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Biochemistry

Formation of γ-glutamylcyst(e)ine *in vivo* is catalyzed by γ-glutamyl transpeptidase

(glutathione/γ-glutamyl cycle/cystine transport)

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ABSTRACT **These studies indicate that γ-glutamylcyst(e)ine, found in the urine of a patient with γ-glutamyl transpeptidase deficiency and also in the urine of experimental animals injected with glutathione or with inhibitors of γ-glutamyl transpeptidase, is formed by the action of γ-glutamyl transpeptidase. The evidence demonstrates that transpeptidation between glutathione and cystine occurs *in vivo* and also that this reaction constitutes a significant physiological function of the enzyme. The appearance of large amounts of γ-glutamylcyst(e)ine in the urine seems to reflect an inhibitory effect of glutathione on the transport of γ-glutamylcyst(e)ine into cells. The findings also indicate that conversion of glutathione to γ-glutamylcysteine by hydrolytic cleavage of the COOH-terminal glycine moiety of glutathione (or analogous cleavage of glutathione disulfide) is not a quantitatively significant pathway. The results reported here show that γ-glutamyl transpeptidase activity is not completely absent in a patient found to have a deficiency of this enzyme and that the activity of the enzyme is not abolished in experimental animals treated with potent γ-glutamyl transpeptidase inhibitors.**

Animals treated with potent inhibitors of γ-glutamyl transpeptidase (1, 2) and a patient having severe γ-glutamyl transpeptidase deficiency (2) excrete large (millimolar) amounts of glutathione, γ-glutamylcysteine, and cysteine in their urine.* It is well known that γ-glutamylcysteine is formed intracellularly by the action of γ-glutamylcysteine synthetase (3), and it is possible that this dipeptide might be translocated from cells into the plasma and thus into the glomerular filtrate and urine. However, such a mechanism to explain the origin of urinary γ-glutamylcysteine is unlikely because there are two highly active intracellular enzymes—glutathione synthetase (3) and γ-glutamyl cyclotransferase (4)—that are capable of acting on this peptide. Indeed, in the disease 5-oxoprolinuria, there is excessive γ-glutamylcysteine formation, but this dipeptide is effectively converted to 5-oxoproline and cysteine by the action of γ-glutamyl cyclotransferase; very little, if any, excess γ-glutamylcysteine appears extracellularly (5). Possible alternative mechanisms for the origin of urinary γ-glutamylcyst(e)ine include (*i*) cleavage of the Cys-Gly bond of glutathione or of glutathione disulfide and (*ii*) transpeptidation between glutathione and cyst(e)ine. The latter pathway would be possible in the presence of an inhibitor of the transpeptidase (or in a patient with transpeptidase deficiency) provided that a small amount of active transpeptidase persists. It is notable that cystine is an excellent acceptor substrate of the transpeptidase (6, 7). In the present work, we have obtained evidence indicating that urinary γ-glutamylcyst(e)ine is formed by the action of γ-glutamyl transpeptidase.

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EXPERIMENTAL

Materials. Amino acids, GSH, glutathione disulfide (GSSG), and dithiothreitol were obtained from Sigma. 2-Vinylpyridine and 4-vinylpyridine were obtained from Aldrich. L-(α*S*,5*S*)-α-Amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) was obtained, through the courtesy of L. J. Hanka, from Upjohn. NCS strain male mice (25–30 g) were obtained from The Rockefeller University. L-γ-Glutamyl-(*o*-carboxy)-phenylhydrazide was prepared as described (1, 8). [^{14}C]GSH was prepared from [1-^{14}C]glycine (New England Nuclear) and γ-glutamylcysteine (9) by the action of rat kidney glutathione synthetase (10). The product was oxidized to GSSG with azodicarboxylic acid(bis)dimethylamide (diamide) and purified by chromatography on Dowex 1 by the procedure described for bis-L-γ-glutamylcystine (9, 11). [^{35}S]GSH with 10 mM dithiothreitol (New England Nuclear) was added to a large excess of 100 mM GSH at pH 7; aliquots of the resulting solution, containing <0.1 mM dithiothreitol, were administered to mice.

