Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 707–711, February 1983
Biochemistry

Transport and direct utilization of γ-glutamylcyst(e)ine for glutathione synthesis

(increased kidney glutathione levels/cystine/buthionine sulfoximine)

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Contributed by Alton Meister, November 5, 1982

ABSTRACT **Administration of γ-glutamylcystine or of γ-glutamylcysteine disulfide to mice leads to significantly increased levels of glutathione in the kidney as compared to controls given glutamate plus cysteine (or cystinylbisglycine). Studies with γ-glutamylcystine selectively labeled with ^{35}S in either the internal or external S atom indicate preferential utilization of the γ-glutamylcysteine moiety of this compound for glutathione synthesis. Mice depleted of glutathione by treatment with buthionine sulfoximine do not significantly use the disulfides γ-glutamylcystine or γ-glutamylcysteine disulfide but do use γ-glutamylcysteine for glutathione synthesis. These findings suggest a pathway in which γ-glutamylcystine, formed by transpeptidation between glutathione and cystine, is transported and reduced by transhydrogenation with glutathione to cysteine and γ-glutamylcysteine; the latter is used directly for glutathione synthesis. The findings show transport of γ-glutamyl amino acids, indicate an alternative pathway of glutathione synthesis, and demonstrate a means of increasing kidney glutathione levels.**

Glutathione (GSH) is synthesized by the consecutive actions of γ-glutamylcysteine synthetase and GSH synthetase, which catalyze reactions 1 and 2, respectively (1).

L-Glutamate + L-cysteine
$$+ \text{ATP} \rightleftharpoons \text{L-}\gamma\text{-Glu-L-CysH} + \text{ADP} + P_i \quad [1]$$

$$\text{L-}\gamma\text{-Glu-L-CysH} + \text{glycine} + \text{ATP} \rightleftharpoons \text{GSH} + \text{ADP} + P_i \quad [2]$$

Reaction 1 is feedback inhibited by GSH (2), and this effect of GSH apparently regulates the upper level of GSH in the kidney and probably also in other tissues (3, 4). Other studies indicate that kidney (and possibly other) cells have a transport mechanism for γ-glutamyl amino acids that is not shared by free amino acids (5). Thus, transport of γ-glutamylmethionine sulfone into kidney after its administration to mice was found to be (*i*) more rapid than that of the corresponding free amino acid, (*ii*) not significantly inhibited by free amino acids, (*iii*) inhibited by other γ-glutamyl amino acids, and (*iv*) inhibited by increased levels of extracellular GSH (6). It has also been found that inhibition of γ-glutamyl transpeptidase (or a marked deficiency of this enzyme) is associated with substantial urinary excretion of GSH, γ-glutamylcysteine, and cysteine moieties (as identified by chromatography after reduction with dithiothreitol and derivatization with 2-vinylpyridine) (7). The data indicate that the formation of γ-glutamylcyst(e)ine *in vivo* is catalyzed by γ-glutamyl transpeptidase (6). The finding of urinary γ-glutamylcyst(e)ine seems to indicate that the rate of formation of γ-glutamylcyst(e)ine exceeds that of its transport into cells, presumably because such transport is inhibited by the increased extracellular levels of GSH associated with decreased γ-glutamyl transpeptidase activity.

In the present work, we examined directly the transport of γ-glutamylcystine [γ-Glu-$(Cys)_2$] and related compounds in studies in which these compounds were administered to mice. The findings show that intact γ-glutamylcyst(e)ine is transported into kidney and used directly for GSH synthesis.*

EXPERIMENTAL PROCEDURES

Thiolyte MB (monobromobimane) was obtained from Calbiochem–Behring. Methanol was obtained from Waters Associates. L-[^{35}S]Cystine was obtained from New England Nuclear. The other compounds were obtained from Sigma and Aldrich. DL-Buthionine-(*SR*)-sulfoximine was prepared as described (9, 10). We thank R. Donald Allison for the γ-glutamyl transpeptidase.

