*Proceedings of the National Academy of Sciences*
Vol. 67, No. 3, pp. 1248–1255, November 1970

# The γ-Glutamyl Cycle: A Possible Transport System for Amino Acids*

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*Communicated August 7, 1970*

**Abstract.** Evidence is presented that rat kidney contains enzymes that catalyze the synthesis and utilization of glutathione; these reactions, which involve the uptake and release of amino acids from γ-glutamyl linkage, constitute a cyclical process which is termed "the γ-glutamyl cycle." The γ-glutamyl cycle has properties that fulfill the requirements of an amino acid transport system. Thus, γ-glutamyl transpeptidase may function in translocation and γ-glutamylcysteine synthetase and glutathione synthetase may catalyze energy-requiring "recovery" steps in transport. These and other considerations suggest that glutathione serves a carrier function in amino acid transport.

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It has long been known that kidney preparations catalyze the degradation of glutathione[1], and that γ-glutamyl transpeptidase[2] plays an important role in this process. This enzyme catalyzes the transfer of the γ-glutamyl moiety of glutathione to a variety of α-amino acids:

$$\text{glutathione} + \text{amino acid} \rightarrow \gamma\text{-glutamylamino acid} + \text{cysteinylglycine} \quad (1)$$

Purified preparations of γ-glutamyl transpeptidase also catalyze the liberation of glutamate from glutathione and γ-glutamylamino acids. The broad amino acid specificity of reaction (1) has been amply demonstrated[3–7]. The enzyme is widely distributed in animal tissues, kidney being most active. It is notable that the enzyme is bound to tissue particles and that purified preparations of the enzyme contain as much as 30% carbohydrate.[8,9]

γ-Glutamyl cyclotransferase (γ-glutamyl lactamase), which catalyzes reaction (2)[10],

$$\gamma\text{-glutamylamino acid} \rightarrow \text{pyrrolidone carboxylate} + \text{amino acid} \quad (2)$$

acts rapidly on the γ-glutamyl derivatives of glutamine, alanine, and glycine. When both transpeptidase (TP) and cyclotransferase (CT) are present the γ-glutamyl derivatives of virtually all of the protein amino acids are degraded by the following pathway[11]:

$$\gamma\text{-Glu-amino acid} + \gamma\text{-Glu-amino acid} \xrightarrow{\text{TP}} \gamma\text{-Glu-}\gamma\text{-Glu-amino acid} + \text{amino acid} \quad (3a)$$

$$\gamma\text{-Glu-}\gamma\text{-Glu-amino acid} \xrightarrow{\text{CT}} \text{pyrrolidone carboxylate} + \gamma\text{-Glu-amino acid} \quad (3b)$$

γ-Glutamyl cyclotransferase is also widely distributed; it is found to a considerable extent in kidney, and a very highly purified preparation has been obtained from brain [11]. In contrast to the transpeptidase, the cyclotransferase is found in the soluble fraction of tissues; three isozymic forms of the human brain enzyme have been found[11].

That the kidney contains substantial amounts of γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase—and nevertheless maintains an appreciable concentration of glutathione—suggested to us that this organ might also possess the enzymatic equipment[12,13] needed for the synthesis of glutathione. Such expectation has been realized, as reported here, with the finding that rat kidney contains a high concentration of γ-glutamylcysteine synthetase and also of glutathione synthetase. The presence of these enzymes in kidney thus makes possible a series of catalytic events involving the synthesis and degradation of glutathione, and the coupled uptake and release of free amino acids from γ-glutamyl linkage. These reactions are steps in a cyclical process (Fig. 1), to which we shall refer as the "γ-glutamyl cycle."