The 2-vinylpyridine derivative of L-cysteine (Cys-VP) was prepared by dissolving 3.04 g (25 mmol) of L-cysteine in 50 ml of 10% (vol/vol) methanol. This solution, under nitrogen, was adjusted to pH 7, and then 3.25 ml (27.5 mmol) of 2-vinylpyridine was added. After 60 min at 25°C the mixture was flash evaporated to yield a clear oil. Two portions of methanol (50 ml each) were added and removed by successive flash evaporations. The residue was then dissolved in the minimum volume of warm methanol; on cooling, white crystals appeared, which were collected, washed with small amounts of cold methanol and ether, and then dried over P_2O_5. The product, *S*-β-(2-pyridylethyl)-L-cysteine, was obtained in 75% yield; mp 210–212°C [lit. 165–166°C (12)]. A second crop of product was readily obtained from the mother liquor by evaporation; the overall yield was nearly quantitative. The corresponding derivative of GSH (GS-VP) was prepared similarly (mp 190–191°C). Both compounds gave the expected C, H, and N analysis and eluted as single peaks when chromatographed on a Durrum model 500 amino acid analyzer. A solution of L-γ-glutamyl-*S*-β-(2-pyri-

Abbreviations: GSH and GSSG, glutathione and glutathione disulfide, respectively; GS-VP, Cys-VP, γ-Glu-Cys-VP, and Cys-VP-Gly, 2-vinylpyridine derivatives of glutathione, cysteine, γ-glutamylcysteine, and cysteinylglycine, respectively; AT-125, L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid.

* These compounds, which were identified and quantitated in urine samples that were treated with dithiothreitol and then with 2-vinylpyridine, are present in part in disulfide form. Definite conclusions about their initial state of oxidation cannot yet be drawn. Although there is good evidence that glutathione is translocated out of cells chiefly as reduced glutathione (GSH), the extensive occurrence of extracellular nonenzymatic transhydrogenation and oxidation reactions complicates interpretations.

dylethyl)-L-cysteine (γ-Glu-Cys-VP) was prepared by adding 2 μl of 2-vinylpyridine to 100 μl of 1 mM L-γ-glutamyl-L-cysteine containing 2 mM dithiothreitol. After 60 min at 25°C, aliquots of the solution were diluted with 5% (wt/vol) 5'-sulfosalicylic acid and were used as standards for amino acid analysis. The 2-vinylpyridine derivative of cysteinylglycine (Cys-VP-Gly) was prepared similarly. With the standard Durrum physiological fluids elution procedure, the elution times of γ-Glu-Cys-VP, GS-VP, Cys-VP, and Cys-VP-Gly were 72, 80, 167, and 187 min, respectively. The corresponding values for the 4-vinylpyridine derivatives, which were prepared by the procedure used for γ-Glu-Cys-VP were, respectively, 80, 88, 186, and 195 min. Norleucine elutes at 64 min.

Methods. The activity of GS-VP as a γ-glutamyl donor in transpeptidation was determined at pH 8 in reaction mixtures containing 10 mM γ-glutamyl donor, 25 mM L-[^{14}C]methionine, 175 mM Tris·HCl buffer, and purified rat kidney γ-glutamyl transpeptidase (13). The formation of γ-glutamyl[^{14}C]methionine was determined at 5-min intervals (14). The observed rates were 76, 42, and 40 nmol/min, respectively, when GSH, GSSG, and GS-VP were used as γ-glutamyl donors.