Rockefeller NCS male mice (18–25 g) were sacrificed by decapitation and exsanguination. The kidneys were rapidly removed, decapsulated, and homogenized in either 1% picric acid or 5% sulfosalicylic acid at 5 vol/g. After centrifugation of the homogenates (Beckman Microfuge B, 2 min), the total glutathione [GSH + glutathione disulfide (GSSG)] of the supernatants was determined by the GSSG reductase/dithiobis(nitrobenzoic acid) recycling assay (11, 12). The specific activity of GSH was determined by HPLC (13) as follows: 400 μl of sample, 100 μl of 7.8 M *N*-ethylmorpholine, 445 μl of water, and 25 μl of 100 mM Thiolyte MB were allowed to react for 20 min at 25°C in the dark. The reaction was stopped by addition of 30 μl of glacial acetic acid (final volume, 1 ml). The samples were analyzed immediately or stored frozen in the dark for up to 2 days. A portion (200 μl) was analyzed on a C_{18} μBondapak column (Waters; 0.39 × 25 cm), using two M6000A Waters pumps, a Waters WISP autoinjector, and a Gilson Spectraglo fluorometer (OPA filters). The flow rate was 1.5 ml/min and the eluent was solvent A [14.2% (vol/vol) methanol/0.25% acetic acid (adjusted to pH 3.9 by adding NaOH)]. After each run, the column was treated with solvent B [90% methanol/0.25% acetic acid (pH 3.9)] and reequilibrated with solvent A. Fractions collected were 1.5 ml. Radioactivity was determined by scintillation counting.

L-γ-Glutamyl-L-cysteine disulfide [$(\gamma\text{-Glu-Cys})_2$] was prepared by a modification of the procedure of Strumeyer and Bloch (14) in which a column containing agarose-carboxypeptidase was used. L-γ-Glutamyl-L-cystine was synthesized by reaction of L-γ-glutamyl-L-cysteine with L-cystine thiosulfonate (15, 16). The product, which was purified by Dowex-1 formate chromatography (16), was eluted with 0.15 M formic acid.

L-γ-Glutamyl-L-cysteine (γ-Glu-CysH) used for injection was prepared by reduction of the corresponding disulfide by

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* A preliminary report of some of these findings was presented at the meeting of the American Society of Biological Chemists (8).

1 M dithiothreitol containing 1 mM EDTA (pH 8). After standing for 60 min at 25°C, the solution was adjusted to pH 2 with formic acid and extracted three times with 2 vol of water-saturated ethyl acetate (17). The solution was evaporated under reduced pressure to dryness. Solutions were prepared and neutralized immediately prior to use.

L-γ-Glu-L-Cys L-[^{35}S]Cys was synthesized as follows. L-[^{35}S]Cystine (20 μmol; 2 mCi; 1 Ci = 3.7×10^{10} becquerels) and 0.2 mmol of L-γ-glutamylcysteine disulfide were dissolved in 1 ml of 100 mM NH_4HCO_3 (pH 8.0) containing 2.1 mM EDTA. Dithiothreitol (2 mmol) was dissolved in the solution by warming at 55°C for 10 min. The reduction was allowed to proceed at 25°C. After 60 min, 0.4 ml of 88% formic acid was added (pH 2), and the solution was extracted five times with 3-ml portions of water-saturated ethyl acetate. The residual ethyl acetate was removed by blowing a stream of dry nitrogen over the solution at 30°C for 10 min. The pH was adjusted to 8 with NH_4OH, and $FeSO_4$ (≈1 mg) was added. Oxygen was bubbled into the mixture for 60 min. Formic acid (88%; 0.75 ml) was added and the solution was carefully evaporated. The residue was dissolved in 10 ml of water and chromatographed on a Dowex-1 formate column (2.1 × 11 cm); the product, which was eluted with 0.15 M formic acid, had a specific radioactivity of 22,200 cpm/nmol.

