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Transport of γ-glutamyl amino acids: Role of glutathione and γ-glutamyl transpeptidase

[kidney/L- and D-γ-glutamyl-(*o*-carboxy)phenylhydrazide/buthionine sulfoximine/cell membrane]

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ABSTRACT **This work relates to the hypothesis that one of the mechanisms that mediates amino acid translocation across cell membranes involves the action of membrane-bound γ-glutamyl transpeptidase on intracellular glutathione and extracellular amino acids to form γ-glutamyl amino acids. According to this idea, the latter are translocated into the cell where the γ-glutamyl moiety is removed to yield free amino acids. Previous studies in this laboratory showed that intracellular glutathione is translocated out of many cells. We have now directly examined the transport of γ-glutamyl amino acids into tissues in the mouse by use of the model substrate L-γ-glutamyl-L-[^{14}C]methionine sulfone. Of 11 tissues examined, only the kidney showed strong and preferential uptake of the substrate. A substantial amount of the administered L-γ-glutamyl-L-[^{14}C]methionine sulfone was found intact in the kidney; the total uptake of this compound was greater (by about 2-fold) than that of free L-methionine sulfone. Studies with a number of other γ-glutamyl amino acids and γ-glutamyl compounds indicate that the kidney has a relatively specific transport system for γ-glutamyl amino acids. Small but significant amounts of γ-glutamylmethionine sulfone were found in the liver and pancreas, suggesting that other tissues may also have this system. Transport of γ-glutamylmethionine sulfone into the kidney was inhibited by inhibitors of glutathione synthesis and of γ-glutamyl transpeptidase. The results suggest that both the transpeptidase and glutathione may be involved in transport of γ-glutamyl amino acids.**

Results of recent studies on glutathione metabolism in which the function of the γ-glutamyl cycle was studied *in vivo* are consistent with the idea that the cycle serves as one of the systems that mediates translocation of amino acids across cell membranes (1–5). According to this hypothesis (see ref. 5 for background), amino acids are translocated across the cell membrane as the corresponding γ-glutamyl amino acids, which are formed by the action of membrane-bound γ-glutamyl transpeptidase on glutathione and amino acids. Glutathione is predominately intracellular, whereas a major fraction of the cellular γ-glutamyl transpeptidase is on the external surface of cell membranes. The finding of an enzyme on one side of a membrane and of its major substrate on the other has been illuminated by the discovery that intracellular glutathione is translocated out of many cells. *In vivo* studies on mice in which γ-glutamyl transpeptidase was inhibited by administration of L- or D-γ-glutamyl(*o*-carboxy)phenylhydrazide and in which glutathione synthesis was inhibited by giving prothionine sulfoximine indicated that translocation of glutathione occurs across cell membranes in kidney and in other organs (3). Studies on human lymphoid cells that have markedly different γ-glutamyl transpeptidase activities (6) and on human fibroblasts (7), as well as observations on isolated perfused liver preparations (8), have provided additional evidence for efflux of glutathione from cells, and it is now apparent that translocation of intracellular glutathione across the cell membrane is probably a property of many if not most cells.*

Evidence that γ-glutamyl amino acids are effectively translocated into certain cells was obtained in studies in which it was found that administration to mice of an inhibitor of 5-oxoprolinase led to accumulation of 5-oxoproline in the tissues and that administration of both the inhibitor and amino acids led to increased 5-oxoproline accumulation (9). Subsequent studies showed that administration of large amounts of amino acids to mice led to decreased glutathione levels and to increased levels of 5-oxoproline in the kidney (1). The level of 5-oxoproline in control mouse kidney and the increased level found after inhibition of 5-oxoprolinase were both substantially decreased when an inhibitor of γ-glutamyl cyclotransferase was given (1). These findings are consistent with translocation of γ-glutamyl amino acids into the cell followed by cleavage of the γ-glutamyl amino acids intracellularly by γ-glutamyl cyclotransferase to form amino acids and 5-oxoproline. There is as yet no direct evidence for translocation of γ-glutamyl amino acids formed by the transpeptidase *in vivo*. However, when γ-glutamyl-α-aminobutyrate is given to rats, it is apparently directly incorporated into kidney ophthalmic acid (10). Administration of γ-glutamylmethionine sulfoximine to mice is followed by the appearance of substantial amounts of intracellular γ-glutamylmethionine sulfoximine in the kidney; furthermore, the uptake of methionine sulfoximine by the kidney is significantly greater after administration of γ-glutamylmethionine sulfoximine than after administration of methionine sulfoximine itself (1).