The activity of Cys-VP as a γ-glutamyl acceptor in the transpeptidase reaction was assayed spectrophotometrically (15). The reaction mixtures contained 175 mM Tris·HCl buffer (pH 8.0), 1 mM D-γ-glutamyl-*p*-nitroanilide, γ-glutamyl transpeptidase, and various amounts of acceptor amino acid. L-Cystine, L-cysteine, and Cys-VP exhibited apparent K_m values of 0.19, 10, and 30 mM, respectively. The V_{max} values for L-cysteine and Cys-VP were greater than that for L-cysteine by 1.33- and 1.94-fold, respectively. Cys-VP is not a substrate of purified rat kidney γ-glutamylcysteine synthetase when substituted for L-α-aminobutyrate in the ADP assay method (16).

Glutathione, GS-VP, amino acids, and γ-glutamyl transpeptidase inhibitors were administered to mice as neutral solutions. The bladders of the mice were emptied of urine before injection. Following injection, urination was induced by applying direct pressure to the abdomen; urine samples were collected for 15–60 min after injection. GSH and its metabolites were quantitated by amino acid analysis (Durrum D-500) after reduction with dithiothreitol and conversion to the 2- or 4-vinylpyridine derivative (2). When mice were given GS-VP or Cys-VP, samples of the collected urine were analyzed both with and without prior reduction and treatment with 2-vinylpyridine.

Various mouse tissues were assayed for enzymatic activities capable of cleaving glycine from GSH as follows: Samples of tissue (0.4–0.7 g) were obtained from one or more mice. The tissues were homogenized in 5 vol of 100 mM KCl, and each homogenate was divided into two approximately equal portions. One portion was reserved as "crude homogenate"; the other was centrifuged for 5 min (Beckman Microfuge) and the supernatant was saved. To inactivate γ-glutamyl transpeptidase in the samples, portions (675 μl) of the crude homogenate and supernatant fractions were mixed with 75 μl of 50 mM AT-125 and incubated at 30°C for 30 min or for longer periods as described below (see Table 4). For each tissue, portions of the crude and supernatant fractions were assayed before and after AT-125 treatment. The reaction mixtures (final volume, 500 μl) contained 5 mM [*glycine*-1-^{14}C]GSH, 10 mM dithiothreitol, 200 mM buffer (see below), and 200 μl of tissue extract. The mixtures were maintained at 30°C; at 30 sec, 60 min, 120 min, and 180 min, 100-μl portions were removed and added to 2-ml portions of ice-cold 20 mM acetic acid. The quenched solutions were centrifuged and the supernatants were applied to small (0.5 × 7 cm) columns of Dowex-1-acetate. The columns were eluted with 4 ml of 20 mM acetic acid, and the entire effluent (≈6 ml) (which contains [^{14}C]glycine but not [^{14}C]GSH) was collected. Two-milliliter portions of the effluent were submitted to liquid scintillation counting. The buffers used for the incubations were potassium phosphate (pH 4.5), potassium phosphate (pH 7.0), and Tris·HCl (pH 8.0). Thus, each tissue studied required 12 incubation mixtures (i.e., homogenate and supernatant, with and without AT-125 treatment, in each of 3 buffers); controls in which tissue extract was omitted were also run. The tissue preparations were similarly assayed for activities capable of cleaving glycine from GSSG. The incubation mixtures were as described above except that 2.5 mM [*glycine*-1-^{14}C]GSSG was substituted for GSH and dithiothreitol was omitted. In all cases, the specific activity of the glycine residue(s) in GSH or GSSG was ≈45,000 cpm/μmol.

RESULTS

Because GSH accumulates extracellularly in γ-glutamyl transpeptidase deficiency and also after inhibition of this enzyme, it seemed of interest to determine the effects of administering a substantial amount of GSH. In the experiments described in Fig. 1, mice were injected with GSH and their urine was collected for 1 hr and then analyzed (after reduction and conversion to the 2-vinylpyridine derivative) for GSH, γ-glutamylcysteine, cysteine, and cysteinylglycine. Substantial amounts of these compounds were found. The finding of urinary excretion of γ-glutamylcysteine and cysteine after giving GSH is thus comparable with the observations made on the patient with transpeptidase deficiency and in the experiments in which inhibitors of γ-glutamyl transpeptidase were given to mice. The finding of γ-glutamylcysteine is consistent with either (*i*) hydrolytic cleavage of the COOH-terminal glycine moiety of GSH or (*ii*) transpeptidation between GSH and cyst(e)ine (2).

