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Biochemistry of Glutamate: Glutamine and Glutathione

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Glutamate and two of its γ-linked derivatives, glutamine and glutathione, play central roles in the metabolism of amino acids and ammonia. In this chapter, I will present an outline of the metabolism of glutamate in mammalian tissues and review some recent studies on glutamate metabolism and on the function of the γ-glutamyl moiety that have been carried out in our laboratory.

OUTLINE OF GLUTAMATE METABOLISM

The scheme given in Fig. 1 summarizes what appear to be the major enzyme-catalyzed reactions involved in the metabolism of glutamate. α-Ketoglutarate may be converted to glutamate by reductive amination catalyzed by glutamate dehydrogenase (reaction 2) and by transamination reactions involving a number of amino acids (reaction 3) (26). The quantitative significance of the glutamate dehydrogenase reaction in the formation of glutamate in mammals requires additional study. If the relatively high K_m value for ammonia of mitochondrial liver glutamate dehydrogenase reflects its *in vivo* affinity for ammonia, one may seriously question a biosynthetic role for this enzyme. On the other hand, glutamate dehydrogenase might be linked *in vivo* with an enzyme such as glutaminase (reaction 5) so as to make the amide nitrogen atom of glutamine directly available for the synthesis of glutamate and thus also for the formation of the α-amino groups of amino acids. [There is at this time no evidence for the presence of glutamate synthase in mammalian tissues, but the presence in mammals of this enzyme or of a complex representing its catalytic equivalent is not excluded (17)]. Liver carbamyl phosphate synthetase I (reaction 7) is a major catalyst for ammonia utilization in this organ (19,42). This enzyme requires N-acetylglutamate for activity and the rate and extent of synthesis of this cofactor may play a role in the regulation of carbamyl phosphate synthesis. Glutamate is also formed in the degradation of arginine, ornithine, proline, and histidine (26). Ammonia is also formed in other reactions, for example, in the deamination of adenosine 5′-monophosphate (24).

Glutamate is used directly for protein synthesis; thus, the α-carboxyl group of this amino acid is activated by a specific aminoacyl tRNA synthetase (8,22). A quantita-

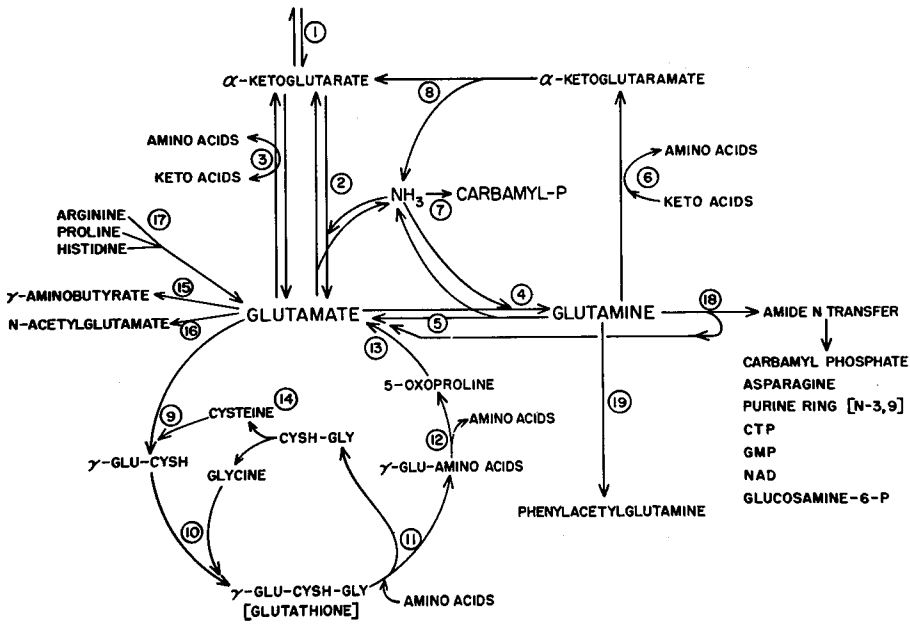


FIG. 1. An outline of glutamate metabolism in mammalian tissues. 1, Reactions of the citric acid cycle; 2, glutamate dehydrogenase; 3, glutamate transaminases; 4, glutamine synthetase; 5, glutaminase; 6, glutamine transaminase; 7, carbamyl phosphate synthetase (liver); 8, α-keto acid ω-amidase; 9, γ-glutamyl cysteine synthetase; 10, glutathione synthetase; 11, γ-glutamyl transpeptidase; 12, γ-glutamyl cyclotransferase; 13, 5-oxoprolinase; 14, cysteinylglycinase; 15, glutamate decarboxylase; 16, glutamate N-acylase; 17, various enzymes involved in the degradation of these amino acids; 18, glutamine amidotransferases known to occur in mammalian tissues; and 19, phenylacetyl glutamine synthetase (Acyl-CoA-L-glutamine N-acyltransferase).

tively minor, but physiologically significant, pathway of glutamate metabolism involves decarboxylation to γ-aminobutyrate, a putative neurotransmitter. There is growing evidence that both γ-aminobutyrate and glutamate are of importance as inhibitory and excitatory neurotransmitters, respectively (12). As discussed below, glutamate is a precursor of two γ-glutamyl compounds of major biochemical importance: glutamine and glutathione. Glutamine occurs both intra- and extracellularly, but glutathione has a predominantly intracellular localization.