In the present studies we have examined the transport of γ-glutamyl amino acids into the organs of mice. The model substrate L-γ-glutamyl-L-[^{14}C]methionine sulfone [L-Met(SO_2)] was chosen because this substrate, a close analog of γ-glutamylglutamine, was expected to be metabolized relatively slowly. Studies on 11 tissues of the mouse indicate that γ-glutamylMet(SO_2) and otherγ-glutamyl amino acids are transported intact into the kidney; under the conditions used, such transport occurred to a much lesser extent in the other tissues examined. The data thus far obtained show that there is an apparently specific transport site in the kidney for L-γ-glutamyl-L-amino acids.

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Abbreviation: L-Met(SO_2), L-methionine sulfone.

* Although several earlier reports suggested that glutathione (and its disulfide) is translocated across cell membranes, the possibility that the glutathione found extracellularly was related to oxidative or other types of cell damage could not be excluded.

EXPERIMENTAL

Materials. L-[*methyl*-^{14}C]Methionine was obtained from New England Nuclear and converted to L-[^{14}C]Met(SO_2) with hydrogen peroxide (11). D- and L-γ-Glutamyl-L-α-aminobutyrate, L-γ-glutamyl-L-[^{14}C]Met(SO_2), L-γ-glutamyl-DL-α-methyl-α-aminobutyrate, and L-γ-glutamyl-DL-β-aminobutyrate were prepared from the appropriate *N*-phthaloylglutamic anhydride and amino acid essentially as described (12). L-γ-Glutamyl-L-methionine, L-γ-glutamyl-L-alanine, and L-β-aspartyl-L-alanine were obtained from Vega-Fox, (Tucson, AZ). L-α-Glutamyl-L-alanine and L-γ-glutamyl-L-glutamine were obtained from Sigma and Calbiochem, respectively. L- and D-γ-Glutamyl(*o*-carboxy)phenylhydrazides (3) and DL-buthionine-*SR*-sulfoximine (13) were prepared as described. L-Methionine-*SR*-sulfoximine was obtained from Sigma. Male mice (NCS strain; 25–30 g) were obtained from The Rockefeller University.

Methods. The compounds were administered to the mice as described in the text. The animals were killed by decapitation and exsanguination, and the tissues of interest were quickly removed, rinsed in ice water, blotted dry, weighed, and homogenized (Potter–Elvehjem) in 5 vol of 1% picric acid. The homogenates were centrifuged to yield supernatants that were assayed for glutathione (14) and fractionated on Dowex-1 (acetate). In the latter procedure, 0.5–1.0 ml of the picric acid supernatant was applied to small (0.5 × 3 cm) columns of Dowex-1 (acetate). L-[^{14}C]Met(SO_2) (and other neutral compounds) were eluted with 5 ml of 0.2 M acetic acid; then, L-γ-glutamyl-L-[^{14}C]Met(SO_2) (and other acidic compounds) were eluted with 5 ml of 1 M formic acid. Radioactivity in the eluents was determined by liquid scintillation counting. In some cases, the L-γ-glutamyl-L-[^{14}C]Met(SO_2) fraction was applied to small (0.5 × 3 cm) columns of Dowex 50 (H^+). Radioactive metabolites not containing an amino group (e.g., keto acids) were eluted with 8 ml of water, and L-γ-glutamyl-L-[^{14}C]Met(SO_2) was then eluted with 6 ml of 3 M NH_4OH. The presence in the latter eluent of L-γ-glutamyl-L-[^{14}C]Met(SO_2) in the amount indicated by the contained radioactivity was confirmed by amino acid analysis on a Durrum analyzer.

