochondria from rats fed a normal-protein meal ($p<0.05$). The maximal velocities for these data were 20.8 ± 5.2 and 32.9 ± 4.66 nmoles/min/mg protein for high-protein and normal-protein meals, respectively, which were not statistically different.

Sensitivity to inorganic phosphate of flux through glutaminase

Inorganic phosphate (Pi) has been shown to be an activator of glutaminase when the activity is measured in either intact mitochondria (Joseph and McGivan, 1978b) or in disrupted mitochondria (McGivan et al., 1980). In intact mitochondria from glucagon-treated rats the sensitivity of glutaminase to Pi is greatly increased (Lacey et al., 1981). We therefore examined whether an increase in the phosphate sensitivity was involved in the stimulation of glutaminase by

Fig. 4.3. Flux through glutaminase in rat liver mitochondria as a function of glutamine concentration. Rats were fed the normal-protein diet for 3-4 days before being given a high-protein (●) or normal-protein (O) meal for two hours. The concentration of Pi in the incubations was 10 mM. Results are means ± S.E.M. of 3 separate experiments. The curve of best fit (sigmoidal curve) was arrived at as described in **Chapter 2**. Results were compared using a Student t-test ($p<0.05$): * significantly different from the rate in mitochondria from rats receiving the normal-protein meal.


Flux through glutaminase
(nmoles/min/mg protein)
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Glutamine (mM)
*
*
*

a high-protein meal. This was tested in both high-protein-fed rats (**Fig. 4.4a**) and normal-protein-fed rats (**Fig. 4.4b**). In each case a marked increase in the sensitivity of glutaminase flux to Pi was evident in mitochondria from the rats fed a high-protein meal (left-shift of the curves). A summary of the kinetic analysis of these data is presented in **Table 4.1**. In mitochondria from rats fed on the high-protein diet and given a high-protein meal, the K_a for phosphate was 1.7 mM compared to a value of 12.3 mM for rats receiving a normal-protein meal. In rats fed the normal-protein diet, and given a high-protein or normal-protein meal, the K_a values were 1.0 mM and 24.1 mM, respectively. Thus a single high-protein meal markedly enhances the sensitivity of glutamine hydrolysis in isolated mitochondria toward phosphate such that one-half the maximal rate of flux through glutaminase is reached at a much lower Pi concentration than in mitochondria from rats given a normal-protein meal.

<u>The concentration of Pi in the matrix of mitochondria from rats fed on high-protein and normal-protein meals</u>

Mitochondria from rats fed a high-protein meal exhibited stimulated rates of flux through glutaminase even when the incubation medium did not contain Pi (**Fig. 4.4**). This could conceivably be explained by a higher matrix Pi concentration in these mitochondria compared to those from rats fed a normal-protein meal. A difference in matrix phosphate content

Fig. 4.4. The effect of a high-protein meal on the phosphate activation of flux through glutaminase in rat liver mitochondria. Rats were fed a high-protein (a) or normal-protein (b) diet for 3-4 days before being given a high-protein (●) or normal-protein (O) meal for two hours. The concentration of glutamine in the incubations was 20 mM. Results are means ± S.E.M. of 4 separate experiments. Results were compared using a Student t-test ($p<0.05$): * significantly different from the rate in mitochondria from rats receiving the normal-protein meal.

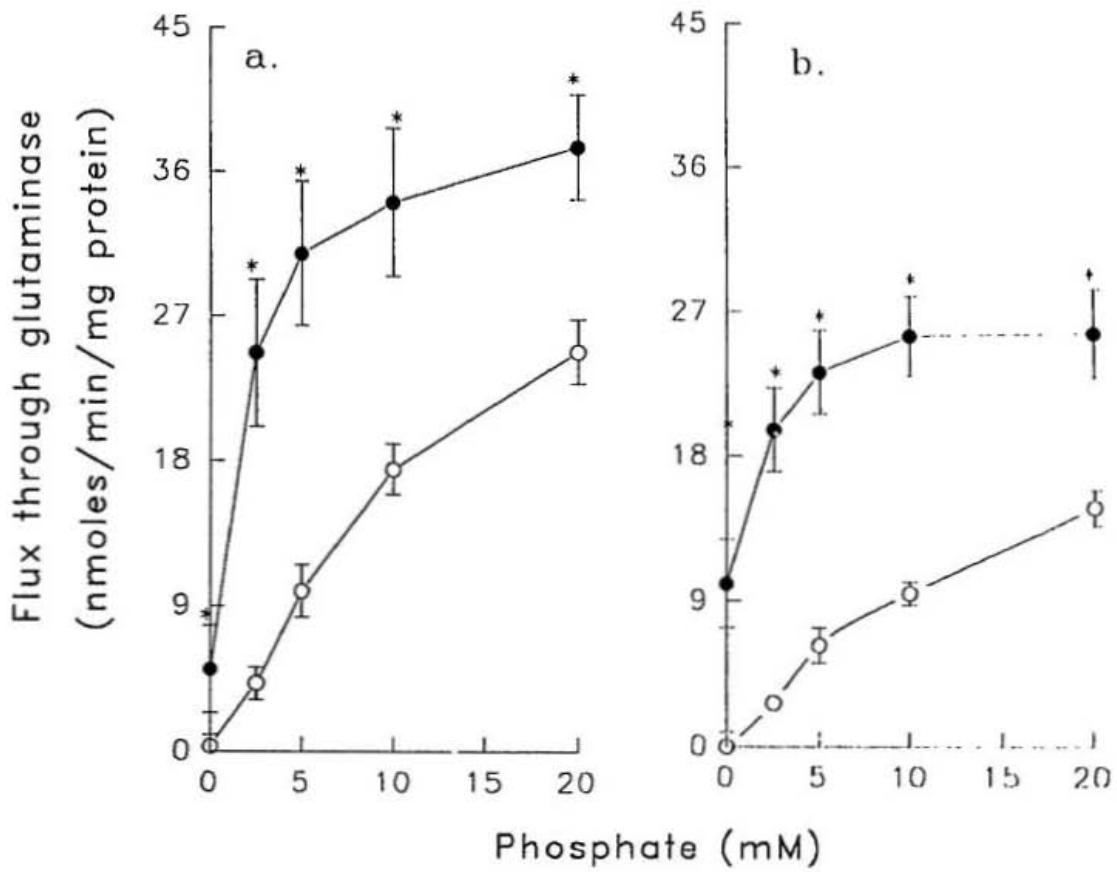

Flux through glutaminase
(nmoles/min/mg protein)
45
36
27
18
9
0
a.
b.
0
5
10
15
20
Phosphate (mM)

Table 4.1. Effect of high protein feeding on the phosphate requirement of glutaminase in isolated liver mitochondria. The phosphate required for half-maximum activation of glutaminase (K_a) and the maximum velocity (V_{max}) were calculated for the data presented in **Fig.4.4** as described in **Chapter 2**. Results are mean±S.E.M.
* significantly different from value for rats fed same diet but given a normal-protein meal.

Diet	Meal	K_a (mM)	V_{max} (nmol/min/mg)
HP	HP	1.74 ± 0.42*	40.37 ± 3.21
HP	NP	12.29 ± 0.28	42.71 ± 5.30
NP	NP	24.08 ± 4.64	30.74 ± 5.82
NP	HP	1.03 ± 0.29*	27.52 ± 2.85

when incubated in media of different phosphate concentrations would also explain the different phosphate sensitivities observed for glutaminase flux in these mitochondria. However, it was found that the difference in sensitivity of flux through glutaminase toward added phosphate did not involve a difference in their abilities to accumulate matrix Pi (**Table 4.2**). In mitochondria incubated in the absence of added phosphate, the matrix phosphate concentration was found to be slightly higher in mitochondria from rats fed the high-protein meal. When phosphate was present in the incubations, mitochondria were found to develop a four to six-fold higher concentration of phosphate in the matrix compared to levels in the medium. However, matrix phosphate in mitochondria from rats fed the high- and normal-protein meals did not differ, either at a concentration of 2.5 mM, which is close to that of the cytosol, or at 10 mM, the external concentration used in the majority of the incubations to assess flux through glutaminase. In agreement with the findings reported in **Chapter 3**, mitochondrial matrix volume was similar for mitochondria isolated from rats fed the high-protein and normal-protein meals and was independent of the phosphate concentration of the incubation medium.

Effect of ammonia activation

Ammonia is an important activator of glutaminase. It is possible that the effect of high-protein-feeding could be the

Table 4.2. The concentration of inorganic phosphate in the matrix of mitochondria isolated from rats fed a high- or normal-protein meal. Rats were fed a normal-protein diet for 3-4 days before being given the high-protein or normal-protein meal for two hours. Phosphate (Pi) content of mitochondria was that associated with the mitochondrial pellet minus a correction for extramatrix space. Mitochondrial matrix phosphate concentration was the matrix Pi content divided by the matrix volume. Results are Means $\pm$ S.E.M. of 4 experiments. Results were compared using a Student t-test ($p<0.05$): * significantly different from the level in mitochondria isolated from rats fed a NP meal incubated in the same medium.