METABOLISM OF GLUTAMINE

The conversion of glutamate to glutamine, catalyzed by glutamine synthetase, is of great significance in the utilization of ammonia. This reaction takes place in a number of mammalian tissues, e.g., liver, brain, kidney, muscle, and intestine (28). Glutamine, which is widely distributed in mammalian tissues, is not only an essential building block of proteins, but is a central compound in nitrogen

metabolism. Glutamine functions in the uptake, storage, and formation of ammonia; the homeostatic control of amino acid balance; the synthesis of the purine and pyrimidine moieties of nucleic acids, ATP and other nucleotides, and the amide groups of the pyridine nucleotide coenzymes; the formation of amino sugars; and the biosynthesis of a number of amino acids and other compounds of biological importance. Although glutamine is not a dietary essential amino acid, it is nevertheless usually the amino acid present in highest concentration in mammalian blood plasma. Glutamine is also present in high concentrations in mammalian tissues. Glutamine, which crosses cell membranes more readily than glutamate, appears to serve as a transport form of both glutamate and ammonia. The physiological function of glutamine varies depending on the tissue or cell. For example, in the kidney, glutamine is a major source of energy and of urinary ammonia (3). The role of glutamine in the brain seems related to the synaptic functions of glutamate and γ-aminobutyrate. In certain plants, glutamine is a major storage form of nitrogen, and in a number of microorganisms glutamine functions as an essential intermediate in the assimilation of nitrogen. It seems notable that in man (and in some of his close relatives) glutamine is coupled with phenylacetate to form phenylacetylglutamine, a so-called detoxication reaction that does not occur in most mammals (37,56).

The conversion of glutamine to glutamate is effected by several enzymes, including glutamine synthetase, which catalyzes a reversible reaction (23); such reversal is probably not of physiological importance since the equilibrium of this reaction lies distinctly in the direction of synthesis. Glutamine is hydrolyzed to glutamate and ammonia by glutaminase, a reaction of particular importance in the kidney, but one which takes place in other tissues as well. There is also a rather heterogeneous group of enzyme-catalyzed reactions in which the amide nitrogen atom of glutamine is utilized (with concomitant formation of glutamate) in reactions leading to the formation of new compounds. These reactions are catalyzed by the glutamine amidotransferases, and 13 such catalytic activities have thus far been identified (6,31). Glutaminase may be considered as a glutamine amidotransferase in which the amide group is transferred to a hydrogen ion. Glutamine amidotransferases are involved in the biosynthesis of both the purine and pyrimidine rings and in the introduction into these rings of certain amino groups. The amidotransferases also catalyze reactions leading to the synthesis of several amino acids, including asparagine, arginine, and (in bacteria) of glutamate (glutamate synthase), tryptophan, histidine, and *p*-aminobenzoate. A glutamine amidotransferase is also involved in the conversion of fructose-6-phosphate to glucosamine-6-phosphate and of deamido-NAD to NAD.

Although recent biochemical studies tend to emphasize the reactions catalyzed by the glutamine amidotransferases, reactions involving the α-amino group of glutamine are probably also of considerable metabolic and physiological significance. Reactions of the latter type are catalyzed by the physiologically coupled enzymes glutamine transaminase and α-keto acid ω-amidase (9–11,35). There are several separate glutamine transaminases that exhibit high affinity for glutamine and