Table 1. Tissue uptake of radiolabeled compounds after administration of L-γ-glutamyl-L-[^{14}C]Met(SO_2) [L-γ-Glu-Met(SO_2)] and L-[^{14}C]Met(SO_2)

Tissue	Total radiolabeled compounds found, μmol/g*		
	After L-γ-Glu-[^{14}C]Met(SO_2)	After L-[^{14}C]-Met(SO_2)	Ratio
Kidney	5.16 ± 0.19	2.46 ± 0.55	2.10
Lung	0.33 ± 0.02	0.35 ± 0.04	0.94
Testis	0.12 ± 0.02	0.18 ± 0.01	0.67
Muscle	0.13 ± 0.01	0.20 ± 0.04	0.65
Heart	0.29 ± 0.02	0.46 ± 0.04	0.63
Pancreas	2.06 ± 0.26	3.34 ± 0.18	0.62
Large intestine	0.23 ± 0.02	0.40 ± 0.07	0.57
Liver	0.58 ± 0.04	1.14 ± 0.05	0.51
Spleen	0.32 ± 0.02	0.64 ± 0.06	0.50
Brain	0.04 ± 0.005	0.08 ± 0.01	0.50
Small intestine	0.29 ± 0.01	0.63 ± 0.19	0.46

L-γ-Glu-[^{14}C]Met(SO_2) and L-[^{14}C]Met(SO_2) were administered to mice at doses of 0.5 mmol/kg (≈0.5 μCi) by subcutaneous injection of 100 mM solutions. The animals were killed 30 min after injection. Data reflect the sum of all soluble radioactive compounds found in the tissues specified.

* Based on the specific radioactivity of injected compound. Shown as means ± SD.

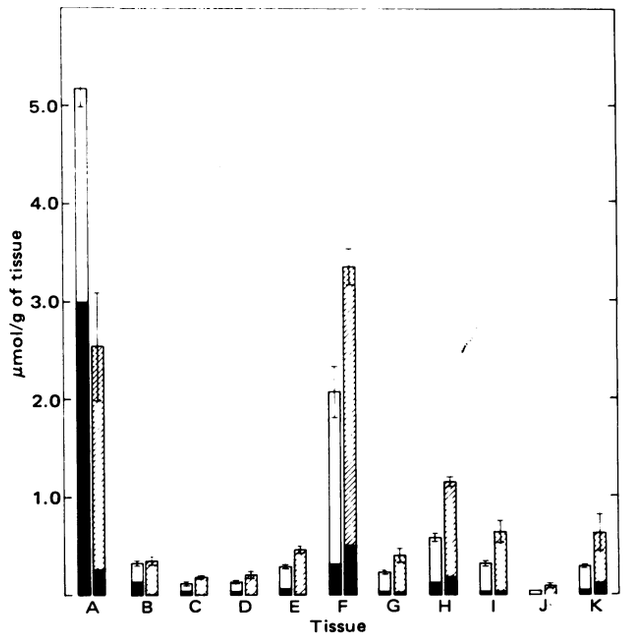

FIG. 1. Tissue uptake of radiolabel after administration of L-γ-glutamyl-L-[^{14}C]Met(SO_2) or L-[^{14}C]Met(SO_2) (left or right bar of each pair). Compounds were administered subcutaneously in doses of 0.5 mmol/kg, and animals were killed 30 min later. Tissues analyzed were: A, kidney; B, lung; C, testis; D, muscle; E, heart; F, pancreas; G, large intestine; H, liver; I, spleen; J, brain; K, small intestine. The black area of each bar represents the radioactivity found as γ-glutamyl-[^{14}C]Met(SO_2). The white and crosshatched areas represent [^{14}C]Met(SO_2). In the kidney a small percentage (10%) of radioactivity attributed in the figure to γ-glutamyl-[^{14}C]Met(SO_2) was due to other acid metabolites (e.g., α-keto acids); in the other tissues, acidic metabolites accounted for at least half of this fraction. Data are shown as means ± SD.