		Mitochondria		
Meal	Medium Pi (mM)	Pi content (nmol/mg protein)	Volume (µl/mg protein)	Matrix Pi (mM)
High-protein	0	5.85 ± 0.7	1.35 ± 0.10	4.3 ± 0.3*
	2.5	35.89 ± 6.0	1.42 ± 0.12	20.8 ± 1.4
	10	87.10 ± 9.7	1.44 ± 0.13	45.1 ± 1.6
Normal-protein	0	4.00 ± 0.8	1.39 ± 0.03	2.9 ± 0.5
	2.5	30.35 ± 2.4	1.35 ± 0.10	17.2 ± 2.1
	10	80.96 ± 5.6	1.35 ± 0.14	41.1 ± 4.3

result of a higher ammonia content of mitochondria from these animals. However, incubation done in the presence of varying ammonium chloride concentrations ruled out this possibility (**Fig. 4.5**). The stimulatory effect of high-protein-feeding on flux through glutaminase is evident, even when flux is maximally activated by ammonia.

Glutaminase activity in broken mitochondria is not changed by a single high-protein meal

The stimulation of flux through glutaminase in intact mitochondria isolated from rats fed a high-protein meal did not involve an increase in total enzyme activity as measured in membranes of disrupted mitochondria (**Fig. 4.6**). We chose to disrupt the mitochondria by freezing and thawing because this procedure does not significantly release glutaminase from the mitochondrial membrane and the preparation of enzyme most closely resembles that in intact mitochondria (McGivan et al., 1980). In these experiments, rats fed the normal-protein diet for 3-4 days were given a normal-protein or high-protein meal for 2 hours, liver mitochondria were isolated, membranes prepared by freezing-thawing and glutaminase assayed. The rates of flux through glutaminase in intact mitochondria from these rats were 7.7 and 36.3 nmol/min.mg mitochondrial protein, respectively ($p < 0.05$). Yet no difference was found in the glutaminase activity assayed in the broken mitochondrial preparations. Moreover,

Fig. 4.5. The effect of a high-protein meal on the ammonia activation of flux through glutaminase in rat liver mitochondria. Rats were fed the normal-protein diet for 3-4 days before being given a high-protein (●) or normal-protein (○) meal for two hours. The concentration of Pi in the incubations was 10 mM. Results are means ± S.E.M. of 3 separate experiments. Results were compared using a Student t-test ($p<0.05$): * significantly different from the rate in mitochondria from rats receiving the normal-protein meal.

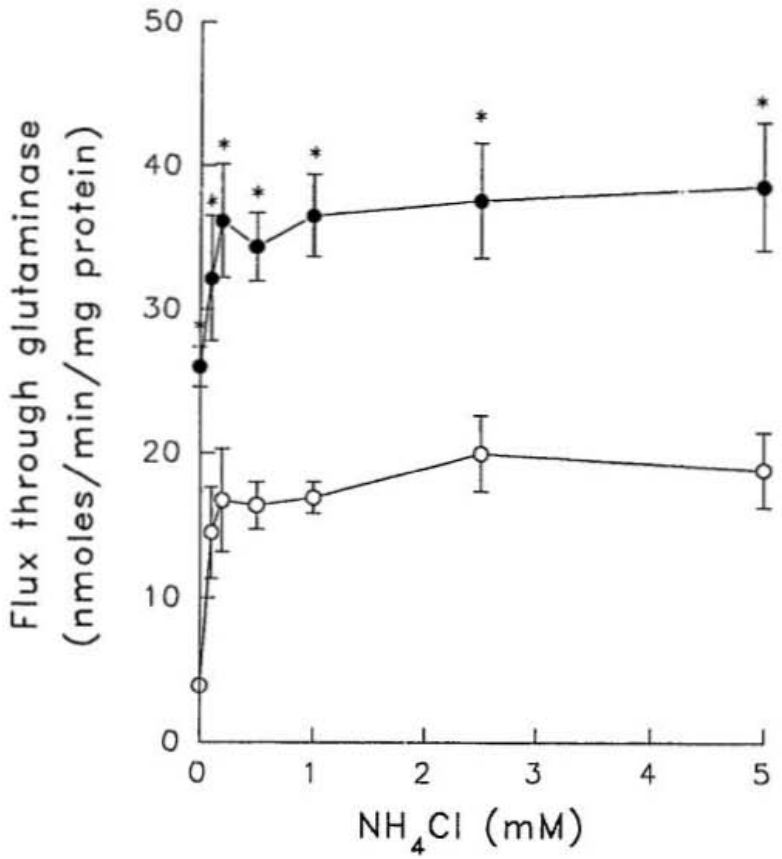
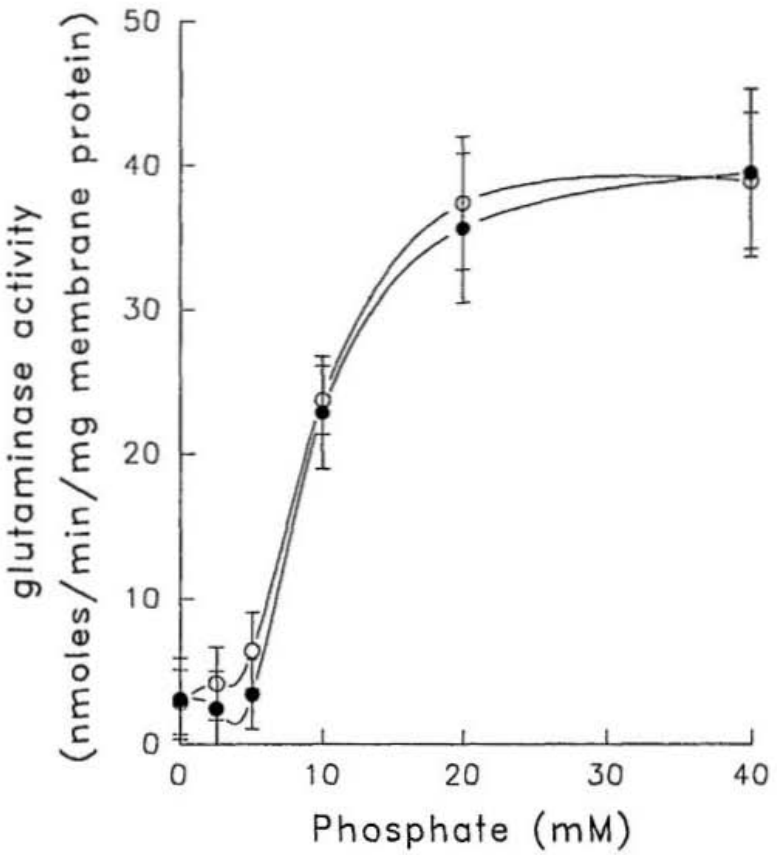

Flux through glutaminase
(nmoles/min/mg protein)
50
40
30
20
10
0
0
1
2
3
4
5
NH4Cl (mM)

Fig. 4.6. The effect of a single high-protein meal on glutaminase activity as measured in liver mitochondrial membranes. Rats fed the normal-protein diet for 3-4 days were given a high-protein (●) or normal-protein (O) meal for two hours. Mitochondrial membranes were incubated in the presence of 20 mM glutamine. Results are means ± S.E.M. for 3 separate experiments.


glutaminase activity
(nmoles/min/mg membrane protein)
50
40
30
20
10
0
0
10
20
30
40
Phosphate (mM)

the difference in affinity for phosphate, exhibited by intact mitochondria from rats fed the high- and normal-protein meals was not evident in the membrane preparations.

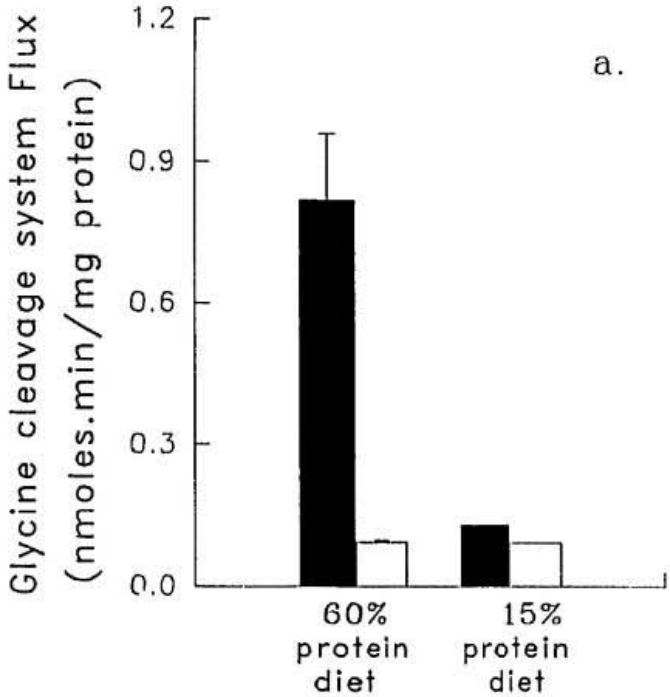
Flux through glutaminase and flux through the glycine cleavage system in mitochondria from rats fed lactalbumin diets