L-γ-Glu-L-[^{35}S]Cys L-Cys was synthesized as follows. L-[^{35}S]Cystine (30 μmol; 3 mCi), L-γ-glutamyl-*p*-nitroanilide (15 μmol), and sodium phosphate (300 μmol; pH 8.3) in a final volume of 7.5 ml was placed at 37°C for 5 min. The reaction was initiated by addition of 0.038 unit of rat kidney γ-glutamyl transpeptidase (18). The reaction was terminated after 15 min by adding 3.75 ml of 70 mM acetic acid. The γ-glutamyl[^{35}S]cystine formed was purified by chromatography on a Dowex-1 acetate column (1.9 × 12.6 cm). After washing with water and 0.1 M acetic acid, this product was eluted with 1.5 M ammonium acetate (pH 7). The unreacted [^{35}S]cystine was recovered. The γ-glutamyl[^{35}S]cystine (≈4 μmol) was further purified by chromatography on a Dowex 50 (H^+) column (3.1 × 25.5 cm) and lyophilized. The γ-glutamyl[^{35}S]cystine was dissolved in 10 ml of 10 mM NH_4HCO_3 (pH 8) containing 2 mM EDTA and 0.2 ml of 2-mercaptoethanol. After standing at 25°C for 60 min, the solution was acidified by adding 1 ml of 4 M formic acid, and then it was lyophilized. The residue was dissolved in 24 ml of 10 mM formic acid; 2 mmol of L-cystine thiosulfonate was added and the solution was stirred for 14 hr at 25°C. L-γ-Glu-L-[^{35}S]Cys L-Cys, which was filtered and purified by Dowex-1 formate chromatography as described above, had a specific radioactivity of 22,200 cpm/nmol.

RESULTS

Administration of glutamate plus cysteine or of glutamate plus cystinylbisglycine [$(Cys\text{-}Gly)_2$] to mice led to moderate rises in the kidney levels of GSH; thus, levels of 4.4–5.4 μmol/g were observed after such treatment as compared to 4.4 μmol/g for untreated mice. In contrast, after administration of γ-glutamylcystine or of γ-glutamylcysteine disulfide, we found kidney GSH levels in the range 6–7 μmol/g. In these studies, the maximal increase was found 30–60 min after the compounds were given (Fig. 1). That only a small rise in kidney GSH levels occurred after giving glutamate plus cysteine or glutamate plus cysteinylbisglycine may be ascribed to inhibition of γ-glutamylcysteine synthetase by GSH. On the other hand, administration of γ-glutamylcystine or of γ-glutamylcysteine disulfide, followed by reduction, would yield γ-glutamylcysteine, which can be used directly as a substrate of GSH synthetase; this pathway bypasses the feedback regulated step of GSH synthesis.