RESULTS

In the experiments described in Table 1, mice were injected subcutaneously with either L-γ-glutamyl-L-[^{14}C]Met(SO_2) or with L-[^{14}C]Met(SO_2), and, after 30 min, the radioactivity present in various organs was determined. After administration of L-γ-glutamyl-L-[^{14}C]Met(SO_2), the largest uptake of label was found in the kidney. The uptake of label by the kidney was far greater after L-γ-glutamyl-L-[^{14}C]Met(SO_2) than after L-[^{14}C]Met(SO_2). In contrast, the uptake of label by the other tissues examined was somewhat greater after L-[^{14}C]Met(SO_2) was given. It is of interest that, after administration of the labeled compounds, radioactivity was effectively taken up by the pancreas under these conditions.

The findings summarized in Fig. 1 are from studies in which the deproteinized tissue extracts obtained from injected animals were separated by chromatography on Dowex-1 (acetate) to yield a neutral fraction [containing Met(SO_2)] and an acidic fraction [containing γ-glutamylMet(SO_2)]. The left-hand bar of each pair in Fig. 1 gives the amounts of the two fractions (based on the specific radioactivity of the administered compound). About 60% of the radioactivity found in the kidney was present in the acidic fraction, and the remainder was in the neutral fraction; the major labeled components of these fractions were found by amino acid analysis to consist of γ-glutamyl Met(SO_2) and Met(SO_2), respectively.† When the acidic fraction was chromatographed on Dowex 50 (H^+), about 87% of the

† L-γ-Glutamyl-L-Met(SO_2) is a substrate of cytosolic γ-glutamyl cyclotransferase. Thus, some of the Met(SO_2) found is probably derived from intracellular γ-glutamyl Met(SO_2).

Table 2. Effect of various compounds on renal uptake of L-γ-glutamyl-L-[^{14}C]Met(SO_2)

Compound	Dose, mmol/kg	Kidney γ-Glu-Met(SO_2) μmol/g	% of control
None	—	2.55 ± 0.30	[100]
L-Glutamine	5	2.60 ± 0.17	102
L-Met(SO_2)	5	1.64 ± 0.15	64
L-Glutamate	5	1.86 ± 0.17	73
L-γ-Glu-L-Met	5	0.548 ± 0.02	21
L-γ-Glu-L-Gln	5	0.840 ± 0.11	33
L-γ-Glu-L-α-aminobutyrate	5	1.10 ± 0.08	43
D-γ-Glu-L-α-aminobutyrate	5	2.24 ± 0.10	88
L-γ-Glu-D-α-aminobutyrate	5	2.31 ± 0.21	91
L-γ-Glu-DL-(α-CH_3)α-aminobutyrate	5	0.702 ± 0.12	28
L-γ-Glu-DL-β-aminobutyrate	10	0.608 ± 0.05	24
L-γ-Glu-L-Ala	5	1.23 ± 0.32	48
L-β-Asp-L-Ala	5	1.82 ± 0.16	71
L-α-Glu-L-Ala	5	2.17 ± 0.10	85

The compounds were administered, at the doses indicated, to mice by subcutaneous injection. At 10 min later, L-γ-Glu-L-[^{14}C]Met(SO_2) was administered at a dose of 0.4 mmol/kg by subcutaneous injection at a second site. The animals were killed 40 min after the initial injection, and the kidneys were analyzed. Data are shown as means ± SD.

radioactivity was retained; the small amount not adsorbed by Dowex 50 (H^+) probably includes metabolites of Met(SO_2), such as its α-keto acid analog. In the other tissues, very small amounts of label were found in the acid fraction. About half of the acidic fraction obtained from liver was retained on Dowex 50 (H^+), suggesting the presence of some γ-glutamyl Met(SO_2), but clearly this amount is much lower than that found in the kidney. Similarly, a very small amount of the label of the acidic fraction obtained from pancreas was retained by Dowex 50 (H^+).

The right-hand bars shown in Fig. 1 give data on the acidic and neutral fractions obtained from the tissues of animals injected with L-[^{14}C]Met(SO_2). In all instances, the amount of label found in the acidic fraction was low and largely attributable to acidic metabolites of Met(SO_2).