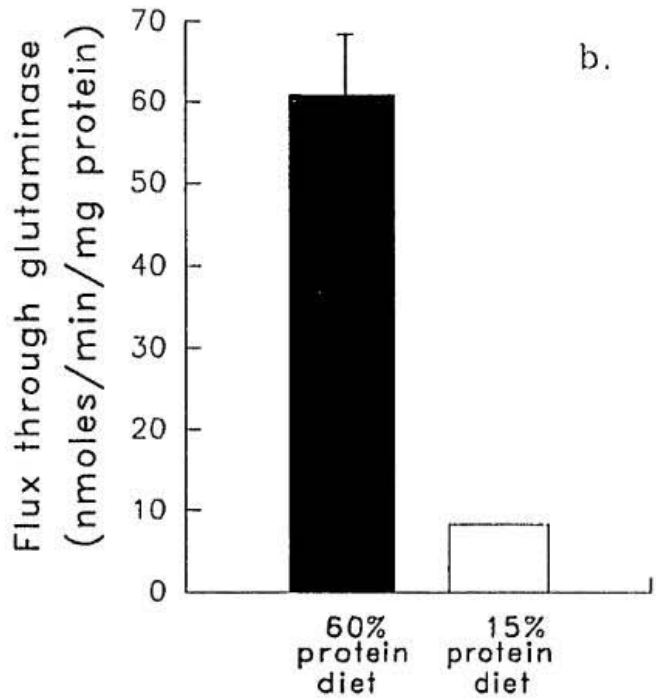
So far it has been shown that flux through glutaminase and flux through the glycine cleavage system is stimulated in mitochondria from rats fed a high-protein diet or meal. This, however, was shown using only one type of protein, casein. Therefore, it was important to show that this was a general effect of protein-feeding and not due to a particular characteristic of casein. That this was indeed the case is illustrated in **Fig. 4.7** where flux through glutaminase and flux through the glycine cleavage system were measured in mitochondria from rats fed 15% and 60% lactalbumin diets (see **Chapter 2**). In these experiments, glycine cleavage system flux and glutaminase flux were markedly elevated in mitochondria from rats fed the 60% lactalbumin diet compared to fluxes in mitochondria from rats fed the 15% lactalbumin diets.

Succinate oxidation in mitochondria from rats fed high-protein meals

Our findings thus far reveal that ingestion of a high-

Fig. 4.7. Flux through glutaminase and the glycine cleavage system in mitochondria from rats fed 15% and 60% lactalbumin diets. Rats were fed on the 15% and 60% lactalbumin diets for 6 days. Flux through the glycine cleavage system (a.) was measured in the KCl-based medium in the absence (□) or presence (■) of phosphate (2.5 mM). For flux through glutaminase (b.) glutamine and phosphate were present at 20 mM and 10 mM, respectively. Results are the mean ± S.E.M. of four separate experiments (60% lactalbumin diet) or the mean of two experiments (15% lactalbumin diet).


Glycine cleavage system Flux
(nmoles.min/mg protein)
1.2
0.9
0.6
0.3
0.0
a.
60%
protein
diet
15%
protein
diet


Flux through glutaminase
(nmoles/min/mg protein)
70
60
50
40
30
20
10
0
b.
60%
protein
diet
15%
protein
diet

protein meal activates the glycine cleavage system, glutaminase, and citrulline synthesis in liver mitochondria. Succinate oxidation is another mitochondrial parameter reported to increase after glucagon injection (Yamazaki, 1975). In our hands we found that glucagon injection increased succinate oxidation by 42% in subsequently isolated mitochondria (**Table 4.3**). However, succinate oxidation rate was not increased in mitochondria from rats fed a high-protein meal.

Discussion

Flux through glutaminase was stimulated in mitochondria from rats fed a single high-protein meal for two hours. Citrulline synthesis was also stimulated in these mitochondria. The situation was very much like that reported for the glycine cleavage system in the previous chapter, in that the protein content of the last meal was the single important determinant of rate of flux through glutaminase and in the rate of citrulline synthesis. Thus it is evident that glutaminase and the urea cycle enzymes have the ability to respond very rapidly to the level of amino acids entering from the diet, an event that may be mediated by hormones.

The stimulatory effect of a high-protein meal on flux through glutaminase does not involve an increase in glutaminase protein, since activity measured in broken mitochondria, was unchanged from controls (**Fig. 4.6**).

Table 4.3. Succinate oxidation in mitochondria from rats fed a high- or normal-protein meal. Rats fed the normal-protein diet for 2-3 days were given a high- or normal-protein meal for 2 hours. Rats fed the chow diet were injected with glucagon (0.1 mg/100 g body weight) or 0.9% saline/0.05% bovine serum albumin. Results are the mean ± S.E.M. for 5 separate experiments or the means of 2 experiments.

	Succinate oxidation (ng-Atoms O/min.mg)
High-protein meal	206.0 ± 11.1
Normal-protein meal	188.6 ± 11.2
Glucagon-injected	240.4
Saline-injected	170.5

Instead the stimulation of glutaminase after a high-protein meal is due to an activation process which, in large part, appears to be mediated by an increase in the enzyme's affinity for phosphate. Flux through glutaminase in intact miitochondria exhibited a remarkable reduction in its K_a for phosphate after a high-protein diet or meal (See **Table 4.1**). The precise nature of the phosphate activation is unknown. Mitochondria accumulate phosphate several-fold in relation to the external concentration (Akerboom et al., 1978; Hutson et al., 1992). It was possible that the increased affinity of glutaminase flux for phosphate was due to an increased ability of these mitochondria to accumulate phosphate, a possibility consistent with the finding that the increased affinity of glutaminase for phosphate dissappears upon disruption of the mitochondria by freezing and thawing (**Fig. 4.6**). However, direct measurement of the phosphate content in the matrix of the mitochondria showed that this was not the case (**Table 4.2**). In these experiments, a small increase in the phosphate content of mitochondria was detected after a high-protein meal if the mitochondria were incubated in the absence of added phosphate. This initial higher phosphate content likely accounts for the glutaminase flux observed in these mitochondria when incubated without added phosphate (see **Fig. 4.4**). However, when phosphate is included in the incubation at near physiological concentration (2.5 mM) or at the standard incubation concentration (10 mM), no difference

in the matrix phosphate concentration was found to exist. These findings are consistent with that reported for the phosphate activation of glutaminase flux in mitochondria from glucagon-injected rats (Lacey et al., 1981).

The stimulatory effect of a high-protein meal on flux through glutaminase is not the result of an increased content of ammonia in these mitochondria. In incubations where flux was stimulated by addition of ammonia, the effect of the meal was still evident, even when ammonia was present at optimal concentrations (**Fig. 4.5**). It is probable that the activation of glutaminase by a high-protein meal and by ammonia represent different mechanisms or sites of action. Similar findings have been reported in perfused liver where the activating effect of ammonia and glucagon on glutaminase were found to be additive (Häussinger et al., 1983).

Mitochondria from rats fed high-protein meals for two hours exhibit high rates of citrulline synthesis. This rapid stimulation is likely due to the activation of CPS I, which is an important regulatory enzyme in urea synthesis. Rapid activation of CPS I has been reported to occur within 15 minutes of an amino acid load (Stewart and Walser, 1980), an effect which was attributed to an increase in N-acetylglutamate, its obligatory activator. Increases in N-acetylglutamate closely correlate with the stimulation of citrulline synthesis in mitochondria from rats fed for a single day on a high-protein diet (Morimoto et al., 1990) and

it is likely that an increase in this substance leads to the stimulation of citrulline synthesis following a two hour high-protein meal. A rapid activation of citrulline synthesis following a high-protein meal means that the amino acid nitrogen associated with the meal can be converted to urea.

The stimulation of glutamine and glycine catabolism and citrulline biosynthesis by a high-protein meal have similar characteristics to that elicited by glucagon. The increase in glucagon in the circulation during ingestion of a high-protein meal (Robinson et al., 1981) is likely to be an important signal in the stimulation of mitochondrial amino acid metabolism at this time. However, events after a high-protein meal may be more complex as a variety of hormonal changes will occur. Evidence that glucagon alone may not be the only signal affecting these mitochondria is illustrated by the experiments on succinate oxidation where no affect of the meal was observed (**Table 4.3**). This emphasises the complexity of the in vivo situation and the need to consider factors in addition to glucagon.