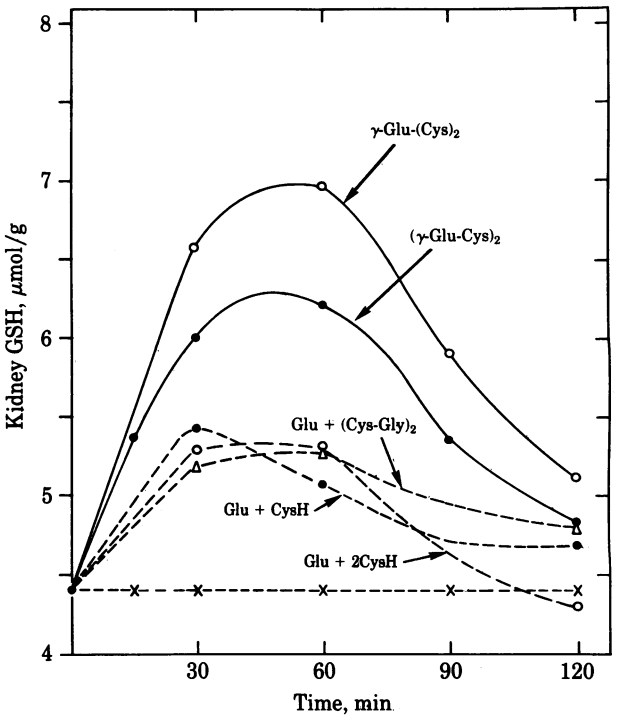

FIG. 1. Kidney GSH levels after administration of L-γ-glutamyl-L-cystine, L-γ-glutamyl-L-cysteine disulfide, and other compounds. Solutions (adjusted to pH 7) of γ-glutamylcystine (2 mmol/kg), γ-glutamylcysteine disulfide (1 mmol/kg), L-glutamate (2 mmol/kg) plus L-cysteine (2 mmol/kg), L-glutamate (2 mmol/kg) plus L-cysteine (4 mmol/kg), or L-glutamate (2 mmol/kg) plus L-cystinylbisglycine (1 mmol/kg) were injected into mice subcutaneously. At the indicated intervals the kidneys were homogenized in 1% picric acid, and GSH was determined. Values for untreated controls are indicated by ×. The points are average values obtained in studies on five to seven mice (SD ± 5%).

This interpretation of the findings is supported by studies in which the incorporation of ^{35}S into GSH was determined after administration of internally labeled γ-glutamylcystine (i.e., γ-Glu-[^{35}S]Cys Cys) and externally labeled γ-glutamylcystine (i.e., γ-Glu-Cys [^{35}S]Cys). Data obtained in these studies (Table 1) show that the incorporation of ^{35}S into GSH was substantially greater when the internally labeled compound was given. That the internal cysteine residue of γ-glutamylcystine is more effectively incorporated into GSH than the external cysteine residue of this compound is consistent with preferential

Table 1. Labeling of kidney GSH after administration of γ-Glu-[^{35}S]Cys Cys and γ-Glu-Cys [^{35}S]Cys

Exp.	Compound given	Kidney GSH, cpm/μmol
1	γ-Glu-[^{35}S]Cys Cys	3,350
	γ-Glu-Cys [^{35}S]Cys	1,980
2	γ-Glu-[^{35}S]Cys Cys	6,690
	γ-Glu-Cys [^{35}S]Cys	3,010

The mice were injected subcutaneously with solutions of the selectively ^{35}S-labeled compounds and killed 15 min later. The kidneys were homogenized in 5% sulfosalicylic acid and centrifuged, and the supernatants were analyzed for GSH and ^{35}S. The doses of labeled compounds (containing 9×10^5 cpm) were 2 mmol/kg (Exp. 1) and 1.5 μmol/kg (Exp. 2). The results are the average values (SD ± 5%) obtained in studies on three mice.

utilization of the γ-glutamylcysteine moiety of γ-glutamylcystine for GSH synthesis. The specific radioactivity of GSH after giving γ-Glu-[^{35}S]Cys Cys was about twice that obtained after giving γ-Glu-Cys [^{35}S]Cys with both high and low doses of these compounds.

In the experiments described in Fig. 2, mice were pretreated with buthionine sulfoximine, a selective inhibitor of γ-glutamylcysteine synthetase (9, 10, 19); 10 hr later, the effect of administration of various compounds on the kidney GSH levels was determined. In experiment *A*, administration of γ-glutamylcysteine disulfide was found to produce only a small increase in the level of GSH, whereas giving the reduced form, γ-glutamylcysteine, led to a very substantial (3-fold) increase in the level of kidney GSH. In experiment *B*, administration of γ-glutamylcysteine disulfide and of γ-glutamylcystine increased the kidney level of GSH only slightly, but again a large increase in the level of GSH was observed after giving γ-glutamylcysteine. Control studies in which glutamate and cysteine were given did not increase kidney GSH level significantly. The findings indicate that, in animals treated with buthionine sulfoximine whose kidney levels of GSH are markedly reduced, there is relatively little utilization of administered γ-glutamylcystine and of γ-glutamylcysteine disulfide, or of glutamate plus cysteine. In contrast, such animals are evidently able to effectively utilize γ-glutamylcysteine for GSH synthesis. This suggests that utilization of the disulfide compounds requires an appreciable intracellular level of GSH, which is presumably required for reduction of the administered disulfides. These results demonstrate that, despite the inhibition of γ-glutamylcysteine synthetase, kidney GSH levels are increased due to transport of γ-glutamylcysteine and its direct utilization by GSH synthetase.