To explore this problem further, we used a model system involving the 2-vinylpyridine derivatives of glutathione and cysteine. As described above (see *Methods*), γ-glutamyl transpeptidase effectively uses Cys-VP as an acceptor substrate and GS-VP as a γ-glutamyl donor. Table 1 summarizes the results of experiments in which GS-VP and Cys-VP were administered to mice. When GS-VP was injected (experiment 1), there was urinary excretion of GS-VP, γ-Glu-Cys-VP, Cys-VP, and Cys-VP-Gly. This result is, in general, similar to that found after the injection of underivatized GSH (see Fig. 1). When Cys-VP was given together with GSH (experiment 2), the urine contained substantial amounts of both Cys-VP and γ-Glu-Cys-VP. When only Cys-VP was administered (experiment 3), a small but significant amount of γ-Glu-Cys-VP appeared. As GS-VP was not formed in experiments 2 and 3, it is unlikely that the formation of γ-Glu-Cys-VP can be ascribed to cleavage of the glycine moiety of the corresponding GSH derivative. The findings thus

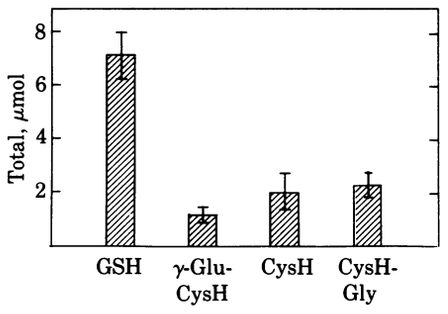

FIG. 1. Effect of administration of GSH on the urinary excretion of γ-glutamylcysteine and related compounds. Mice were injected intraperitoneally with GSH (2.5 mmol/kg) and their urine was collected for 60 min and analyzed as described in *Methods*. Values ± SD (n = 6).

Table 1. Effect of administration of the 2-vinylpyridine derivatives of cysteine and glutathione

Experiment	Compounds given (mmol/kg)	Products found in urine, μmol			
		GS-VP	γ-Glu-Cys-VP	Cys-VP	Cys-VP-Gly
1	GSH-VP (2.5)	2.16 ± 0.75	2.69 ± 0.43	11.9 ± 3.2	1.39 ± 0.59
2	Cys-VP (2.5) + GSH (2.5)	0*	1.67 ± 0.71	9.65 ± 4.4	0
3	Cys-VP (2.5)	0	0.02 ± 0.005	13.9 ± 3.3	0

Compounds were administered to mice by subcutaneous injection of 100 mM solutions. In experiment 2, the injections were made at different sites. Urine was collected for 60 min. Analyses were done on a Durrum amino acid analyzer. Values are mean ± SD.
* The urine also contained 3.4–6.2 μmol of glutathione.

support the view that γ-Glu-Cys-VP arises by transpeptidation between GSH and Cys-VP. In accord with this conclusion is the finding that the excretion of γ-Glu-Cys-VP was substantially greater when both GSH and Cys-VP were given (experiment 2) than when Cys-VP was given alone (experiment 3).

Earlier studies in this laboratory showed that there is considerable urinary excretion of γ-glutamylcysteine after administration to mice of potent inhibitors of γ-glutamyl transpeptidase (1, 2). In the experiment summarized in Table 2, the effects of giving such inhibitors on the metabolism of Cys-VP were determined. Administration of this cysteine derivative to mice treated with the inhibitors led to urinary excretion of substantial amounts of γ-Glu-Cys-VP. This result, which indicates *in vivo* occurrence of transpeptidation, shows that the administration of L-γ-glutamyl-(*o*-carboxyl)phenylhydrazide or of AT-125 does not, under these conditions, abolish the activity of γ-glutamyl transpeptidase.