Chapter 5

Glycine catabolism in hepatocytes and in perfused liver, and glutamine catabolism in hepatocytes, from rats fed diets or meals high in protein

Synopsis

Flux through the glycine cleavage system was studied in hepatocytes and in perfused liver by measuring the release of $^{14}CO_2$ from 1-^{14}C-glycine. Glutaminase flux was studied in hepatocytes by measuring the release of $^{14}CO_2$ from 1-^{14}C-glutamine. The glycine cleavage system and glutaminase were stimulated in hepatocytes from rats fed the high-protein diet for 5 days. When animals fed on the normal-protein diet were given a single, high-protein meal for two hours, hepatocytes from these animals displayed an enhanced ability to catabolise glycine, but no statistically significant effect was observed with respect to glutamine catabolism. However, if the duration of the meal was extended to six hours, both the glycine cleavage system and glutaminase were markedly stimulated. In perfused liver, flux through the glycine cleavage system was, initially, three-fold higher in liver from rats fed a two hour high-protein meal compared to liver from rats fed a normal-protein meal. But during the course of the perfusion, the high rate of glycine catabolism brought about by the high-protein meal was diminished, so that no stimulation was evident after 30 minutes. This was not due to an inadequate oxygenation of liver, or to changes in the glycine specific radioactivity in the tissue.

Introduction

Mitochondrial amino acid metabolism within the liver cell can be regulated by extramitochondrial events such as transport of amino acids across the plasma membrane. It was, therefore, important to determine whether the stimulatory effect of high-protein feeding on hepatic glycine and glutamine metabolism, as measured in intact mitochondria, was also evident in intact cells. Flux through the glycine cleavage system is elevated in liver slices (Matsuda et al., 1973) and in hepatocytes (Petzke et al., 1986) from rats fed a high-protein diet for several days. However, there is no information on the ability of such intact cell preparations to catabolise glycine, when isolated from animals that have ingested a single high-protein meal. This chapter reports experiments where flux through the glycine cleavage system was measured in hepatocytes from rats fed a high-protein diet or given a single high-protein meal. Glycine catabolism was further studied in isolated, perfused liver from rats fed a protein meal.

The measurement of flux through glutaminase in hepatocytes is complicated by the simultaneous operation of glutamine synthetase in the preparations. Methods based on the enzymatic measurement of glutamine disappearance, therefore, may largely underestimate flux through glutaminase (Vincent et al., 1989). Consequently, when cells are

incubated at low, near physiological concentrations of glutamine there is little or no net removal of glutamine. Alternatively, flux can be determined by measuring $^{14}CO_2$ release from 1-^{14}C-glutamine (Baverel and Lund, 1979; Vincent et al., 1989). The validity of this method was shown in **Chapter 2**, and therefore, it was used here to study glutamine metabolism in hepatocytes from animals fed a high-protein diet or meal.

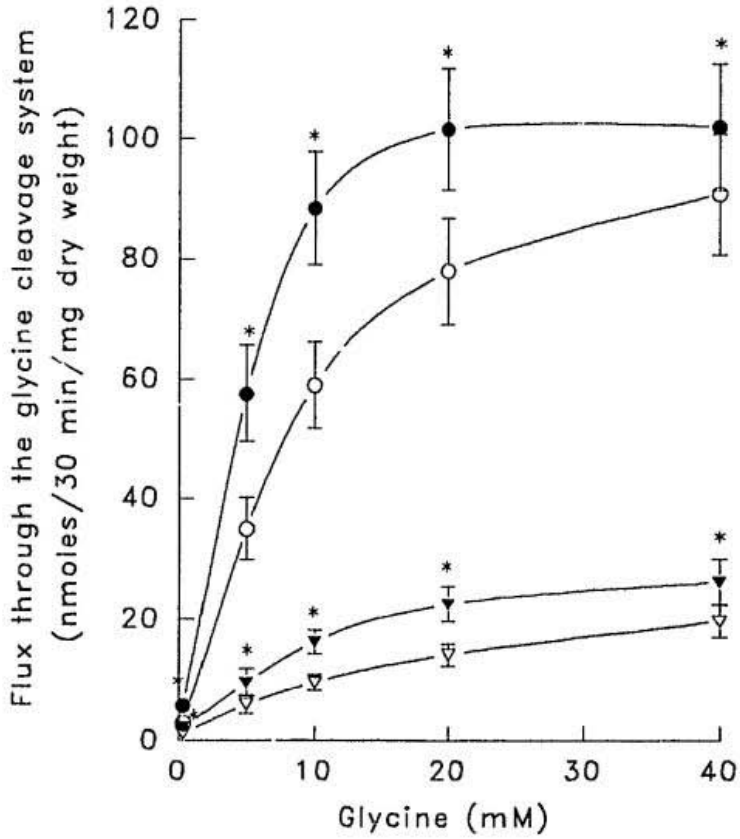
Protocol

The effect of diet on glycine and glutamine catabolism was examined in hepatocytes from rats fed the 15% or 60% casein diet. These animals were held under the normal light-dark cycle and hepatocytes were isolated at 9 am. The effect of a single, high-protein meal on glycine and glutamine catabolism was examined in animals fed the 15 % casein diet for 3-4 days. In these experiments, animals were held under the reverse light-dark cycle and meals began at the start of the dark period (8 am) and lasted for two or six hours.

Results

Flux through the glycine cleavage system was stimulated in hepatocytes from rats fed the high-protein diet for 5 days compared to rates in those from rats fed the normal-protein diet (**Fig. 5.1**). When the incubations were done at a physiological concentration of glycine (0.3 mM), the

Figure 5.1. Flux through the glycine cleavage system in hepatocytes from rats fed high-protein and normal-protein diets. Hepatocytes were isolated from rats fed the high-protein (●,○) or normal-protein diet (▼,▽) for 5 days. The incubations were carried out at concentrations of glycine ranging from 0.3 to 40 mM in the absence (open symbols) or presence (closed symbols) of glucagon (100 nM). The results are the mean ± S.E.M. of 6 separate experiments . Flux through the glycine cleavage system was greater in hepatocytes from high-protein-fed rats compared to hepatocytes from normal-protein-fed rats at each of the glycine concentrations tested (Student t-test; $p < 0,05$). * significantly higher compared to rate in absence of glucagon (paired t-test; $p< 0.05$).


Flux through the glycine cleavage system
(nmoles/30 min/mg dry weight)
120
100
80
60
40
20
0
0
10
20
30
40
Glycine (mM)

stimulation was approximately 2.5-fold (2.94 ± 0.5 nmoles/30 min/mg dry weight versus 1.27 ± 0.17 nmoles/30 min/mg dry weight of cells). The addition of glucagon to the incubations had a stimulatory effect on the glycine cleavage system . At 0.3 mM glycine, this amounted to a stimulation of flux by 96% and 80% in cell preparations from high-protein- and normal-protein-fed animals, respectively.

Flux through the glycine cleavage system was stimulated in hepatocytes from rats fed a single high-protein meal (**Table 5.1**). In the case of a two hour high-protein meal, glycine catabolism was increased by 50% compared to the flux in hepatocytes from rats fed a normal-protein meal. However, a much larger stimulation of flux through the glycine cleavage system was observed if the duration of the meal was extended to 6 hours. The stimulation was especially pronounced in the presence of glucagon where the rate was 9.60 nmoles/30 min/mg protein versus 2.02 nmoles/30 min/mg protein in hepatocytes from rats fed the normal-protein meal.

Flux through the glycine cleavage system was also measured in the isolated, perfused liver of animals not fed, or fed a single high-protein or normal-protein meal for two hours. The livers were perfused at a flow rate of around 60 ml/min which was sufficient to maintain the oxygen tension in the effluent above 100 mm Hg during the 30 minute perfusion (**Appendix A**). Oxygen consumption rate of the livers varied

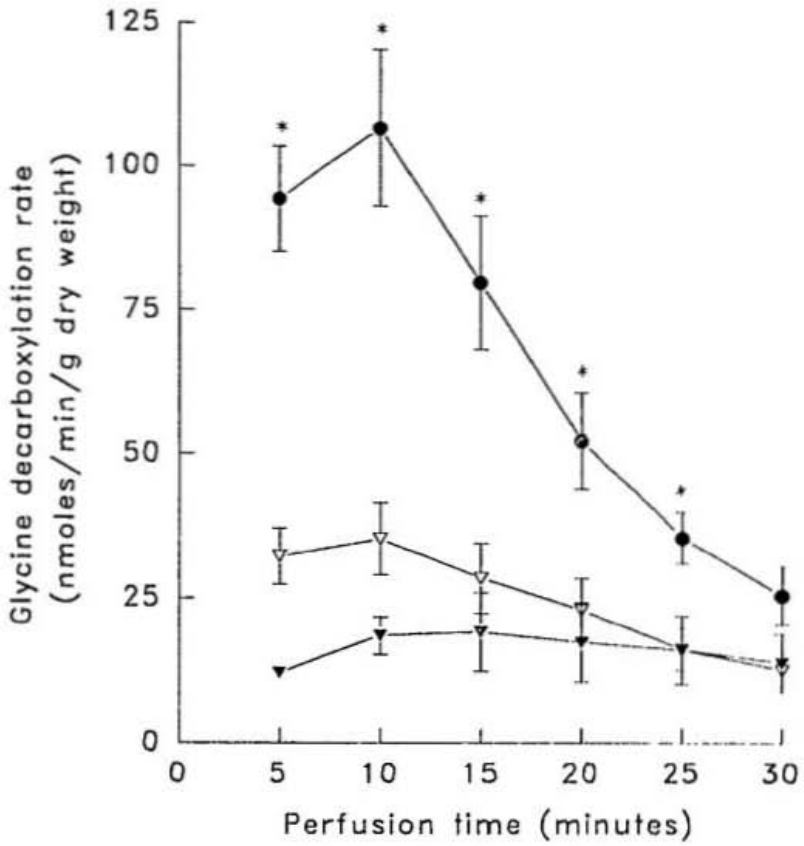
Table 5.1. Flux through the glycine cleavage system in hepatocytes from rats fed high-protein and normal-protein meals. Rats fed the normal-protein diet for 3-4 days were deprived of food for six hours and then given a high-protein or normal-protein meal. After a 2 or 6 hour meal, hepatocytes were isolated and incubated in the absence or presence of glucagon (100 mM) at a glycine concentration of 0.3 mM. The results are the mean ± S.E.M. with the number of experiments in parentheses. * significantly higher than the rate in the absence of glucagon (paired t-test; $p < 0.05$). † significantly higher than the rate in hepatocytes from animals fed the normal-protein meal (Student t-test; $p < 0.05$).