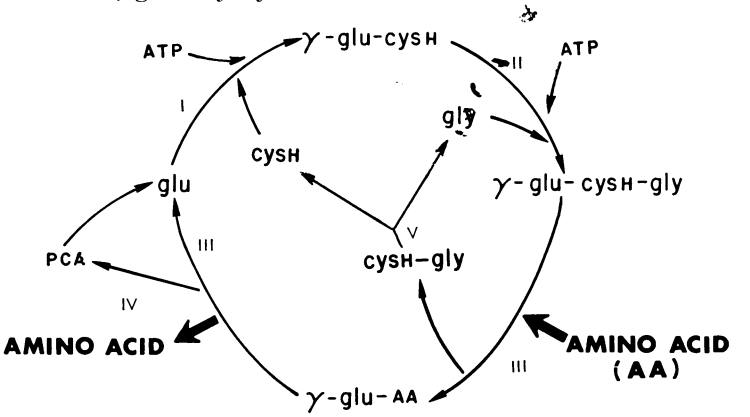


FIG. 1. The γ-glutamyl cycle. *I*, γ-glutamylcysteine synthetase; *II*, glutathione synthetase; *III*, γ-glutamyl transpeptidase; *IV*, γ-glutamyl cyclotransferase; *V*, peptidase. PCA = pyrrolidone carboxylic acid; AA = amino acid.

In this communication we will present and review data which support the view that the γ-glutamyl cycle functions in kidney. We also wish to suggest that the γ-glutamyl cycle or a similar cycle of reactions may function in the transport of amino acids. Although we and others have speculated that γ-glutamyl transpeptidase might possibly be involved in amino acid transport, this now seems much more attractive because the action of this enzyme can now be integrated with the reactions catalyzed by γ-glutamylcysteine synthetase, glutathione synthetase, and γ-glutamylcyclotransferase. The synthetases, which couple ATP cleavage with the formation of peptide bonds, could serve in an energy-requiring recovery phase of transport, leading to synthesis of carrier for amino acid transport.

**Materials and Methods.** L-γ-Glutamyl-*p*-nitroanilide[8], L-γ-glutamyl-L-γ-glutamyl-*p*-nitroanilide[11], [$^{14}$C]L-glutamate, L-α-aminobutyrate, and other amino acids were

obtained as described.[8,11] L-γ-Glutamyl-L-α-aminobutyric acid was synthesized by a modification of the general method of King and Kidd[14].

Sprague-Dawley rats (300–400 g) were decapitated and exsanguinated. The excised tissues were homogenized at 0°C for 2 min at 1600 rpm in 9 vol of 0.15 M KCl containing 0.005 M 2-mercaptoethanol and 0.001 M $MgCl_2$ with a Potter-Elvehjem homogenizer equipped with a motor-driven Teflon pestle. The homogenates were centrifuged at 10,000×*g* for 15 min prior to the determinations of γ-glutamylcysteine synthetase and γ-glutamyl cyclotransferase activities.

γ-Glutamyl transpeptidase activity was determined by the procedure previously described[8,15] in which L-γ-glutamyl-*p*-nitroanilide is used as a substrate; the free *p*-nitroaniline formed was determined spectrophotometrically.

γ-Glutamyl cyclotransferase was determined as described previously[11,16] using L-γ-glutamyl-L-γ-glutamyl-*p*-nitroanilide as substrate. Since this substrate is cleaved by both γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase, a serine–borate buffer mixture (0.04 M L-serine–0.04 M sodium borate; pH 8.0) was substituted for the Tris·HCl buffer used in the earlier work. Under these conditions γ-glutamyl transpeptidase is almost completely inhibited[4], while cyclotransferase activity remains unaffected[17].

γ-Glutamylcysteine synthetase was determined[18] in reaction mixtures of final volume 0.5 ml containing 0.01 M [$^{14}$C]L-glutamate (about 250,000 cpm), 0.01 M ATP, 0.01 M L-α-aminobutyrate, 0.02 M $MgCl_2$, 0.01 M phosphocreatine, creatine phosphokinase (0.03 mg), 0.1 M Tris·HCl buffer (pH 8.2), purified γ-glutamyl cyclotransferase (5 units), and the enzyme solution. After incubation at 37°C for 10–20 min, the mixture was placed at 100°C for 2 min and then cooled in ice and centrifuged. A sample (0.25 ml) of the supernatant solution was added to the top of a small column (1 × 1.5 cm) of Dowex 50 ($H^+$) and the column was washed with water until 2.5 ml of effluent emerged. The $^{14}$C present in the effluent ([$^{14}$C]pyrrolidone carboxylate) was determined by scintillation counting in Bray's solution[19].

A unit of enzyme is defined as the amount that catalyzes the formation of 1 nmol of product per minute under the conditions given above.