Experiments similar to those described in Fig. 2*B* were carried out in which the various compounds were given together with an additional dose of buthionine sulfoximine. As shown in Fig. 2*C*, when γ-glutamylcysteine and buthionine sulfoximine were given together, there was no appreciable increase in the level of kidney GSH. These studies suggest that simultaneous administration of buthionine sulfoximine inhibits transport of γ-glutamylcysteine. As previously discussed (5), buthionine sulfoximine may inhibit the transport of γ-glutamyl amino acids by serving as a γ-glutamyl amino acid analog. Buthionine sulfoximine may also have direct effects on the cell membrane leading to reduced transport.

DISCUSSION

These findings provide additional evidence that γ-glutamyl amino acids are transported into kidney cells. The data also show that the administered disulfides are reduced; reduction probably takes place intracellularly, but it might also occur in or on

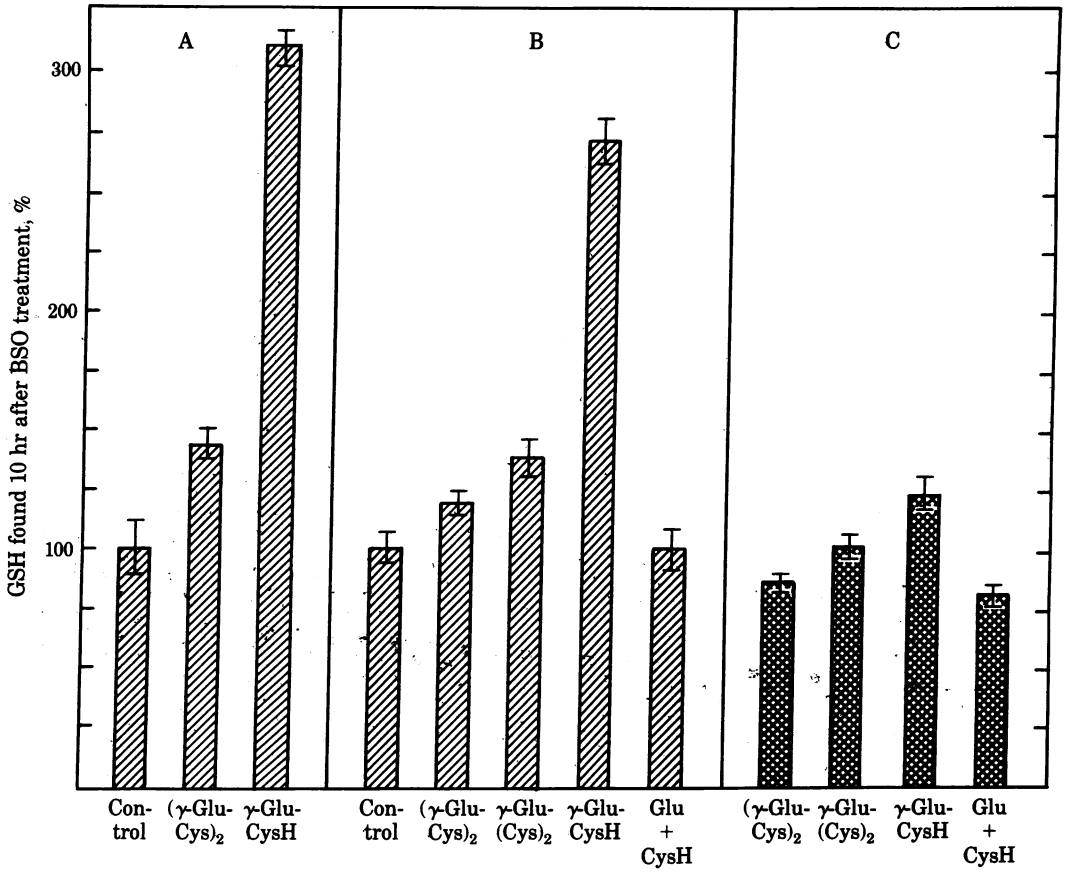

FIG. 2. Effect of administration of γ-glutamylcystine, γ-glutamylcysteine disulfide, and γ-glutamylcysteine on kidney levels of GSH in mice pretreated with buthionine sulfoximine (BSO). DL-Buthionine-(*SR*)-sulfoximine (4 mmol/kg) was administered intraperitoneally as a 100 mM solution to fasted (12 hr) mice 10 hr prior to further treatment. In experiment *A*, γ-glutamylcysteine disulfide (1 mmol/kg) and γ-glutamylcysteine (2 mmol/kg) were given subcutaneously. After 60 min, the kidney GSH levels