Glycine Cleavage System Flux (nmoles/30 min/mg dry weight)			
		(-) glucagon	(+) glucagon
2 hour meal	NP	1.85 ± 0.2 (7)	2.30 ± 0.3* (7)
	HP	2.76 ± 0.3† (8)	3.68 ± 0.4*,† (8)
6 hour meal	NP	1.37 ± 0.2 (4)	2.02 ± 0.2* (4)
	HP	5.82 ± 1.2†	9.60 ± 2.0*,†

between 2.7 and 3.3 nmole O/min/g wet weight, but did not differ among the pre-meal, high-protein , and normal-protein groups (**Appendix B**). In livers of rats fed the high-protein meal, flux through the glycine cleavage system was over 90 nmoles/min/g dry weight during the first 10 minutes of the perfusion, which was about three times the rate observed in livers from animals fed the normal-protein meal (**Fig. 5.2**). The stimulation of flux, however, diminished markedly during the remainder of the perfusion so that by 30 minutes there was no longer a difference in glycine catabolism in livers from high-protein- and normal-protein-fed animals. The flux through the glycine cleavage system in the livers of rats fed normal-protein meals also decreased, but less dramatically. In livers of animals not fed a meal, flux was low and fairly constant throughout the perfusion.

In these experiments flux through the glycine cleavage system was calculated using the specific radioactivity of 1-^{14}C-glycine in the perfusate and not the specific activity of glycine that may have existed in the liver during the perfusion. It was possible, therefore, that the above observations were due to differences in glycine specific radioactivity in liver, although it might be considered that radioactive glycine equilibration could be incomplete early in the perfusion and that rates of glycine decarboxylation would be underestimated here. In any case it was felt important to determine the specific activity of glycine in

Figure 5.2. Glycine decarboxylation rate by perfused livers. Rats fed the normal-protein diet for 3-4 days were deprived of food for six hours and then given a high-protein (●) or normal-protein (▽) meal for two hours. The livers of these animals were perfused for 30 minutes with 0.3 mM glycine (1-^{14}C-glycine ≅ 40 dpm/nmol) as described in the Methods. Livers of control (▼) rats were perfused at the beginning of the meal period. Results are the mean ± S.E.M. of three (controls) or four separate experiments. * significantly higher compared to rate in livers from rats fed a normal-protein meal.


Glycine decarboxylation rate
(nmoles/min/g dry weight)
125
100
75
50
25
0
0
5
10
15
20
25
30
Perfusion time (minutes)

liver tissue during the perfusion of livers from rats fed the high-protein and normal-protein meals. The perfusions were carried out in the same fashion as the previous ones except after 10 minutes a liver lobe was tied-off and removed for assay. As shown in **Table 5.2** there was no difference in the specific radioactivity of glycine in liver tissue sampled at 10 minutes and 30 minutes of perfusion. In addition, no difference existed in the specific activities in livers from rats fed the different meals. These findings support the initial observations of a large stimulation of flux through the glycine cleavage system in livers from rats fed the high-protein meal. The actual rates reported for flux, however, are likely to be underestimates since it was observed that the specific radioactivity of glycine in liver was approximately one-half the value in the perfusate, suggesting a significant dilution of the label in the tissue (**Table 5.2**). A similar dilution of glycine specific radioactivity has been reported to occur in isolated hepatocytes (Jois et al., 1989). Finally, the reason why the the stimulation of flux through the glycine cleavage system following a high-protein meal diminishes in perfused liver with time is unknown. However, it is clear that this was not related to an inadequate oxygenation of liver tissue. This is evident from the finding that adenine nucleotide levels in these livers did not change appreciably during the perfusion (**Table 5.3**).

Hepatocytes from rats fed a high-protein diet for 5 days

Table 5.2. Glycine specific radioactivity in perfused liver from rats fed a high-protein or normal-protein meal for two hours. Livers of animals fed a high-protein or normal-protein meal were perfused for 30 minutes with 0.3 mM glycine (1-^{14}C-glycine ≈40 dpm/nmol) as described in the Methods. Liver samples were taken at 10 and 30 minutes of the perfusion for determination of glycine specific radioactivity. The results are the mean ± S.E.M. with the number of experiments in parentheses.

	Glycine specific radioactivity (dpm/nmol)		
	Perfusate	Liver	
		10 min.	30 min.
High-protein	52.0 ± 5.7 (3)	27.7 ± 3.3 (3)	25.0 ± 2.8 (3)
Normal-protein	46.3 ± 4.2 (4)	19.3 ± 1.7 (4)	22.1 ± 1.9 (4)

Table 5.3. Adenine nucleotide content in perfused liver from rats fed a high-protein or normal-protein meal for two hours. Livers of animals fed a high-protein or normal-protein meal were perfused for 30 minutes with 0.3 mM glycine (1-^{14}C-glycine ≈40 dpm/nmol) as described in the Methods. Liver samples were taken at 10 and 30 minutes of the perfusion for determination of adenine nucleotides. The results are the mean ± S.E.M. with the number of experiments in parentheses.

		Adenine nucleotides (μmole/g tissue)		
		ATP	ADP	AMP
High-protein	10 min	2.18 ± 0.05 (3)	0.97 ± 0.05 (3)	0.22 ± 0.04 (3)
	30 min	2.61 ± 0.17 (3)	0.93 ± 0.11 (3)	0.18 ± 0.04 (3)
Normal-protein	10 min	2.20 ± 0.30 (4)	0.97 ± 0.23 (4)	0.32 ± 0.16 (4)
	30 min	2.58 ± 0.57 (4)	0.85 ± 0.11 (4)	0.22 ± 0.09 (4)

exhibited a large increase in glutamine catabolism (**Fig. 5.3**). At 1 mM ammonium chloride this amounted to approximately a 4.5-fold increase in glutaminase flux compared with the rate in hepatocytes from rats fed the normal-protein diet (72.3 nmoles/min/mg protein versus 16.8 nmoles/min/mg protein). The addition of glucagon had a stimulatory effect on glutaminase flux. In hepatocytes from rats fed the normal-protein diet, however, the effectiveness of glucagon depended on the presence of added ammonium chloride. When ammonium chloride was not added to the incubations, glucagon stimulated flux by only 16% compared to values in excess of 70% when ammonium chloride was present. In hepatocytes from rats fed the high-protein diet glucagon effectively stimulated glutaminase flux even in the absence of ammonium chloride.The effect of a high-protein diet on glutaminase flux was evident in hepatocytes from animals fed for only one day on the diet (**Fig. 5.4**). Glutaminase flux in these hepatocytes was the same as in hepatocytes from rats fed the high-protein diet for 5 days. In contrast, there was no significant increase in glutaminase flux in hepatocytes isolated from rats fed a single high-protein meal for 2 hours (**Fig. 5.5a**). However, when rats were fed the meal for 6 hours (**Fig. 5.5b**), hepatocytes from these animals exhibited a large increase in glutaminase flux compared to hepatocytes from rats fed the normal-protein meal (50.8 nmoles/30 min/mg dry weight versus 23.5 nmoles/30 min/mg dry weight, at 1 mM

Figure 5.3. Flux through glutaminase in hepatocytes from rats fed high-protein and normal-protein diets. Hepatocytes were isolated from rats fed the high-protein (●,○) or normal-protein diet (▼,▽) for 5 days. The incubations were carried out at a 1 mM glutamine (1-^{14}C-glutamine ≡ 25 dpm/nmol) and various concentrations of ammonium chloride in the absence (open symbols) or presence (closed symbols) of glucagon (100 nM). The results are the mean ± S.E.M. of 4 separate experiments . Flux through glutaminase was higher in hepatocytes from high-protein-fed rats compared to hepatocytes from normal-protein-fed rats at each concentration of ammonium chloride (Student t-test; $p < 0,05$). * significantly higher compared to rate in absence of glucagon (paired t-test; $p< 0.05$).