**Results. Determination of γ-glutamylcysteine synthetase activity in crude tissue preparations:** The determination of this enzyme activity in crude kidney and other tissue preparations is complicated by the presence of γ-glutamyl transpeptidase and γ-glutamyl cyclotransferase, enzymes which act effectively on γ-glutamylcysteine and other γ-glutamyl amino acids. We have developed a reliable method for the determination of γ-glutamylcysteine synthetase in which an ATP-generating system is added together with an excess of purified γ-glutamyl cyclotransferase. The composition of the assay mixture is given above under *Methods*. In this system the [$^{14}$C]glutamyl moiety of the [$^{14}$C]γ-glutamyl amino acid synthesized (in this case [$^{14}$C]γ-glutamyl-α-aminobutyrate) is converted to [$^{14}$C]pyrrolidone carboxylate in accordance with the following reactions, catalyzed by γ-glutamylcysteine synthetase (reaction 4a) and γ-glutamyl cyclotransferase (reaction 4b), respectively:

$$[^{14}\text{C}]\text{L-glutamate} + \text{ATP} + \text{L-}\alpha\text{-aminobutyrate} \rightarrow [^{14}\text{C}]\text{L-}\gamma\text{-glutamyl-L-}\alpha\text{-aminobutyrate} + \text{ADP} + P_i \quad (4a)$$

$$[^{14}\text{C}]\text{L-}\gamma\text{-glutamyl-L-}\alpha\text{-aminobutyrate} \rightarrow [^{14}\text{C}]\text{pyrrolidone carboxylate} + \text{L-}\alpha\text{-aminobutyrate} \quad (4b)$$

The [$^{14}$C]pyrrolidone carboxylate is separated from the remaining [$^{14}$C]glutamate on a column of Dowex 50 ($H^+$) and the [$^{14}$C]pyrrolidone carboxylate present in the eluate is determined. Under these conditions, product formation is propor-

tional to time and amount of enzyme over 30 min. As indicated in Table 1, when ATP is omitted, there is virtually no product formed; maximal activity was achieved only when an ATP-generating system was also present. Omission of L-$\alpha$-aminobutyrate led to marked reduction of product formation; the presence of some amino acids in the homogenate probably explains the formation of a very small amount of product in the absence of added L-$\alpha$-aminobutyrate. When $\gamma$-glutamyl cyclotransferase was not added to the assay mixture, only about 25% as much product was formed; the formation of some product in the absence of added cyclotransferase may be ascribed to the presence of this enzyme in the crude kidney preparation used.

Application of this assay method to purified preparations of $\gamma$-glutamylcysteine synthetase gives values that are in close agreement with those obtained for inorganic phosphate formation.

TABLE 1. *Determination of $\gamma$-glutamylcysteine synthetase activity; effect of omission of components of the assay system.*

| Component of assay system omitted* | Pyrrolidone carboxylate ($\mu$mol/ml/hr) |
|---|---|
| None | 74.8 |
| Kidney extract | 0 |
| ATP + phosphocreatine + creatine kinase | 0.4 |
| Phosphocreatine + creatine kinase | 61.7 |
| L-$\alpha$-Aminobutyrate | 1.2 |
| $\gamma$-Glutamyl cyclotransferase | 21.9 |

* The composition of the assay system is given in the text; the reaction was started by addition of 0.025 ml (0.15 mg of protein) of rat kidney extract; incubated for 10 min at 37°C.

TABLE 2. *Activities of the enzymes of the $\gamma$-glutamyl cycle in kidney.*

| Enzyme | Activity (nmol/min/mg protein) |
|---|---|
| $\gamma$-Glutamylcysteine synthetase | 168 |
| Glutathione synthetase | 50* |
| $\gamma$-Glutamyl transpeptidase | 460 |
| $\gamma$-Glutamyl cyclotransferase | 286 |

* Estimated; see the text.

**Purified rat kidney $\gamma$-glutamylcysteine synthetase:** A highly purified preparation of this enzyme has been obtained from rat kidney by a procedure (unpublished) involving fractionation with ammonium sulfate, gel filtration, and chromatography on DEAE-cellulose. The enzyme was purified about 40-fold over the homogenate in approximately 40% yield; the specific activity of the purified enzyme is 9000 units/mg of protein, or about 100 times more active than the previously described preparations of the enzyme from hog liver[20,21] and bovine lens[22]. The purified enzyme, which is homogeneous on ultracentrifugation and acrylamide gel electrophoresis, appears to constitute 2–3% of the soluble protein of rat kidney homogenate. The purified enzyme loses activity when stored at 0°C, but is promptly reactivated by brief exposure to dithiothreitol. The exquisite sensitivity of the enzyme to sulfhydryl reagents is indicated by the fact that it is markedly inhibited by *p*-chloromercuribenzoate, *p*-chloromercuribenzene sulfonate, and iodoacetamide.