were determined. In experiment *B*, γ-glutamylcysteine disulfide (1 mmol/kg), γ-glutamylcystine (2 mmol/kg), γ-glutamylcysteine (2 mmol/kg), or glutamate (2 mmol/kg) plus cysteine (2 mmol/kg) were given as stated for experiment *A*. After 20 min, the GSH levels were determined. In experiment *C*, the compounds were administered exactly as in experiment *B* except that the mice were simultaneously injected with buthionine sulfoximine (4 mmol/kg) intraperitoneally. The control values for experiments *A*, *B*, and *C* (0.67–0.89 μmol/g) were normalized to 100%. The brackets on the bars indicate the SD (obtained in studies on three to five mice).

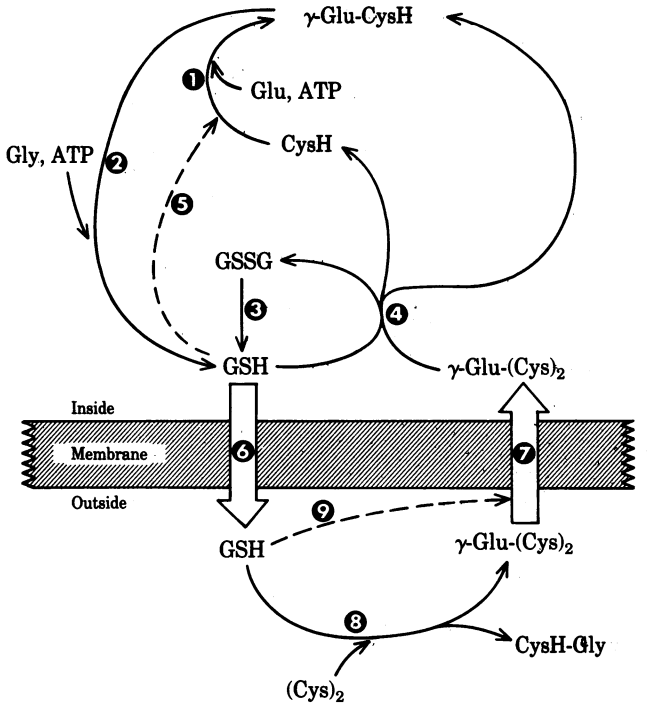

FIG. 3. Metabolism and transport of GSH and γ-glutamylcystine by the kidney. 1, Synthesis of γ-glutamylcysteine from glutamate and cysteine by γ-glutamylcysteine synthetase; 2, synthesis of GSH from γ-glutamylcysteine and glycine by GSH synthetase; 3, reduction of GSSG by GSSG reductase; 4, reduction of γ-glutamylcystine by transhydrogenation with GSH; 5, feedback inhibition of γ-glutamylcysteine synthetase by intracellular GSH; 6, transport of GSH out of cell; 7, transport of γ-glutamylcystine into cell; 8, formation of γ-glutamylcystine from GSH and cystine by γ-glutamyl transpeptidase; 9, inhibition of transport of γ-glutamylcystine by high levels of extracellular GSH. γ-Glutamylcyst(e)ine is also a substrate of γ-glutamylcyclotransferase (21), which would convert it to cyst(e)ine and 5-oxoproline.