Table 2 gives data from studies in which the uptake of L-γ-glutamyl-L-[^{14}C]Met(SO_2) by tissues was examined in animals given both L-γ-glutamyl-L-[^{14}C]Met(SO_2) and another compound. There was relatively little effect on the uptake of the labeled compound when free amino acids were given simultaneously. Significantly decreased uptake was observed, however, when the L-γ-glutamyl derivative of L-methionine, L-glutamine, L-α-aminobutyrate, DL-α-methyl-α-aminobutyrate, L-alanine, or DL-β-aminobutyrate was simultaneously administered, a result consistent with competition for uptake. Very little such competition was found with L-α-glutamyl-L-alanine, L-β-aspartyl-L-alanine, D-γ-glutamyl-L-α-aminobutyrate, or L-γ-glutamyl-D-α-aminobutyrate. These observations suggest that: (*i*) the transport site for L-γ-glutamyl L amino acids is relatively specific for the L-γ-glutamyl moiety and for a COOH-terminal amino acid of the L configuration, and (*ii*) a number of L-γ-glutamyl L-amino acids are effectively transported.

Studies were also carried out in which known inhibitors of γ-glutamyl transpeptidase and of γ-glutamylcysteine synthetase were utilized (Table 3). It is of interest that both the L and D isomers of γ-glutamyl(*o*-carboxy)phenylhydrazide decreased significantly the uptake of L-γ-glutamyl-L-[^{14}C]Met(SO_2). Both isomers of the hydrazide are effective inhibitors of γ-glutamyl transpeptidase (3), and these effects are thus consistent with a role of the transpeptidase in the transport process (see *Discussion*). Although the L isomer of the hydrazide might possibly serve as a competitive inhibitor of transport, one would not expect the D isomer to have such an effect because D-γ-glutamyl-L-α-aminobutyrate did not markedly affect uptake (Table 2). Buthionine sulfoximine and also methionine sulfoximine markedly inhibited the renal uptake of L-γ-glutamyl-L-[^{14}C]Met(SO_2) (Table 3). Buthionine sulfoximine might serve as an analog of a γ-glutamyl amino acid (13) and therefore competitively inhibit transport of γ-glutamyl amino acids; however, methionine sulfoximine does not appear to be an effective γ-glutamyl amino acid analog. Both methionine sulfoximine and buthionine sulfoximine markedly inhibit glutathione synthesis, the latter being far more potent in its inhibitory effect on γ-glutamylcysteine synthetase (13). These considerations and the finding that the sulfoximines have apparently parallel effects on both uptake of γ-glutamylMet(SO_2) and on the level of glutathione in the kidney (Table 3) suggest that the inhibitory effects of the sulfoximines on γ-glutamyl amino acid transport are probably related to glutathione depletion.

DISCUSSION

These experiments support the view that the kidney has a transport site for γ-glutamyl amino acids. Such a transport mechanism may also be present to some extent in other tissues as well, especially in anatomic locations known to possess relatively high concentrations of γ-glutamyl transpeptidase [e.g., choroid plexus (15), ciliary body (16), and jejunum (17, 18)]. It seems notable that a low but detectable uptake of γ-glutamylMet(SO_2) was found in the liver and pancreas. The present studies (Table 2) indicate that a number of L-γ-glutamyl L amino acids are transported and that several related structures containing L-β-aspartyl, L-α-glutamyl, D-γ-glutamyl, or COOH-terminal D amino acids do not readily interact with the L-γ-glutamyl L amino acid site.

The studies with L- and D-γ-glutamyl(*o*-carboxy)-

Table 3. Effect of γ-glutamyl cycle enzyme inhibitors on renal uptake of L-γ-glutamyl-L-[^{14}C]Met(SO_2)

Exp.	Inhibitor	Dose, mmol/kg	Kidney γ-Glu-Met(SO_2) μmol/g	%	Kidney glutathione μmol/g	%
1	None	—	2.55 ± 0.30	[100]	2.38 ± 0.15	[100]
2	L-γ-Glu-(*o*-carboxy)phenylhydrazide	0.5	1.11 ± 0.07	44	—	
3	D-γ-Glu-(*o*-carboxy)phenylhydrazide	1.25	1.28 ± 0.09	50	—	
4	Buthionine sulfoximine	4	0.476 ± 0.04	19	0.694 ± 0.03	29
5	Buthionine sulfoximine	4	0.492 ± 0.12	19	0.512 ± 0.07	21
6	Methionine sulfoximine	2	0.947 ± 0.04	37	0.960 ± 0.10	40