certain α-keto acids. Glutamate-aspartate and glutamate-alanine transaminases, and the transaminases that catalyze various α-ketoglutarate-amino acid transamination reactions (including those that involve branched-chain and aromatic amino acids) do not act on glutamine at significant rates. The glutamine transaminase reactions, like other transamination reactions, are freely reversible, but in contrast to reactions such as those catalyzed by glutamate-aspartate transaminase, glutamine transaminases catalyze reactions that proceed, under physiological conditions, in the direction of glutamine utilization rather than its synthesis. The steady-state concentrations of α-ketoglutaramate in mammalian tissues are relatively low (13), and it appears that, in the presence of α-keto acid ω-amidase, the open-chain form of α-ketoglutaramate formed in the transamination of glutamine undergoes rapid enzyme-catalyzed deamidation yielding α-ketoglutarate. Therefore, the transamination of glutamine is essentially irreversible *in vivo,* and it follows that its metabolic role must be associated with the utilization of glutamine, formation of ammonia, and the utilization of certain α-keto acids for the synthesis of the corresponding amino acids. Although there is relatively little evidence that the glutamine transaminase-ω-amidase pathway has a major role in ammoniagenesis, it may contribute to ammonia formation to some extent. The most plausible idea concerning the physiological role of glutamine transaminases is that they function in the conversion of α-keto acids to amino acids. The major degradative pathways of many amino acids lead initially to the formation of the corresponding α-keto acids, but there are some notable exceptions, including, for example, phenylalanine and methionine (26). The concentrations of amino acids in mammalian tissues probably fluctuate depending on nutritional and other factors, and it therefore appears likely that certain amino acids are temporarily accumulated in amounts that exceed those necessary for the synthesis of proteins and for the formation of other products. When such amino acids accumulate, they may undergo transamination to the corresponding α-keto acids catalyzed by α-ketoglutarate-amino acid transamination reactions; indeed, it has long been known that such reactions occur in a number of mammalian tissues. Under these circumstances, the carbon chains of amino acids, such as phenylalanine, tyrosine, and methionine, might be lost by excretion or degradation and therefore would become unavailable for protein synthesis. It is of metabolic importance to retain these essential carbon chains, and it appears that the glutamine transaminases function in this salvage process. The transamination reactions between glutamine and α-keto acids are driven by the removal of the α-keto acid product (α-ketoglutaramate), and the metabolic balance tends to be further stabilized by the formation of ammonia, which can be used for glutamine synthesis. Thus, one can picture a mechanism by which the glutamine transaminases serve as part of a homeostatic mechanism for the preservation of amino acid balance. Such a process may be illustrated by the following example:

$$\text{phenylpyruvate} + \text{glutamine} \rightarrow \alpha\text{-ketoglutaramate} + \text{phenylalanine}$$
$$\alpha\text{-ketoglutaramate} \rightarrow \alpha\text{-ketoglutarate} + NH_3$$
$$\text{glutamate} + NH_3 + ATP \rightarrow \text{glutamine} + ADP + P_i$$

Sum: phenylpyruvate + glutamate + ATP → phenylalanine + α-ketoglutarate + ADP + P_i

The overall reaction involves a large free energy change and the formation of phenylalanine. These considerations emphasize the metabolic importance of glutamine transaminase and glutamine synthetase in amino acid formation and in the recovery of the carbon chains of certain amino acids.

It has also been suggested that the glutamine transaminases function in transport phenomena. Possibly, certain amino acids are transported into or out of cells or intracellular organelles as the corresponding α-keto acids; in such a system, the α-keto acid might be formed on one side of the membrane and reaminated on the other. This concept is, of course, consistent with a large body of data showing that there is extensive deamination and reamination of most of the amino acids in mammalian species. The initial studies in this area were performed by Schoenheimer and colleagues, who found that when $^{15}NH_4^+$ or ^{15}N-labeled amino acids were given to rats, the isotope appeared in almost all of the amino acids (52). It is also known that the α-keto analogs of most of the amino acids can replace the corresponding L-amino acids in the diet (26). In studies on patients with phenylketonuria, it was found that the administration of glutamine led to substantially decreased urinary excretion of phenylpyruvate (36). This result can be explained by the occurrence of glutamine-phenylpyruvate transamination and suggests that a metabolic abnormality accompanied by an accumulation of an α-keto acid can be corrected, at least partially, by a mechanism involving transamination. Recent attempts have been made to treat patients with "nitrogen accumulation diseases" by the administration of α-keto acids (4,7,25,46,49–51,65–67). Thus, patients with chronic renal failure, who accumulate urea and other nitrogen-containing compounds, were improved by administration of mixtures containing the α-keto acid analogs of the essential amino acids. Similarly, studies on obese patients undergoing a starvation therapy showed that administration of α-keto acids decreased the loss of nitrogen and apparently improved the efficiency of utilization of amino acids. It seems probable that therapy with α-keto acids decreases the formation of urea by diverting nitrogen from the carbamyl phosphate synthetase pathway into the pathways leading to formation of glutamate and glutamine. The accumulated evidence indicates that mammalian tissues have a homeostatic metabolic mechanism for preserving amino acid balance in which the dietary nonessential amino acids, especially glutamine, function to maintain the tissue levels of the other amino acids by preventing the loss of essential carbon chains. The glutamine transaminase-ω-amidase system seems to be a physiologically significant catalyst in this process.