the cell membrane by transhydrogenation with GSH that is transported out of the cell. This idea is consistent with one of the suggested functions of GSH transport (20). The scheme given in Fig. 3 summarizes current information about GSH metabolism and transport. GSH, synthesized intracellularly (reactions 1 and 2), is transported (pathway 6) to membrane-bound γ-glutamyl transpeptidase (8), which catalyzes formation of γ-glutamylcystine from GSH and extracellular cystine. Transport of γ-glutamylcystine into the cell (pathway 7) is accompanied by or is followed by reduction (by transhydrogenation with intracellular or transported GSH) to cysteine and γ-glutamylcysteine (reaction 4). These are substrates of γ-glutamylcysteine synthetase (1) and GSH synthetase (2), respectively. In the present work administered γ-glutamylcyst(e)ine was shown to be utilized directly by GSH synthetase, thus bypassing the GSH feedback-inhibited pathway (5) catalyzed by γ-glutamylcysteine synthetase (1). High levels of extracellular GSH inhibit transport of γ-glutamyl amino acids (6). The inhibitory phenomena indicated in the scheme (5 and 9) may play a role in regulation of GSH synthesis and transport.

The results (Table 1) show that GSH is more highly labeled when internally labeled γ-glutamylcystine is administered, indicating that the cysteine moieties of γ-glutamylcystine are not metabolically equivalent. If γ-Glu-$(Cys)_2$ were split by transpeptidase, the cysteine moieties would be metabolically equivalent. It is also possible that γ-glutamylcyst(e)ine might be cleaved intracellularly by γ-glutamyl cyclotransferase, leading to formation of 5-oxoproline and cyst(e)ine. If this were the sole pathway, the cysteine moieties would be expected to be metabolically equivalent. These reactions may occur, but the data show that they are not the sole pathways of γ-Glu-$(Cys)_2$ transport and metabolism.

It is possible that in the experiment (Table 1, Exp. 2) in which a low dose of γ-Glu-$(Cys)_2$ (37.5 nmol per mouse) was given, reduction occurs in the blood plasma to produce γ-glutamylcysteine and cysteine. However, because the data show that the relative labeling of GSH was about the same with a large or a small dose of the labeled γ-glutamylcystines, it seems unlikely that very much reduction occurred in blood plasma. The mice receiving the high dose would require high reducing equivalents in the plasma, which contains only about 30 μM GSH (19, 22).

Studies on the interaction of GSH and amino acids with γ-glutamyl transpeptidase show that a major fraction of transported GSH undergoes transpeptidation and that amino acids such as L-cystine and L-glutamine are among the most active in transpeptidation (18, 21, 23, 24). It is notable that the apparent K_m value for L-cystine is about 30 μM (plasma levels are about 20–200 μM) (25, 26). The findings thus indicate that (*i*) γ-glutamylcystine and other γ-glutamyl amino acids are formed *in vivo* (6) and *in vitro* under conditions that approximate physiological ones (24), and (*ii*) γ-glutamyl amino acids are transported intact.

The marked increase in kidney GSH that accompanies administration of γ-glutamylcysteine, its disulfide, and γ-glutamylcystine suggests that these compounds might be used to increase GSH levels in the kidney and possibly other tissues. We have also noted that administration of GSH itself has this effect, which is probably mediated through formation of γ-glutamylcyst(e)ine. It seems less likely that intact GSH is transported into the cells. In recent studies on GSH transport in human lymphoid cells, no evidence for GSH uptake was obtained (27), and repletion of cellular GSH (previously depleted by suspension of the cells in medium containing buthionine sulfoximine) after addition of GSH externally was shown to involve extracellular GSH breakdown, transport of the products of degradation, and intracellular resynthesis (unpublished data).

The authors acknowledge the skillful technical assistance of Ms. Melanie Chun. This research was supported in part by grants from the American Cancer Society and the National Institutes of Health.

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