**Activity and specificity of the enzymes of the $\gamma$-glutamyl cycle in kidney:** As indicated in Table 2, extracts of rat kidney contain substantial amounts of the

enzymes required for the synthesis and utilization of γ-glutamyl amino acids. If one takes the rate found for γ-glutamylcysteine synthetase as equivalent to the rate of glutathione synthesis, it may be calculated that rat kidney can catalyze the synthesis of 17 mmol (or about 5 g) of glutathione per gram of kidney per day!

It is difficult to determine exactly the amount of glutathione synthetase in crude preparations of rat kidney because of the presence of the transpeptidase and cyclotransferase, which decrease the concentrations of both the γ-glutamyl amino acid reactant and the product. We have established the presence of glutathione synthetase and attempted to estimate the amount of activity present in kidney by incubating kidney extract with γ-glutamyl-α-aminobutyrate, $NH_2OH$, ATP, and $Mg^{++}$ essentially as in previous studies on the yeast enzyme[23]; in the present experiments the concentration of γ-glutamyl-α-amino butyrate was increased to 0.04 M. In three separate experiments values of 19.1, 13.3, and 15.5 nmol of hydroxamate product were found per min per mg of protein. Since substitution of glycine for hydroxylamine has been found to increase the product formation by about 3-fold[23], we tentatively estimate that the glutathione synthetase activity is at least 50 nmol/min per mg of protein.

The amino acid specificity of γ-glutamyl transpeptidase is very broad; however, no activity has been observed with proline or hydroxyproline, and there is relative little activity toward aspartate, glutamate, and glycine. The most active amino acids are methionine, glutamine, cysteine, and arginine. As stated above, γ-glutamyl cyclotransferase is most active with the γ-glutamyl derivatives of glutamine, alanine, and glycine; when the transpeptidase is also present, virtually all of the γ-glutamyl amino acids are attacked. γ-Glutamylcysteine synthetase exhibits fairly high specificity; thus, cysteine may be replaced without decrease in activity by α-aminobutyrate, but the other protein amino acids are much less active or inactive.

**Discussion.** The presence in kidney of four highly active enzymes that synthesize and utilize γ-glutamyl amino acids suggests that these catalysts and their substrates are involved in a significant physiological process. Although it has been speculated that γ-glutamyl compounds may be directly involved in protein synthesis, efforts to demonstrate their participation have not been successful, and there is now a considerable body of detailed information about protein synthesis. It seems unlikely that the reactions of the γ-glutamyl cycle are involved in the metabolism of the folate compounds, since none of the enzymes act on γ-glutamyl derivatives in which the glutamyl α-amino group is substituted. The possibility that the enzymes of the γ-glutamyl cycle are involved in amino acid transport seems worthy of serious consideration. Current hypotheses about active transport[24] favor a multistep process in which a membrane-bound component, probably a protein, interacts with the substrate to be transported. This step is followed by a translocation in which the substrate or substrate–carrier complex moves to the inside of the cell; dissociation or release of the substrate then occurs. There is much evidence for the existence of an energy-requiring recovery step; the need for energy in amino acid transport has been amply documented[25–28]. The γ-glutamyl cycle seems to possess the requirements of such an amino acid transport system. Thus, γ-glutamyl transpeptidase, which appears to be mem-

brane-bound, combines with both free amino acid and carrier (either glutathione or γ-glutamylcysteine) and could function in the translocation process. The γ-glutamyl amino acid formed might then be brought into the cell and the amino acid released, possibly by the hydrolytic activity of the transpeptidase or by the action of γ-glutamyl cyclotranferase. It may be noted that glutathione is not a substrate for the cyclotransferase[11]; thus, glutathione, which is resistant to degradation within the cell, could serve as the carrier substrate for the membrane-bound transpeptidase. Additional phenomena are associated with the reactions of the γ-glutamyl cycle. Thus, transpeptidation produces cysteinylglycine; it is known from many studies that this peptide is readily cleaved by kidney preparations to its constituent amino acids, which are substrates for the energy-requiring reactions catalyzed, respectively, by γ-glutamylcysteine synthetase and glutathione synthetase. Little seems to be known about the metabolic fate of pyrrolidone carboxylate[29]; however, recent studies by P. G. Richman in our laboratory and by P. R. Krishnaswamy (personal communication) indicate that pyrrolidone carboxylate is converted to carbon dioxide by kidney slices. (Very recent work by P. VanDerWerf in our laboratory indicates that the main product of the metabolism of L-pyrrolidone carboxylate in kidney slices is glutamate.)