METABOLISM OF GLUTATHIONE

The findings reviewed above support the view that conversion of glutamate to glutamine is of crucial importance in various biosynthetic processes, transport phenomena, preservation of amino acid balance, and ammonia metabolism. How-

ever, glutamate is also converted to another γ-glutamyl compound of major metabolic significance, namely, glutathione (γ-glutamylcysteinylglycine). Glutamate is used, together with cysteine and glycine for the biosynthesis of glutathione. The enzymatic synthesis and degradation of glutathione take place by a cyclic metabolic pathway, the γ-glutamyl cycle (Fig. 1; reactions 9–14). This pathway was elucidated in our laboratory through studies on the two enzymes that catalyze the synthesis of glutathione and by experimental work that demonstrated that 5-oxoproline is a quantitatively significant metabolite of glutathione; 5-oxoproline was also found in mammalian tissues and body fluids (27,33,38,61–64). Although 5-oxoproline was previously known to be formed in the degradation of glutathione by tissue preparations, the significance of this finding was not apparent, and the possibility was seriously considered that the formation of 5-oxoproline under these conditions might be an artifact. Although mammalian tissues and body fluids contain a measurable steady-state concentration of 5-oxoproline, this compound does not normally accumulate to an appreciable extent. The discovery of the enzyme 5-oxoprolinase (Fig. 1, reaction 13) showed that there is a metabolic link between the reactions involved in the degradation of glutathione and those that catalyze its synthesis, making it possible to visualize the γ-glutamyl cycle.

Glutathione is synthesized in many mammalian tissues, including liver, kidney, brain, intestine, lens, muscle, and the erythrocyte (30). Highly purified preparations of γ-glutamylcysteine synthetase and glutathione synthetase have been obtained from several sources, and the mechanisms of actions of these enzymes have been extensively examined (29). The initial step in the breakdown of glutathione is catalyzed by γ-glutamyl transpeptidase, which catalyzes transfer of the γ-glutamyl moiety of glutathione (and other γ-glutamyl compounds) to amino acid and other acceptors. γ-Glutamyl transpeptidase is bound to membranes of various epithelial cells, for example, proximal renal tubules, jejunal villi, choroid plexus, ciliary body, visual receptor cells, retinal epithelium, and cerebral astrocytes and their capillaries (34). Highly purified preparations of γ-glutamyl transpeptidase have been obtained from kidney and other tissues (33). The cysteinylglycine formed in the transpeptidase reaction is cleaved by widely distributed peptidase activity to form cysteine and glycine. The γ-glutamyl amino acids formed by transpeptidation may be substrates for additional transpeptidation reactions and may be converted to 5-oxoproline and the corresponding amino acids by the soluble enzyme, γ-glutamyl cyclotransferase. Highly purified preparations of the cyclotransferase have been obtained from brain (40), liver (1,39), and kidney (57). The 5-oxoproline formed in the reaction catalyzed by the γ-glutamyl cyclotransferase is converted to glutamate by 5-oxoprolinase:

$$\text{5-oxo-L-proline} + \text{ATP} + 2\text{H}_2\text{O} \rightarrow \text{L-glutamate} + \text{ADP} + \text{P}_i$$

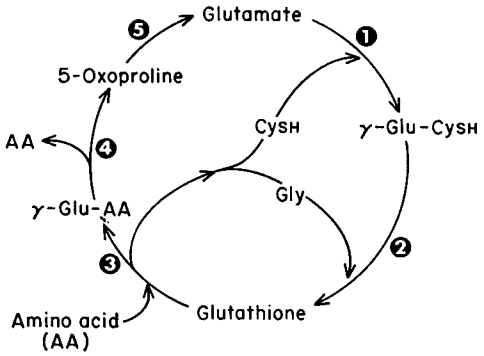
5-Oxoprolinase activity is widely distributed, having been found in kidney, liver, brain, and other mammalian tissues, and a highly purified preparation of the enzyme has been obtained from rat kidney (60).

Although the initial formulation of the γ-glutamyl cycle was derived largely from

enzyme data, there is now excellent evidence that the reactions of the γ-glutamyl cycle take place *in vivo*. Indeed, each of the reactions has been demonstrated by metabolite-labeling studies or through use of specific enzyme inhibitors. It is notable that the turnover of glutathione, as measured by the incorporation of labeled glutamate or labeled 5-oxoproline into glutathione, is substantially higher in mouse kidney than in liver (54); this result reflects the relatively higher activities of the γ-glutamyl cycle enzymes in kidney than in the liver. The rate of incorporation of 5-oxoproline into glutathione is similar to that of glutamate incorporation. After administration of labeled glutamate, labeled 5-oxoproline is found in kidney and liver (55). Further evidence for the *in vivo* function of the cycle has come from studies in which animals were treated with a competitive inhibitor of 5-oxoprolinase (L-2-imidazolidone-4-carboxylate) (63,64). Treatment of mice with this inhibitor decreases their ability to convert labeled 5-oxoproline to labeled respiratory carbon dioxide. Such mice accumulate 5-oxoproline in several tissues, including liver, kidney, and brain. Mice treated with the inhibitor together with various amino acids accumulate substantial amounts of 5-oxoproline in their tissues. An increase in the accumulation of 5-oxoproline after administration of amino acids can be explained in terms of the γ-glutamyl cycle, since it would be expected that the increase in amino acid concentration produced by administering amino acids would be accompanied by increased utilization of glutathione in transpeptidation and thus by increased formation of 5-oxoproline. Additional evidence that the γ-glutamyl cycle functions *in vivo* has come from studies on patients with the inborn error of glutathione metabolism, 5-oxoprolinuria; in this condition, there is a modified γ-glutamyl cycle associated with a marked deficiency of glutathione synthetase activity (32,68).