In the experiments described in Table 3, mice were injected with [^{35}S]GSH (experiment 1 and 2) or with [^{35}S]GSH and unlabeled L-cysteine (experiment 3 and 4). The urine samples subsequently obtained were treated with dithiothreitol and 4-vinylpyridine and a portion of the derivatized samples was chromatographed on a Durrum analyzer in the usual manner to obtain quantitative ninhydrin values for the peaks corresponding to the 4-vinylpyridine derivatives of GSH, γ-glutamylcysteine, and cysteine. Another portion of each sample was chromatographed in exactly the same manner except that fractions of the effluent were collected at 2-min intervals for the determination of radioactivity. From the data thus obtained, the specific radioactivities of the peaks corresponding to GSH, γ-glutamylcysteine, and cysteine were obtained. In experiments 1 and 2, the specific radioactivities of urinary γ-glutamylcysteine and cysteine were somewhat lower than that of GSH. This result is probably not consistent with the cleavage of glycine from GSH to form γ-glutamylcysteine but is consistent with the formation of γ-glutamylcyst(e)ine by transpeptidation between GSH and a cyst(e)ine pool formed predominantly by the *in vivo* metabolism of [^{35}S]GSH. In experiments 3 and 4, in which [^{35}S]GSH was injected 5 min after unlabeled L-cysteine was administered, the specific activity of the urinary GSH was substantially higher than that of γ-glutamylcysteine and cysteine. This result excludes cleavage of glycine from GSH to form γ-glutamylcysteine as a quantitatively major pathway and can be explained by transpeptidation between GSH and cyst(e)ine.

The results described above, which establish the role of transpeptidation in γ-glutamylcyst(e)ine formation, do not exclude the possibility that direct cleavage of glycine from GSH (or GSSG) also occurs. To investigate this possibility, homogenates of several mouse tissues were examined for the presence of activities capable of releasing [^{14}C]glycine from [*glycine*-^{14}C]GSH and [*glycine*-^{14}C]GSSG. The assays were conducted at pH values of 4, 7, and 8 on the homogenates and on the supernatant solutions obtained by centrifugation of the homogenates (see *Methods*). The release of [^{14}C]glycine was determined before and after treatment for 30 min with 5 mM AT-125. Under these conditions, the release of glycine was less than 10, 55, and 81 nmol/hr per 200 μl of tissue preparation (from liver, spleen, or muscle) prior to AT-125 treatment at pH 4, 7, and 8, respectively. The corresponding maximal values found after treatment with AT-125 were, 7, 31, and 27 nmol/hr. The results with GSH and GSSG were in all cases similar. Somewhat higher values were found in similar studies with kidney and pancreas; however, both tissues are rich in transpeptidase and it is probable that the product formed was cyst(e)inyl[^{14}C]glycine rather than [^{14}C]glycine (cysteinylglycine and glycine co-elute from Dowex-1; see *Methods*). With longer periods of preincubation with AT-125, the release of [^{14}C]glycine decreased progressively to values (after 120 min) in the range 1–15 nmol/hr per 200 μl of tissue preparation [equivalent to ≈35 mg of tissue (Table 4)]. These studies suggest that, although an enzymatic activity capable of releasing glycine from GSH may be present in these tissues, the amount of activity is of a rather low order. For comparison, mouse kidney and liver contain transpeptidase activity equivalent to ≈5 mmol/hr per 35 mg of tissue and 700 nmol/hr per 35 mg of tissue, respectively (17).

Table 2. Effect of inhibitors of γ-glutamyl transpeptidase on metabolism of the 2-vinylpyridine derivative of cysteine

Compounds given	Products found in the urine, μmol			
	γ-Glu-Cys-VP	Cys-VP	GS-VP	GSH
Cys-VP + L-OC	0.70 ± 0.11	14.9 ± 8.1	0	0.98 ± 0.33
Cys-VP + AT-125	0.98 ± 0.16	8.52 ± 1.6	0	1.62 ± 0.45

L-γ-Glutamyl-(*o*-carboxy)phenylhydrazide (L-OC; 0.5 mmol/kg) and AT-125 (2.5 mmol/kg) were given by subcutaneous and intraperitoneal injection, respectively. Cys-VP (2.5 mmol/kg) was given by subcutaneous injection 15 min later. Urine was collected for 1 hr. γ-Glu-Cys-VP, Cys-VP, and GS-VP were quantitatively determined by amino acid analysis. Glutathione was quantitated by amino acid analysis after treatment of the sample with 2-vinylpyridine. Values are mean ± SD (n = 3 or 4).