There are several additional considerations which are consistent with the hypothesis that the γ-glutamyl cycle functions in amino acid transport. In particular, it seems notable that histochemical studies of γ-glutamyl transpeptidase have shown that this enzyme is localized in the brush border of the proximal convoluted tubule, which is believed to be the major site for reabsorption of amino acids[30,31]. Membrane-bound γ-glutamyl transpeptidase might be the major site at which competitive phenomena between amino acids occur. The ability of the enzyme to interact with most of the protein amino acids has been noted above, and it is reasonable to expect competition between various amino acids for the enzyme. Such competition would be consistent with *in vivo* studies which have shown that infusion of almost any individual amino acid leads to fairly generalized amino aciduria[32,33]. *In vivo* studies suggest that the renal reabsorption mechanisms for amino acids are not completely specific[34], and that either there is no single mechanism for all the amino acids or that there are widely varying affinities for various amino acids[32]. Infusion of glutamate or aspartate has been reported to be much less effective in producing aminoaciduria than glutamine, asparagine, and several other neutral amino acids[32]; this is consistent with the finding that γ-glutamyl transpeptidase is much more active toward glutamine and asparagine than toward the corresponding dicarboxylic amino acids. There is evidence that proline and glycine are transported by mechanisms different from those responsible for the transport of other neutral amino acids[35-37]; note that proline is not a substrate of γ-glutamyl transpeptidase and that glycine is a relatively poor substrate. We do not suggest that the γ-glutamyl cycle is responsible for all amino acid transport phenomena. It may be significant, however, that the amino acid constituents of the γ-glutamyl cycle participate in reactions which might also serve transport functions. Thus, glycine and cysteine participate in "cycles" of their own, which involve, respectively, three and four of the reactions of the γ-glutamyl cycle. For ex-

ample, extra cellular glycine might react with membrane-bound γ-glutamylcysteine to form glutathione, which in turn would yield, by the action of γ-glutamyl transpeptidase, cysteinylglycine; this is hydrolyzed within the cell to glycine and cysteine. The dibasic amino acids and cystine may be transported by an additional mechanism[34,35,38-40]. Such a system might be related to the existence of isozymic forms of the several enzymes of the cycle or to other phenomena (binding proteins, etc.).

Poisoning by heavy metals is associated with generalized aminoaciduria and deposition of metal in the proximal convoluted tubules[41]; such poisoning may involve the blocking of sulfhydryl groups of various proteins. Aminoaciduria also follows treatment with iodoacetamide or iodoacetate; although these reagents might affect a number of sulfhydryl-containing systems, it may be significant that γ-glutamycysteine synthetase is extremely sensitive to these and other sulfhydryl reagents.

Although the tissue distribution of glutathione synthetase remains to be fully explored, it is evident that the kidney contains substantial amounts of this enzyme as well as very high levels of other enzymes of the γ-glutamyl cycle. This is entirely consistent with the specialized function of the kidney in the reabsorption of amino acids. However, γ-glutamyl transpeptidase, γ-glutamyl cyclotransferase, and γ-glutamylcysteine synthetase are widely distributed in animal tissues. The transpeptidase is localized in the apical portion of the epithelial cells covering the jejunal villi, the external secretory portion of the pancreas, the glandular epithelium of the seminal vesicles, epididymus, fallopian tubes, endometrium, lactating breast, prostate, bronchi, and submaxillary glands[42-46], in the ependymal cells and the epithelium that covers the leaves and villi of the choroid plexus, and in the endothelium of brain capillaries[47]. It is tempting to speculate from these observations that the γ-glutamyl cycle may play a role in the transport or secretory functions of many tissues, and that the ubiquitous occurrence of glutathione reflects its central role in amino acid transport.

* Supported in part by a grant from the National Science Foundation.

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