In an effort to further elucidate the *in vivo* function of the γ-glutamyl cycle, we have looked for specific inhibitors of the several reactions of the cycle and also for analogs of the substrates that would function in some, but not all, of the reactions of the cycle. Although most of these reactions involve the γ-carboxyl group of glutamate, we reasoned that the several enzymatic reaction pathways must be different and that they are probably therefore associated with differences in the enzyme-bound conformations of the glutamate carbon chain at the active sites of the various enzymes. We attempted to exploit such expected differences among the enzymes by variation of substrate structure. We found that suitable modification of the glutamyl moiety of the substrates can indeed produce the desired results, i.e., effective and specific inhibitors or nonmetabolizable analogs at each of the steps of the γ-glutamyl cycle (15).

The enzymes required for the two-step synthesis of glutathione also catalyze the synthesis of the naturally occurring analogs, ophthalmic and norophthalmic acids, compounds in which the cysteine moiety is replaced by α-aminobutyrate and alanine, respectively. As indicated in Fig. 2, glutathione synthetase exhibits a much broader specificity towards substrates in which the glutamyl group is modified than does γ-glutamylcysteine synthetase. It is notable that glutathione synthetase is active towards D-γ-glutamyl-L-α-aminobutyrate; in contrast, D-glutamate is not an



Substrate Analogs	ENZYMES ❶ γ-Glu-CySH Synthetase	❷ GSH Synthetase	❸ γ-Glu Trans-peptidase	❹ γ-Glu Cyclo-transferase	❺ 5-Oxoprolinase
D-Glu	0,↓	+	+	0,↓	0
α-Methyl-Glu	(+)	+	+	0	0
N-Methyl-Glu	+	+	0	0	0
β-Glu	+	+	0	0,↓	
β-Methyl-Glu	(+),↓	+,↓	0	+	0,↓
γ-Methyl-Glu	0,↓	+	0	0	(+),↓

FIG. 2. Interaction of glutamate and glutamyl analogs with enzymes of the γ-glutamyl cycle. CySH, cysteine; GSH, glutathione; ↓, > 50% inhibition; +, > 10% as active as L-Glu; (+), 1–10% as active as L-Glu; 0, < 1% as active as L-Glu. (From Griffith and Meister, ref. 15.)

appreciable substrate for peptide synthesis by γ-glutamylcysteine synthetase. γ-Glutamylcysteine synthetase is markedly inhibited by β-methylglutamate and γ-methylglutamate, as well as by D-glutamate. Studies on γ-glutamyl transpeptidase have shown that this enzyme is significantly active with analogs containing D-glutamyl and α-methylglutamyl residues, but is not active with substrates that have N-methylglutamyl, β-glutamyl, β-methylglutamyl, or γ-methylglutamyl groups. We found previously that D-γ-glutamyl-*p*-nitroanilide is a good substrate of the transpeptidase; because D-amino acids are not acceptor substrates, D-γ-glutamyl-*p*-nitroanilide may be used in a convenient assay procedure that precludes autotranspeptidation (58,59). α-Substituted amino acids are not acceptors of the γ-glutamyl group, and, therefore, autotranspeptidation does not occur with α-methyl-L-glutamyl-α-aminobutyrate (21). The findings given in Fig. 2 thus indicate that it might be possible to achieve *in vivo* synthesis of the analogs of glutathione (e.g., those containing *N*-methylglutamyl or β-glutamyl residues), which would be inactive in transpeptidation. It would be of interest to learn whether such analogs might be active in reactions involving the sulfhydryl group. Of the several substrate analogs examined, only γ-(β-methyl)-glutamyl-L-α-aminobutyrate

is a substrate of γ-glutamyl cyclotransferase. That glutamyl derivatives modified at the α-carbon atom (e.g., D-glutamyl and α-methylglutamyl) are not active seems to reflect the requirement in the cyclotransferase reaction of bringing the α-carbon atom quite close to the catalytic center of the active site. Requirements for size and alignment at the active site of this enzyme would be expected to be stringent. These studies have led to the finding of two good inhibitors of γ-glutamyl cyclotransferase, i.e., β-aminoglutaryl-L-α-aminobutyrate and D-γ-glutamyl-L-α-aminobutyrate. 5-Oxoprolinase exhibits activity towards piperidone-6-carboxylate and the 3- and 4-hydroxy derivatives of 5-oxoproline. As stated above, L-2-imidazolidone-4-carboxylate is a potent competitive inhibitor *in vitro;* this compound is also active *in vivo.*