The inhibitors were administered to mice subcutaneously (Exps. 2 and 3) or intraperitoneally (Exps. 4–6). After 10 min (in Exps. 2–4) or after 130 min (in Exps. 5 and 6), the mice were given L-γ-glutamyl-L-[^{14}C]Met(SO_2) (0.4 mmol/kg) subcutaneously at a second site. The mice were killed 30 min after injection of the labeled material and the kidneys were analyzed. Data are shown as means ± SD

phenylhydrazide (Table 3) suggest that the action of γ-glutamyl transpeptidase may be involved in transport of L-γ-glutamyl L amino acids. A direct effect of the transpeptidase on translocation of γ-glutamyl amino acids needs to be considered. An alternative possibility is that the transpeptidase acts by facilitating the translocation of glutathione. Earlier studies in this laboratory showed that administration of inhibitors of γ-glutamyl transpeptidase decreases the rate at which glutathione is translocated from the kidney. Thus, administration of 6-diazo-5-oxo-L-norleucine, L-serine plus borate, or γ-glutamyl-(*o*-carboxy)phenylhydrazide leads to a decrease in the rate at which the renal glutathione level declines after inhibition of glutathione synthesis by prothionine sulfoximine (1, 3). Whereas the mechanism by which glutathione is translocated still requires study, these observations suggest that the translocation of glutathione is significantly facilitated by the action of the transpeptidase, whose activity may then lead to establishment of a glutathione concentration gradient across the cell membrane. The findings seem to be consistent with the view that transport of γ-glutamyl amino acids into the cell may be linked to translocation of glutathione out of the cell. Perhaps such reciprocal movement of glutathione and γ-glutamyl amino acids across the cell membrane involves interaction of these compounds with the same carrier. The possibility that γ-glutamyl amino acids and glutathione may share the same carrier seems attractive, especially in view of the structural similarities of the transported compounds. The observation that transport of γ-glutamylMet(SO_2) is decreased by γ-glutamyl transpeptidase inhibitors also elucidates the finding of γ-glutamylcysteine in the urine of animals treated with transpeptidase inhibitors (3).

The transpeptidase might also function, as considered previously (1, 3), to convert γ-glutamyl amino acids into γ-glutamyl-γ-glutamyl amino acids, which would then be transported by a separate mechanism. Like that considered above, such a pathway would also require both glutathione and the action of the transpeptidase. It may be significant that γ-glutamyl-γ-glutamyl amino acids are among the most active substrates of γ-glutamyl cyclotransferase (19).

Direct evidence for transport of γ-glutamyl amino acids formed *in vivo* by the action of γ-glutamyl transpeptidase would be desirable. It is interesting, however, to note that recent studies on the toxicity of L-azaserine to several established cell lines revealed a positive correlation between γ-glutamyl transpeptidase activity and sensitivity to azaserine toxicity (20). These experiments led to the conclusion that high sensitivity to azaserine toxicity was a consequence of increased transport of azaserine, facilitated by increased formation of γ-glutamylazaserine. It also seems significant that the toxicity and carcinogenicity of azaserine are greatest in tissues such as kidney and pancreas that have high levels of transpeptidase.

Finally, and quite apart from a consideration of the role that glutathione may play in amino acid transport, it would appear that the activity of the L-γ-glutamyl L amino acid transport site may be usefully employed in directing pharmacologically active compounds (e.g., amino acids, amines, alcohols) to the kidney and perhaps to certain other anatomic sites. In the present studies, for example, administered L-Met(SO_2) was accumulated at highest concentration in the pancreas, whereas L-Met(SO_2) administered as L-γ-glutamyl-L-Met(SO_2) was preferentially delivered to the kidney. Improved survival from acute renal failure in patients treated by intravenous administration of amino acids and glucose (compared to glucose alone) was reported in a prospective double-blind study (21), and intravenous amino acid treatment of rats was found to enhance renal regeneration after acute tubular necrosis (22). It seems possible that administration of amino acids as the γ-glutamyl derivatives could offer a means for efficient delivery of amino acids to the kidney that might be useful in therapy.

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