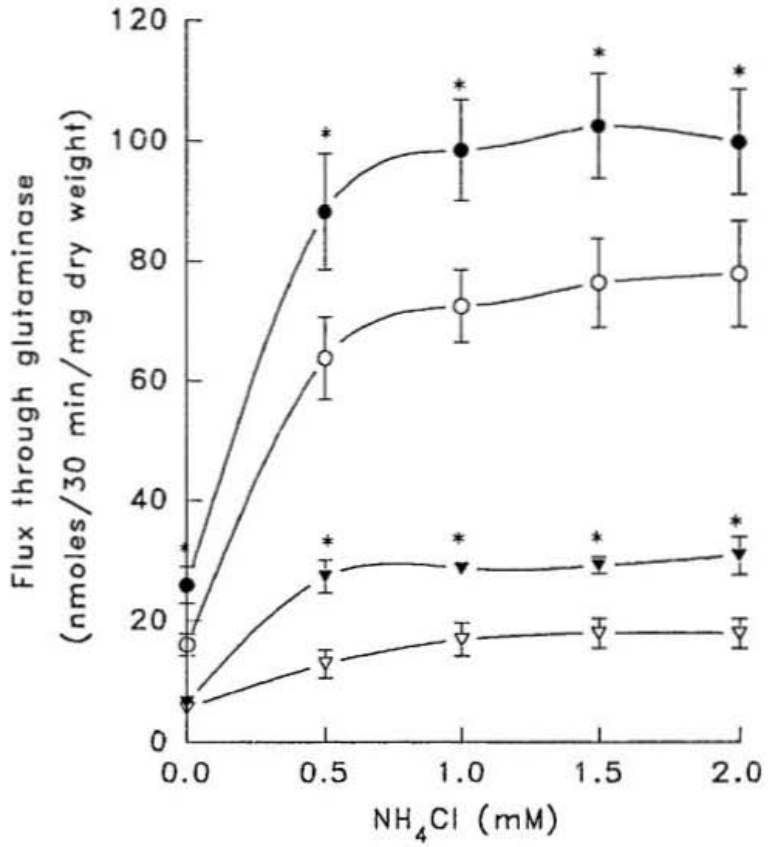

Flux through glutaminase
(nmoles/30 min/mg dry weight)
120
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
NH4Cl (mM)

Figure 5.4. Flux through the glutaminase in hepatocytes from rats fed a high-protein diet for one day. Rats fed the normal-protein diet for 5 days were switched to the high-protein diet for one day. Hepatocytes were incubated with 1 mM glutamine ($1-^{14}C$-glutamine ≡ 25 dpm/nmol) and various concentrations of ammonium chloride in the absence (open symbols) or presence (closed symbols) of glucagon (100 nM). The results are the mean ± S.E.M. of 4 separate experiments. * significantly higher compared to rate in absence of glucagon (paired t-test; $p < 0.05$).

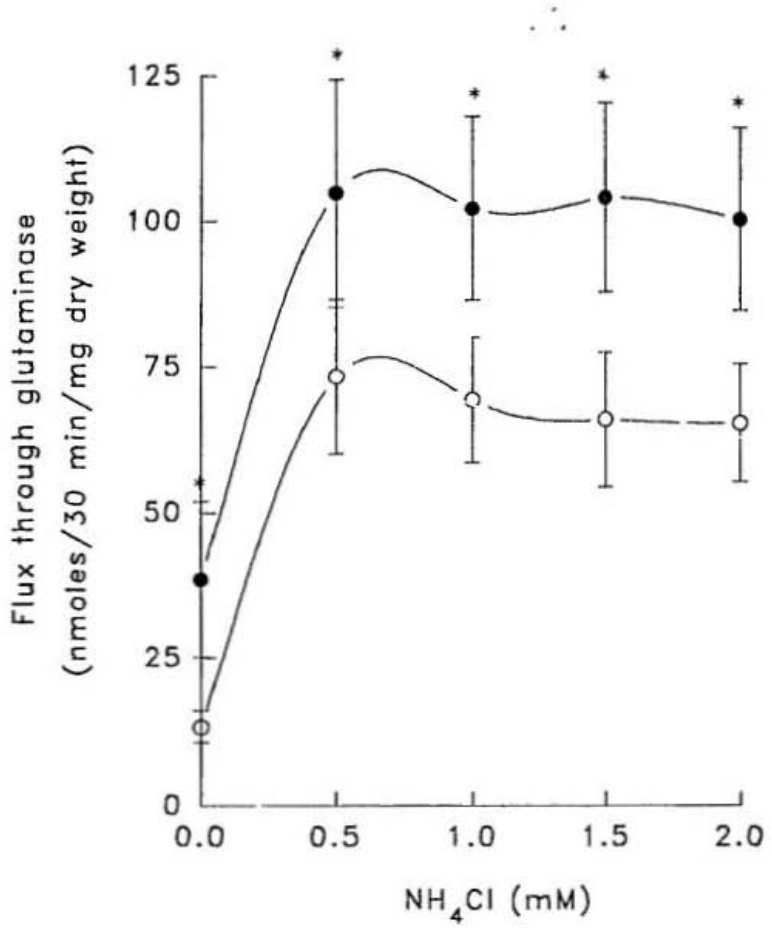
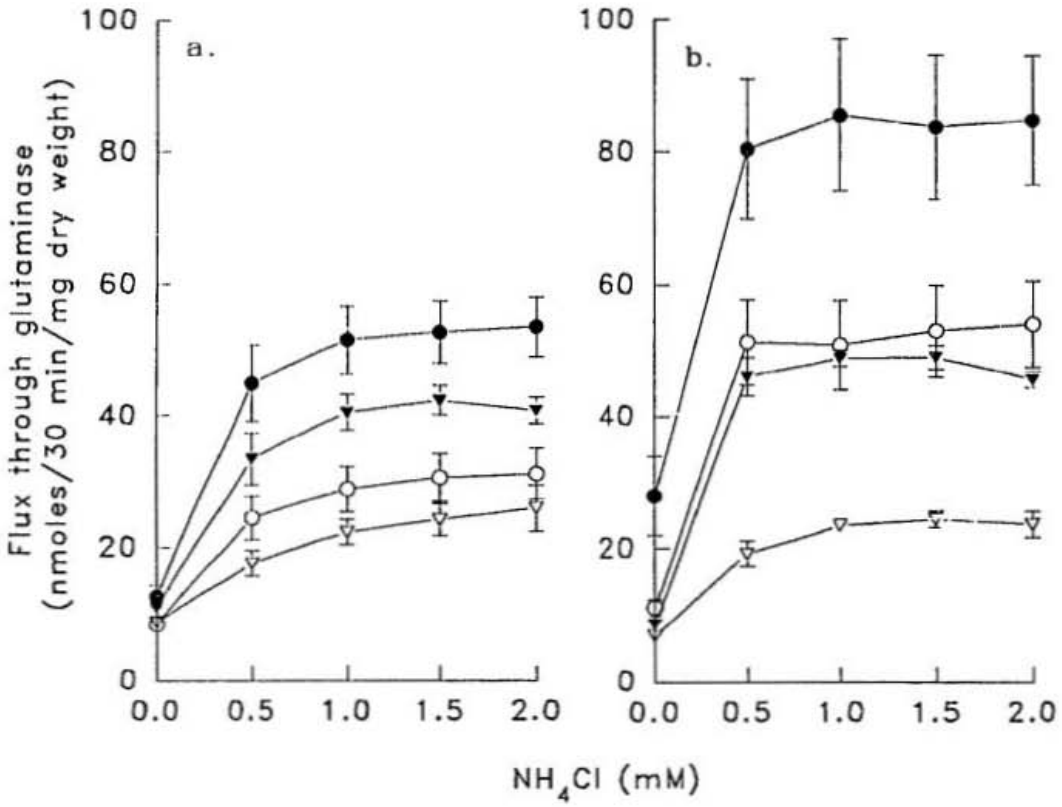

Flux through glutaminase
(nmoles/30 min/mg dry weight)
125
100
75
50
25
0
0.0
0.5
1.0
1.5
2.0
NH4Cl (mM)

Figure 5.5. Flux through glutaminase in hepatocytes from rats fed high-protein and normal-protein meals. Rats fed the normal-protein diet for 3-4 days were deprived of food for six hours and then given a high-protein (●,○) or normal-protein meal (▼,▽). Rats were fed the meals for two hours (a.) or six hours (b.).Hepatocytes were incubated with 1 mM glutamine ($1-^{14}C$-glutamine ≅ 25 dpm/nmol) and various concentrations of ammonium chloride in the absence (open symbols) or presence (closed symbols) of glucagon (100 nM). The results are the mean ± S.E.M. of 8 or 9 separate experiments. Glucagon stimulated glutaminase flux in hepatocytes from the rats fed the various meals at each concentration of ammonium chloride tested (paired t-test; $p < 0.05$). Flux through glutaminase was higher in hepatocytes from rats fed the high-protein meal for six hour compared to hepatocytes from rats fed the normal-protein meal for six hours at each concentration of ammonium chloride tested and in the presence or absence of glucagon (Student t-test; $p < 0.05$).


a.
b.
Flux through glutaminase
(nmoles/30 min/mg dry weight)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
NH4Cl (mM)

ammonium chloride). These findings emphasise the rapidity of the stimulation of flux through glutaminase in livers of animals ingesting high-protein diets or meals. The findings, however, differ from the observation that glutaminase flux, as measured in intact mitochondria, was stimulated within two hours of the start of a high-protein meal (**Chapter 4**). The reason for this discrepancy is unknown.