The studies summarized in Fig. 2 suggest the feasibility of a number of *in vivo* approaches in which selective inhibition of individual reactions of the cycle might be achieved. There is evidence that the synthesis of glutathione is normally controlled by feedback inhibition of γ-glutamylcysteine synthetase by glutathione; this reaction is probably also affected by the tissue concentration of cysteine (43). Decreased tissue levels of glutathione occur after administration of D-glutamate (55), and this amino acid, as well as β-methylglutamate and γ-methylglutamate, would be expected to inhibit glutathione biosynthesis on the basis of the enzyme data. The findings also suggest that it might be possible to introduce *in vivo* a glutathione analog possessing a D-glutamyl or γ-methylglutamyl moiety; this could be accomplished by administering the appropriate carboxyl terminal cysteine dipeptide. The corresponding modified glutathiones would be expected to be substrates for the transpeptidase, but not of the cyclotransferase. Studies with D-γ-glutamyl or L-α-methylglutamyl-L-amino acids might therefore elucidate the extent to which such compounds are hydrolyzed *in vivo* by the action of γ-glutamyl transpeptidase.

It has recently been possible to examine the inhibition of γ-glutamyl cyclotransferase *in vivo* by giving the inhibitor (β-glutamyl-α-aminobutyrate (5,14b). In these studies, mice injected with β-glutamyl-α-aminobutyrate were found to have a moderate, but significant, depression of the steady-state concentration of 5-oxoproline in the kidney. Furthermore, administration of β-glutamyl-α-aminobutyrate prevented the accumulation of 5-oxoproline that occurs after the administration of large amounts of amino acids and that which occurs in the presence of the 5-oxoprolinase inhibitor, 2-imidazolidone-4-carboxylate. These observations provide strong support for the view that 5-oxoproline is formed *in vivo* by the action γ-glutamyl cyclotransferase. The findings with β-glutamyl-α-aminobutyrate, together with those cited above in which animals were treated with L-2-imidazolidone-4-carboxylate, are in accord with the conclusion that γ-glutamyl cyclotransferase and 5-oxoprolinase are, respectively, major *in vivo* catalysts for the formation and utilization of 5-oxoproline.

In considering the physiological function or functions of the γ-glutamyl cycle, it seems of significance that the first step in the degradation of glutathione is catalyzed by a membrane-bound enzyme, and that the reaction catalyzed by γ-glutamyl transpeptidase is greatly stimulated by certain amino acid and peptide acceptors.

The acceptor amino acids are not metabolized in the cycle, but are released unchanged. The other enzymes of the cycle are present in the cytosol. One must consider the possibility that the function of the transpeptidase is to regulate the intracellular concentration of glutathione. However, there is an effective feedback mechanism for the control of glutathione biosynthesis, namely, inhibition of γ-glutamylcysteine synthetase by glutathione (43). Furthermore, the transpeptidase is linked to the membrane, and a variety of studies have indicated that it is readily accessible to externally supplied substrates (33). Therefore, at least some of the transpeptidase appears to be on the outer surface of the membrane; however, one cannot exclude the possibility that it is a transmembrane enzyme. Its location is consistent with a role in the transport of compounds into or out of the cell, but other membrane-related functions, such as those involved in protection and structure, need to be considered.

Earlier studies on amino acid transport, based largely on the kinetics of this process, led to the proposal that the transport of amino acids involves a number of steps, such as the binding of the amino acid to a site on the cell membrane, carrier-mediated translocation, intracellular release of the amino acid from the carrier, and reactivation of the carrier in an energy-requiring process (18,41). The hypothesis that the γ-glutamyl cycle might function in transport followed from the recognition that the cycle has features previously postulated to be involved in amino acid transport. For example, the membrane-bound transpeptidase might mediate the binding of amino acid and its translocation. According to this idea, the enzyme interacts with extracellular amino acid and intracellular glutathione (or perhaps another γ-glutamyl donor derived from glutathione) to yield a γ-glutamyl amino acid which is translocated. (Whether the transpeptidase itself is involved in the translocation process is not yet clear.) Release of the amino acid from its γ-glutamyl carrier within the cell is catalyzed by γ-glutamyl cyclotransferase. The energy-requiring portion of the cycle involves resynthesis of the precursor of the γ-glutamyl carrier, i.e., glutathione. Various modifications of the γ-glutamyl cycle have been suggested; for example, glutamine might play a special role. γ-Glutamylglutamine might be readily formed by transpeptidation; γ-glutamylglutamine is an active γ-glutamyl donor, and the high concentration of glutamine in mammalian body fluids and tissues suggests that this amino acid might be formed. Indeed, there is evidence for the presence of γ-glutamylglutamine in a variety of tissues (20). A prominent role for glutamine in the cycle is also in accord with the relatively restricted specificity of γ-glutamyl cyclotransferase. The most active substrates for this enzyme are γ-glutamylglutamine and a variety of γ-glutamyl-γ-glutamyl amino acids (57).