Table 3. Specific radioactivities of urinary metabolites after [^{35}S]GSH administration

Experiment	Compounds given (mmol/kg)	Specific activity,* cpm/nmol		
		GSH	γ-Glu-Cys	Cys
1	[^{35}S]GSH (2.5)	80	60	72
2	[^{35}S]GSH (2.5)	93	35	47
3	[^{35}S]GSH (2.5) + CysH (5.0)	97	7	18
4	[^{35}S]GSH (2.5) + CysH (5.0)	131	21	17

Mice were injected subcutaneously with [^{35}S]GSH, and urine was collected for 15 min. In experiments 3 and 4, L-cysteine was given intraperitoneally 5 min before injection of [^{35}S]GSH.
* Determined as 4-vinylpyridine derivatives.

Table 4. Effect of preincubation with AT-125 on the release of glycine from GSH and GSSG by preparations of kidney and pancreas

		[^{14}C]Glycine release,* nmol/hr per 200 μl of tissue extract							
		0 min		30 min		60 min		120 min	
Tissue	Substrate	H	S	H	S	H	S	H	S
Kidney	GSH	>1000	>1000	205	149	36	32	5	5
Kidney	GSSG	>1000	>1000	166	115	47	22	8	15
Pancreas	GSH	>1000	>1000	119	67	57	45	6	11
Pancreas	GSSG	370	242	135	23	23	7	1	1

Reaction mixtures (pH 7) and preincubation with AT-125 were as described in *Methods*. A 200-μl aliquot was removed for assay at 30 min; an additional 50 μl of 50 mM AT-125 was then added to the preincubation mixture. At 60 min, another 200-μl aliquot was removed and assayed, and 25 μl of 50 mM AT-125 was then added to the preincubation mixture. At 120 min, a final 200-μl aliquot was removed and assayed.

* [^{14}C]Gly-GSH or [^{14}C]Gly-GSSG. Determined as radioactivity not binding to Dowex-1-acetate (i.e., either glycine or Cys-Gly or both). H, homogenate; S, supernatant.

DISCUSSION

The finding that administration of Cys-VP leads to formation of γ-Glu-Cys-VP offers strong support for the occurrence of transpeptidation *in vivo* and is consistent with the view that the formation of γ-glutamylcysteine that is observed (*i*) after administration of GSH, (*ii*) in the presence of γ-glutamyl transpeptidase deficiency, or (*iii*) after inhibition of γ-glutamyl transpeptidase also takes place by transpeptidation. Although extensive formation of γ-Glu-Cys-VP occurred when both Cys-VP and GSH were given (see Table 1, experiment 2), it is notable that a significant amount of γ-Glu-Cys-VP also appeared after Cys-VP was given alone (see Table 1, experiment 3). This indicates that *in vivo* transpeptidation does not require elevated levels of GSH and that it probably occurs also under physiological conditions. This conclusion is consistent with the high affinity of the transpeptidase for GSH (apparent K_m value, ≈50 μM) (15).