Discussion

The stimulation 0f glycine and glutamine metabolism in hepatocytes from rats fed on the high-protein diet for 5 days is consistent and similar in magnitude to the stimulation of flux through the glycine cleavage system and through glutaminase in isolated mitochondria from such animals (see **Chapter 3** and **Chapter 4**). Since glutaminase (Watford et al., 1985) and glycine cleavage system (Matsuda et al., 1973; Petzke et al., 1986) activities, as well as, P-, H-, and T-protein mRNA levels (Okamura-Ikeda et al. 1991) are increased in animals fed on high-protein diets, it is likely that an increase in enzyme protein levels are involved in the stimulation of these processes.

A number of points can be raised regarding the stimulatory effect of a high-protein meal on flux through the glycine cleavage system. First, a two hour meal results in a 50% increase in glycine cleavage system flux compared to rates in hepatocytes from rats fed a normal-protein meal. In

contrast, a greater than four-fold activation of glycine cleavage system flux occurs if the duration of the meal is extended to 6 hours. This is qualitatively consistent with observations in isolated mitochondria where glycine cleavage system flux was approximately twice the rate after a four hour as compared to a two hour high-protein meal (see last row of **Table 3.3**). These results suggest that activation of hepatic glycine catabolism is not fully developed after only two hours from the start of a high-protein meal. Second, the rate of glycine cleavage system flux following the six hour high-protein meal is twice the rate observed in animals fed the high-protein diet for 5 days. In each situation the addition of glucagon to the incubations led to a similar percentage stimulation of glycine cleavage system flux. These findings suggest that liver cells have a large reserve capacity for glycine catabolism which is elicited during a high-protein meal. The process may involve a conversion of a greater proportion of the enzyme complex to an activated form. Finally, the observations in perfused liver reveal that the stimulatory effect of a high-protein meal on flux through the glycine cleavage system is not stable and is reversed during the course of a perfusion. Thus, during the preparation of hepatocytes the activation of the glycine cleavage system following a 2 hour high-protein meal may be diminished. The reason for this is uncertain but it is likely that, physiologically, these activations require the

continuous presence of the agonist. When this is removed, as during perfusion, the stimulation is lost. Mitochondrial preparation in some way arrests the system in the activated state.

The finding that glutamine metabolism was not stimulated in hepatocytes following a two hour high-protein meal is in contrast to the observations made on glutaminase flux in mitochondria from such animals. The reason for this is unclear but may, in part, be due to the considerable day-to-day variability in flux that was observed during these experiments, suggesting that some condition was not being tightly controlled. It is also possible that the stimulation of glutaminase following a two hour high-protein meal may be lost during the perfusion that is required to prepare hepatocytes, as was shown for the stimulation of glycine catabolism in isolated, perfused liver. Clearly, however, a large stimulation of flux through glutaminase in hepatocytes was evident if the duration of the meal was extended to 6 hours. The reason for this is not immediately obvious, but the effect may be due to an induction of the System N transporter for glutamine.

The findings reported here largely coincide with observations on glycine and glutamine catabolism in mitochondria from rats fed high-protein diets or meals. In addition, the measurement of flux through the glycine cleavage system in isolated, perfused liver reveals that the

stimulatory effect of a high-protein meal is not stable. The effects of hormone treatment on mitochondrial metabolism are regarded as "long-lived" events because of their relative stability during mitochondrial isolation (see Halestrap, 1989). The present findings suggest that in the intact tissue, such effects may be readily reversed.

Chapter 6

Summary and General Discussion

Summary

The major findings of the work presented in this thesis are as follows:

1. Glycine and glutamine catabolism were enhanced in liver mitochondria or hepatocytes from animals fed on a 60% casein diet compared to rates measured in mitochondria or hepatocytes from rats fed on a 15% casein diet.

2. Glycine catabolism was stimulated within two hours of the start of a high-protein meal. The stimulatory effect of a single, high-protein meal was shown in intact mitochondria, hepatocytes, and in intact, perfused liver, although the characteristics of the activation differed slightly in each system. In intact liver mitochondria, a three- to four-fold stimulation of flux through the glycine cleavage system was evident after the two hour meal. This effect required the presence of inorganic phosphate in the incubation medium. In the absence of added phosphate, only low, basal rates of flux were evident. In hepatocytes, there was approximately a 50% increase in flux through the glycine cleavage system following a two hour high-protein meal. A much larger effect was evident when the meal lasted for six hours. In perfused liver, flux through the glycine cleavage system was approximately three-fold higher in livers of rats fed the two

hour high-protein meal compared to flux in livers from rats fed the normal-protein meal. The stimulated rate, however, was diminished during the course of the perfusion.

3. Glutamine catabolism was activated in mitochondria from rats fed a single, high-protein meal for two hours. The stimulatory effect of the meal was associated with a decrease in the concentration of inorganic phosphate required to reach one-half maximal activation of glutaminase flux. This was not due to an increased concentration of phosphate in the matrix of these mitochondria. Glutaminase activity, measured in broken mitochondria, was not increased following the two hour high-protein meal. In these preparations, the phosphate sensitivity of the enzyme was also similar. An effect of the two hour high-protein meal on glutaminase flux could not be demonstrated in hepatocytes. The reason for this is unknown, but a large stimulation of glutaminase flux was evident when the cells were isolated six hours after the start of the meal.

4. The exact mechanism(s) whereby the glycine cleavage system and glutaminase are activated following a high-protein meal is unknown. However, it was shown, by direct measurement, that the effects are not mediated by an increased mitochondrial matrix volume.

5. The stimulation of the glycine cleavage system following a

high-protein meal regarded as "stable" when measured in isolated, intact mitochondria, was readily lost upon perfusion of the liver.

General Discussion

Free amino acids are important in overall nitrogen metabolism, and it is in this form that most of the metabolically active nitrogen is absorbed from the gut and transported to the various tissues following a protein meal. The liver is the first organ encountered by dietary amino acids upon entering the circulation. The liver is the major site of their catabolism, capable of catabolising the twenty or so different amino acids presented to it, with the exception of the branched-chain amino acids, and is the sole site of the urea cycle. The work presented in this thesis describes the activation of the hepatic catabolism of glycine and glutamine following a high-protein meal. A number of key questions regarding the mechanism of these activations remain to be resolved and form the basis of this discussion.

The hepatic metabolism of amino acids following a meal involves two principal events: (1) the repletion of the so-called "labile protein stores" (Garlick et al., 1974; Scornik, 1984) by way of protein synthesis and (2) the catabolism of excess amino acids. In animals fed chronically on diets high in protein, adaptation of hepatic metabolism occurs wherein the activities of the amino-acid-catabolising

enzymes are increased, usually through increases in their tissue concentrations (Krebs, 1972). For example, there is a co-ordinated increase in the levels of the urea cycle enzymes in the livers of animals fed for several days on a high-protein diet (Schimke, 1962). In addition, short-term mechanisms exist to increase the activity of amino acid-catabolising enzymes in response to an excess of amino acids that may occur following an individual protein meal. As discussed in Chapter 1, short-term increases in hepatic enzyme activities are known to occur through two common mechanisms. One mechanism, that of highly inducible enzymes with short half-lives, is exemplified by tyrosine aminotransferase. The amount of such enzyme proteins increases rapidly after a meal and subsequently returns to low levels prior to the next meal (Krebs, 1972). However, it is unlikely that enzyme induction can account for the large stimulation of glycine and glutamine catabolism that occurs within two hours of the start of a high-protein meal. In the case of glutaminase, this contention is supported by the finding that glutaminase activity, measured in broken mitochondria, was not changed by the ingestion of the high-protein meal. The second mechanism involves covalent modification, where activities of enzymes such as branched-chain oxo-acid dehydrogenase are modulated by a reversible phosphorylation/dephosphorylation of enzyme subunits. Ingestion of a high-protein diet, for example, significantly

increases the degree of activation of rat liver branched-chain oxo-acid dehydrogenase, without causing an increase in the amount of the enzyme (Miller et al., 1988). However, regulation of glutaminase or the glycine cleavage system by covalent modification has not been described. In our experiments, the mitochondria were washed three times during the isolation procedure with no particular precautions taken to preserve phosphorylation state. So it would seem the effect is somehow more long-lived than typically labile covalent modification.