One may also consider the possibility of a cycle that requires the cleavage of only 2 ATP molecules per turn; this might take place if the formation of 5-oxoproline were by-passed by hydrolysis (catalyzed by the transpeptidase) of the γ-glutamyl amino acid (33). Even a cycle involving cleavage of only one molecule of ATP is conceivable if there are successive transpeptidation reactions, one of which involves cysteine. A model for exchange diffusion has also been considered in which

enzyme-γ-glutamyl amino acid complexes participate (33). The concept of the γ-glutamyl cycle thus seems to offer a number of possible pathways, based on the function of the γ-glutamyl moiety, for the translocation of amino acids across the cell membrane. Perhaps the transport of amino acids follows more than a single γ-glutamyl cycle pathway. The transpeptidase is most active toward glutamine, cystine, and several other neutral amino acids; these amino acids might be more effectively transported by the cycle than others. Aspartate and proline are poor substrates for the transpeptidase and are probably transported by other systems.

Various blocks or partial blocks of the γ-glutamyl cycle have been studied, and the observations made are consistent with the view that the γ-glutamyl cycle functions *in vivo* (33). Evidently, a block of glutathione synthetase, as found in 5-oxoprolinuria (32,68) does not stop the cycle because γ-glutamylcysteine is good substrate for the transpeptidase. On the other hand, a complete block of γ-glutamyl-cysteine synthetase would be expected to stop the cycle and to produce defects in amino acid transport if the transport hypothesis is correct. Therefore, it is interesting that the patients with a severe block of γ-glutamylcysteine synthetase exhibit aminoaciduria (32,45). A patient reported to have a marked deficiency of γ-glutamyl transpeptidase evidently shows only minor evidence for defective amino acid transport (33,53). It is notable that this patient has substantial glutathionemia and glutathionuria. It seems probable that the presence of glutathione in the urine of this patient reflects its presence in the blood plasma and leakage from the kidney. Perhaps one of the functions of the transpeptidase is to breakdown glutathione that enters the systemic circulation.

Another interpretation is that the genetic defect in this condition is associated with a defect that permits the slow leakage of glutathione from the cell. This patient may have a normal transport mechanism for glutathione, but in the absence of transpeptidase, glutathione appears unchanged outside the cell. Indeed, if the transpeptidase is located on the outer surface of the cell membrane, as seems to be the case, then it is probable that there must normally be transport of glutathione (or of its γ-glutamyl moiety) through the membrane to the enzyme. This follows from the observation that administration of high doses of amino acids to animals leads to a significant decrease in the concentration of glutathione (which is predominantly intracellular) and to a substantial increase in the formation of 5-oxoproline. Such an increase in 5-oxoproline formation is blocked by administration of a specific cyclotransferase inhibitor. Thus, the increased formation of 5-oxoproline and the decrease in intracellular glutathione that occurs after administration of amino acids must be associated with an increase in the intracellular concentration of substrate (γ-glutamyl amino acid) for the cyclotransferase. Such intracellular γ-glutamyl amino acid is presumably formed from intracellular glutathione and extracellular amino acid by the transpeptidase. There must also be transport of γ-glutamyl amino acid into the cell to account for the increase in 5-oxoproline formation. The collected findings support the γ-glutamyl cycle hypothesis for amino acid transport.

Although the presently available data are in general accord with the transport hypothesis, we need to know more about the various quantitative relationships and

about the orientation of the transpeptidase in the cell membrane. It is not yet clear as to whether the transpeptidase itself plays a direct role in translocation or whether it functions to accept glutathione from within the cell and to form γ-glutamyl amino acids, which are then translocated by a separate mechanism.

It should be emphasized that we have not proposed that the γ-glutamyl cycle is the only transport system for amino acids. It may be active only at certain sites and only at certain stages of cellular development; it seems to be more active for certain amino acids than others. Nevertheless, although it has been generally thought that the transport of amino acids (and of a number of other compounds) is mediated by enzymes (or enzyme-like entities—hence, the term "permease" was proposed), the γ-glutamyl cycle hypothesis seems to be the only mechanism thus far suggested for amino acid translocation in which specific enzymes have been implicated.