These findings also show that administration of GSH induces a pattern of urinary excretion that closely resembles those observed in transpeptidase deficiency and after inhibition of transpeptidase. As cystine is an excellent acceptor substrate of γ-glutamyl transpeptidase (6, 7), one must seriously consider the possibility that this amino acid plays a significant role in the formation of γ-glutamylcyst(e)ine observed here and in earlier experiments (1, 2). Previous studies in this laboratory have shown that γ-glutamyl amino acids are transported effectively into renal and probably other cells (18). One would therefore expect that γ-glutamylcysteine and γ-glutamylcystine would be rapidly transported into cells under normal conditions. As noted earlier (6), intracellular reduction of γ-glutamylcystine would form γ-glutamylcysteine and cysteine, both of which are substrates for GSH biosynthesis. Extracellular accumulation and urinary excretion of γ-glutamylcyst(e)ine would occur if it was formed in much higher than normal amounts or if there is competition for transport or metabolism due to elevated levels of GSH. Thus, the formation of γ-glutamylcyst(e)ine may be ascribed to the action of transpeptidase; the appearance of γ-glutamylcyst(e)ine in the urine suggests that under these conditions, the rate of formation of γ-glutamylcyst(e)ine exceeds that of its transport into cells. Other studies in this laboratory have shown that transport of γ-glutamyl amino acids is inhibited by inhibitors of transpeptidase (18) and also by high concentrations of glutathione (unpublished data).

The present studies indicate that neither the patient deficient in transpeptidase nor the experimental animals treated with transpeptidase inhibitors are completely devoid of γ-glutamyl transpeptidase activity. Indeed, other studies in this laboratory in which a model substrate of transpeptidase (1) and labeled glutathione preparations (unpublished data) were used have shown that the inhibitions produced by AT-125 and γ-glutamyl(*o*-carboxy)phenylhydrazide are extensive but not complete. Presumably, in the complete absence of transpeptidase activity, no γ-glutamylcyst(e)ine would be formed extracellularly. The finding that γ-glutamyl transpeptidase is not completely inhibited *in vivo* by potent transpeptidase inhibitors seems to partly explain the apparent discrepancy noted earlier (19) between the calculated amount of GSH translocated from tissues into the plasma and the observed accumulation of plasma GSH in bilaterally nephrectomized animals given transpeptidase inhibitors. It may be noted that even if 90% of the total tissue transpeptidase is inhibited, the residual enzyme activity is very substantial.

The experiments in which mice were injected with [^{35}S]GSH (see Table 3) show that the specific radioactivity of the urinary γ-glutamylcyst(e)ine is lower than that of the urinary GSH. This finding indicates that a pathway of γ-glutamylcyst(e)ine formation involving cleavage of the COOH-terminal glycine residues of GSH or GSSG cannot account for a major fraction of the γ-glutamylcyst(e)ine that is formed. The findings are consistent with the conclusion that γ-glutamylcyst(e)ine is formed by transpeptidation between GSH and cyst(e)ine. Although the experiments were carried out with high concentrations of GSH and cysteine, it is important to note that the physiological levels of GSH and cystine in the plasma are not far from their observed K_m values for γ-glutamyl transpeptidase (15).

In the present studies, we also found no clear evidence for significant formation of γ-glutamylcyst(e)ine by cleavage of glycine from GSH or from GSSG *in vitro*. Thus, incubation of homogenates and centrifuged homogenates of several mouse tissues did not liberate more than small amounts of glycine from either GSH or GSSG in the presence of potent irreversible inhibitor of γ-glutamyl transpeptidase activity. These observations suggest that γ-glutamylcyst(e)ine formation by cleavage of glycine from GSH or GSSG is probably not a major pathway. However, such cleavage cannot be completely excluded because it is known that enzymes with specificities similar to that of carboxypeptidase are widely distributed. The early finding of Maver *et al.* (20, 21) that an enzyme activity in spleen and certain other tissues catalyzed conversion of GSH to γ-glutamylcysteine may possibly now be explained in terms of transpeptidation. It is notable in this connection that Maver *et al.* added cysteine to the tissue preparations used in their studies because it was believed that a reducing agent was required for the presumed hydrolytic reaction. The present results suggest that the added cysteine transpeptidated with GSH.