It is possible that the activation of these processes following a high-protein meal involves alterations in the association of glutaminase and the glycine cleavage system with the inner mitochondrial membrane. This is suggested by the effect of hypotonic conditions on flux through glutaminase (McGivan et al., 1985) and the glycine cleavage system (**Chapter 3**; see also Brosnan et al., 1990) in isolated mitochondria or perfused liver (Pfaller et al., 1993), and by the disappearance of the stimulatory effect of high-protein feeding on glutaminase flux upon disruption of the mitochondrial membrane (**Chapter 4**). McGivan and colleagues (1985), for example, have shown that glutaminase displays different kinetic characteristics when separated from the membrane and suggested that glutaminase may be regulated in situ by a reversible interaction with the mitochondrial membrane. The characteristics of the

stimulation of glutaminase during a high-protein meal are consistent with this proposal, especially with respect to the phosphate activation of the enzyme. In the case of the glycine cleavage system, a change in the association of the complex with the inner membrane could influence the disposition of the various proteins in the glycine cleavage complex toward one another. In particular, the glycine decarboxylase (P-protein) is virtually inactive unless it is coupled with H-protein (Hiraga and Kikuchi, 1980a). Thus, the short-term regulation of the glycine cleavage system could involve membrane-mediated changes in the coupling of these two proteins.

The findings reported here bear strong resemblance to the activation of glycine and glutamine catabolism by hormones, particularly glucagon. Hormones have been shown to result in the activation of a number of mitochondrial processes (see Halestrap, 1989). A fundamental question regarding glucagon and other hormones, which act at the plasma membrane, is how their signal is transmitted to mitochondria. Work from our laboratory suggests that both cAMP and calcium may be important signals in the hormonal stimulation of the glycine cleavage system. The addition of dibutyryl-cAMP, for example, is as effective as glucagon in stimulating flux through the glycine cleavage system in hepatocytes (Jois et al., 1989) and a correlation between intracellular cAMP concentration and flux through the glycine

cleavage system exists (Jois et al., 1990a). The glycine cleavage system in intact mitochondria is also sensitive to changes in the level of free calcium concentration (**Chapter 3**; see also Jois et al., 1990b) and is stimulated by α-adrenergic agonists and vasopressin, which are known to exert their effects by increasing the concentration of free calcium in the cytoplasm (Exton, 1985; Williamson et al., 1985). Similar observations have been made regarding the stimulation of hepatic glutaminase by hormones (Corvera and Garcia-Sainz, 1983). Since glucagon and cAMP have also been shown to elevate cytoplasmic calcium levels (Charest et al., 1983), it is clear that the effects of this hormone on the glycine cleavage system and glutaminase could be mediated by calcium. The relative importance of glucagon, other hormones, or nervous stimuli in the activation of hepatic glycine and glutamine catabolism following a high-protein meal is unclear. However, we consider it likely that the increase in circulating glucagon evident during ingestion of a high-protein meal (Robinson et al., 1981) is an important signal for the activation of these processes.

While we propose that in vivo release of glucagon plays an important role in eliciting the activation of hepatic glycine and glutamine metabolism following a high-protein meal, it is clear from our findings regarding succinate oxidation (**Chapter 4**) that glucagon injection does not completely mimic the high-protein meal situation and

therefore, other factors, in addition to glucagon, should also be considered. Recent work from a number of laboratories suggests that cell volume changes play an important role in regulating hepatic metabolism (see Häussinger and Lang, 1991). Thus an increase in liver cell volume has been shown to stimulate glycogen synthesis (Baquet et al., 1990) and glycolysis (Lang et al., 1989), to inhibit proteolysis (Häussinger et al., 1991), to activate glutaminase and inhibit glutamine synthetase (Häussinger et al., 1990) and, recently, to stimulate flux through the glycine cleavage system (Häussinger et al., 1992). An increase in liver cell volume occurs rapidly upon exposure to insulin, an effect which can be reversed by glucagon (Hallbrucker et al., 1991). An increase in cell volume can also be brought about in hepatocytes or perfused liver by hypotonic conditions or by sodium-driven uptake of amino acids, particularly that of glutamine, glycine, proline, and alanine (Wettstein et al., 1990). In perfused liver for example, glutamine (3 mM) results in about a 6% increase in cell volume with a one-half maximal effect occurring near the physiological portal concentration of 0.6 mM (Wettstein et al., 1990). During a high-protein meal the total concentration of amino acids in the portal blood may increase by more than 5 mM (Fafournoux et al., 1990), and therefore, it is conceivable that sodium-dependent uptake of amino acids by the liver at this time

leads to liver cell swelling, with subsequent activation of cellular metabolism. An increase in liver cell volume has been shown to occur in rats during the fasted-to-fed transition (Agius et al., 1991). In these studies, refeeding with carbohydrate plus casein resulted in a greater increase in liver weight compared to carbohydrate alone, an effect attributed to amino acid-induced cell swelling. However in isolated hepatocytes, glucagon which is known to cause cell shrinkage, stimulates flux through the glycine cleavage system and flux through glutaminase. Therefore, the hormonal activation of these enzymes does not require cell swelling and, indeed, occurs in conditions associated with cell shrinkage. There is, therefore, no compelling evidence to evoke hepatocyte swelling in the physiological activation of glutaminase and the glycine cleavage system.

The disposal of an excess of amino acids after ingestion of a high-protein meal involves the activation of a variety of liver enzymes, both cytosolic and mitochondrial. In this thesis a high-protein meal has been shown to bring about an activation, in mitochondria, of the glycine cleavage system, glutaminase and of citrulline synthesis. These processes appear to be inter-related. Flux through glutaminase, for example, has been reported to provide ammonia for direct utilisation by carbamylphosphate synthase I (Meijer, 1985) and recently glutamine has been shown to be the principle provider of nitrogen for urea synthesis (Nissim et al.,

1992). Thus, these activations may be viewed as part of a coordinated mechanism for regulating hepatic mitochondrial amino acid metabolism, which is physiologically important in permitting the liver to deal with the increased amino acids after a single high-protein meal.

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Appendix A

Flow rate, oxygen pressure, carbon dioxide pressure and pH of effluent during perfusion of livers. Rats fed the normal protein diet for 3-4 days were given a high-protein (HP) or normal-protein (NP) meal for two hours. The livers were then perfused as described in the Methods. Controls were perfused at the beginning of the meal period. Flow was calculated from the weight of effluent collected during a 30 second interval. Other parameters were determined on a blood-gas analyser. Results are the mean ± s.e.m. for the values obtained in the experiments reported in **Fig. 5.2.**

	Time (min)	Flow (ml/min)	O_2 pressure (mm Hg)	CO_2 pressure (mm Hg)	pH
Pre-meal	5	60.0 ± 3.6	301.7 ± 32.5	36.0 ± 1.2	7.37 ± 0.01
	10	56.8 ± 2.3	225.3 ± 32.5	35.3 ± 1.3	7.38 ± 0.01
	15	55.9 ± 3.6	196.7 ± 14.8	35.7 ± 0.6	7.36 ± 0.01
	20	55.7 ± 4.6	172.7 ± 16.0	37.0 ± 1.0	7.35 ± 0.01
	25	54.7 ± 4.2	151.3 ± 15.5	38.0 ± 1.0	7.34 ± 0.01
	30	51.3 ± 4.5	137.3 ± 17.8	38.7 ± 0.7	7.33 ± 0.01
NP meal	5	59.0 ± 2.4	183.3 ± 56.1	39.3 ± 2.8	7.33 ± 0.03
	10	63.1 ± 2.2	178.8 ± 40.0	39.3 ± 2.5	7.34 ± 0.03
	15	61.4 ± 2.3	157.5 ± 46.5	39.8 ± 2.9	7.32 ± 0.03
	20	64.0 ± 1.6	151.5 ± 45.2	40.5 ± 2.6	7.32 ± 0.02
	25	63.5 ± 2.1	151.3 ± 42.2	41.3 ± 2.6	7.31 ± 0.02
	30	61.0 ± 1.4	129.5 ± 38.7	38.3 ± 3.5	7.29 ± 0.03
HP meal	5	60.1 ± 3.8	230.7 ± 63.0	38.3 ± 1.0	7.34 ± 0.01
	10	59.5 ± 2.9	202.4 ± 49.4	36.0 ± 1.3	7.35 ± 0.01
	15	58.2 ± 2.5	174.4 ± 57.6	37.8 ± 1.5	7.33 ± 0.01
	20	59.8 ± 1.5	199.6 ± 49.0	39.2 ± 1.5	7.32 ± 0.01
	25	57.6 ± 2.1	172.6 ± 38.7	40.6 ± 1.7	7.30 ± 0.01
	30	56.9 ± 2.3	105.3 ± 14.8	39.0 ± 2.9	7.31 ± 0.01

Appendix B

Oxygen consumption rate of livers during the perfusions. Rats fed the normal protein diet for 3-4 days and were given either a high-protein (●) or normal-protein (O) meal for two hours. The livers were perfused as described in the Methods. The livers of animals not receiving a meal (∇) were perfused at the beginning of the meal period. Oxygen comsumption rate was calculated as the difference between the influent oxygen pressure (670 to 700 mm Hg) and the effluent oxygen pressure (see **Appendix A**). Results are the mean ± s.e.m. for the values obtained in the experiments reported in **Fig. 5.2.**