Glutathione is used for detoxication reactions, some of which lead to the formation of mercapturic acids. It is generally believed the initial step in the formation of mercapturic acids involves a reaction of a foreign compound with the sulfhydryl group of glutathione within the cell to yield a glutathione adduct; this reaction may occur spontaneously or be catalyzed by glutathione-S-transferases. It is notable that the protein ligandin, a basic dimeric cytoplasmic protein that binds various electrophiles noncovalently and possibly some carcinogens covalently, exhibits glutathione-S-transferase activity (2). Thus, the ligandin system may function in the binding and transport of glutathione and of foreign compounds, and as a catalyst for coupling. The further metabolism of such S-substituted glutathione derivatives involves the removal of the γ-glutamyl group by a reaction that appears to be catalyzed by transpeptidase. This is followed by cleavage of the glycine moiety and acetylation (within the cell) of the amino group of the S-substituted cysteine moiety. Thus, it would appear that reactions of the γ-glutamyl cycle can be utilized in the formation of mercapturic acids and that the formation of mercapturic acids involves transport phenomena.

One must also consider various interrelationships between the γ-glutamyl cycle and other metabolic phenomena, which include the metabolism of glutamate, cysteine, and glycine. The cycle also functions to convert cysteine to an apparently less metabolically active tripeptide form. The intracellular concentration of cysteine is regulated at a level that is substantially lower than those of most of the other amino acids. The release of cysteine from glutathione takes place via the γ-glutamyl cycle, which could function in such a manner as to affect the rate of protein synthesis by providing cysteine, which may under some circumstances be rate limiting. Other aspects of the γ-glutamyl cycle have been considered in detail elsewhere (33).

In the course of studies on the conversion of glutamate to glutathione and to glutamine, we have examined in some detail the inhibition of glutamine synthetase and of γ-glutamylcysteine synthetase by methionine sulfoximine (Fig. 3). Investigation of the mechanisms of these enzymatic reactions indicate that enzyme-bound γ-glutamyl phosphate is an intermediate (28). Studies on glutamine synthetase showed that methionine sulfoximine is phosphorylated by ATP on the enzyme to

(a) Glutamate + ATP + Enzyme $\rightleftharpoons$ Enzyme [γ-glutamyl-P; ADP]

(b) Enzyme [γ-Glu-P; ADP] + NH_3 $\rightleftharpoons$ Enzyme + Glutamine + P_i + ADP

(c) Enzyme [γ-Glu-P; ADP] + Cysteine $\rightleftharpoons$ Enzyme + γ-Glu-cySH + P_i + ADP

(d) Enzyme + Methionine sulfoximine + ATP $\longrightarrow$ Enzyme [Methionine sulfoximine phosphate; ADP]

FIG. 3. Mechanism of the reactions catalyzed by glutamine synthetase—(a), (b) and γ-glutamylcysteine synthetase—(a), (c). Inhibition of both of these enzymes by methionine sulfoximine is described in (d).

form methionine sulfoximine phosphate, which binds tightly to the active site of the enzyme, resulting in irreversible inhibition (47). There is good evidence that methionine sulfoximine inhibits glutamine synthetase by serving as an inhibitory analog of the enzyme-bound tetrahedral intermediate or a transition state formed in the reaction catalyzed by this enzyme (14). Substantially the same type of inhibition by methionine sulfoximine occurs with γ-glutamylcysteine synthetase (44). It is interesting that only one of the four isomers of methionine sulfoximine (L-methionine-S-sulfoximine) inhibits glutamine synthetase and that only the same isomer inhibits γ-glutamylcysteine synthetase. A detailed consideration of the properties of the active sites of glutamine synthetase and γ-glutamylcysteine synthetase suggested the possibility of designing and synthesizing analogs of methionine sulfoximine that would selectively inhibit each of the synthetases. The full details of the reasoning and experimental work involved in these studies have been given elsewhere (14a,16). A number of methionine sulfoximine analogs were synthesized and examined not only for their effects on synthetases, but also for their convulsant activity. It has long been known that methionine sulfoximine induces convulsions in a number of species, and studies in our laboratory showed that of the four diastereoisomers of methionine sulfoximine only L-methionine-S-sulfoximine exhibits convulsant activity (48). Recent studies have led to the finding that α-ethylmethionine sulfoximine, which is a convulsant, inhibits glutamine synthetase effectively, but has no significant effect on γ-glutamylcysteine synthetase (16). Similarly, methionine sulfoximine analogs in which the *S*-methyl group is replaced with bulkier moieties (e.g., S-propyl homocysteine sulfoximine) do not inhibit glutamine synthetase appreciably, but markedly inhibit γ-glutamylcysteine synthetase (14a). Compounds of the latter type are very weak convulsants. The accumulated data support the hypothesis that the induction of convulsions is closely associated with the inhibition of glutamine synthetase rather than inhibition of glutathione synthesis. The structurally modified sulfoximine compounds that have been obtained thus make it possible to selectively inhibit two major pathways of glutamate metabolism, i.e., the one that leads to formation of glutamine (Fig. 1, reaction 4) and that leading to glutathione biosynthesis (Fig. 1, reaction 9). The availability of these one-enzyme inhibitors will hopefully make it possible to pursue in further detail the metabolic and physiological phenomena associated with these pathways of glutamate metabolism.

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