The scheme given in Fig. 2 summarizes our present ideas about GSH metabolism. These have been derived from early work on the γ-glutamyl cycle (22), studies on the specificity of purified γ-glutamyl transpeptidase (6, 7), experiments on the translocation of GSH (1, 23) and on the intraorgan (1) and interorgan (19, 24) cycles of GSH metabolism, observations on a patient with γ-glutamyl transpeptidase deficiency (2), studies of experimental animals treated with potent γ-glutamyl transpeptidase inhibitors (1, 2), studies on the transport of γ-glutamyl amino acids (18), and the present work. According to the scheme, GSH is synthesized intracellularly by the action of γ-glutamylcysteine synthetase (reaction 1) and glutathione synthetase (reaction 2). GSH is translocated (step A) to membrane-bound γ-glutamyl transpeptidase, which catalyzes the transfer

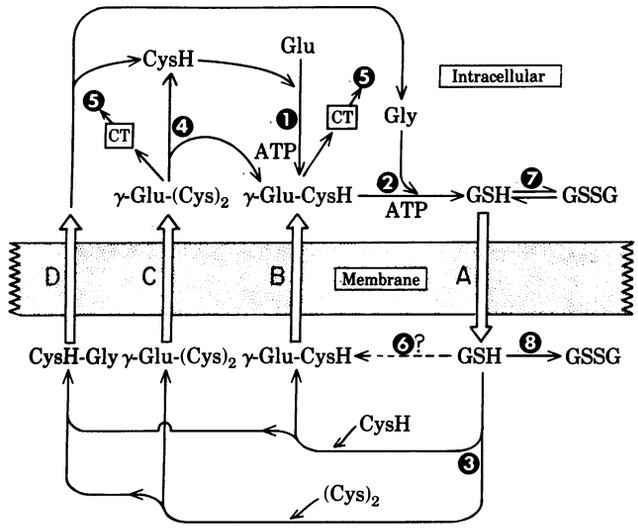

FIG. 2. Summary of the metabolism and transport of γ-glutamylcysteine, GSH, and related compounds. Reactions: 1, γ-glutamylcysteine synthetase; 2, glutathione synthetase; 3, γ-glutamyl transpeptidase; 4, intracellular reduction of γ-glutamylcystine, probably mediated by transhydrogenation with GSH and the activity of glutathione reductase; CT, γ-glutamyl cyclotransferase; 5, 5-oxoprolinase; 6, hydrolytic cleavage of the COOH-terminal glycine moiety of GSH (probably not quantitatively significant); 7, intracellular oxidation of GSH to GSSG catalyzed by several enzymes—e.g., glutathione peroxidase—and intracellular conversion of GSSG to GSH catalyzed by glutathione reductase; 8, extracellular oxidation of GSH to GSSG (probably mainly nonenzymatic; the metabolism of GSSG closely resembles that of GSH).

of the γ-glutamyl moiety to amino acids—e.g., cystine and cysteine—to form the corresponding γ-glutamyl amino acids (reaction 3); these are translocated (steps B and C) into the cells. Some GSH may undergo transpeptidase-catalyzed hydrolysis. γ-Glutamylcysteine may be used directly for GSH biosynthesis (reaction 2) or by γ-glutamyl cyclotransferase (step CT), which converts γ-glutamyl amino acids to the corresponding amino acid and 5-oxoproline. 5-Oxoproline is converted to glutamate by the action of 5-oxoprolinase (reaction 5). The transport of γ-glutamylcystine (step C) into the cell is followed by its reduction to cysteine and γ-glutamylcysteine (reaction 4). Cysteinylglycine formed in the transpeptidation reaction may be transported as such, or the products of its hydrolysis may be transported. Activity capable of cleaving this dipeptide is present within the cell and also on the membrane. Intracellular GSH may be reversibly converted to glutathione disulfide (reactions summarized under 7). On the other hand, the extracellular oxidation of GSH to GSSG, largely a nonenzymatic process (11), is irreversible because there is no source of reducing power. Extracellular GSSG follows metabolic pathways that are closely analogous to those of GSH. The present studies suggest that the conversion of GSH to γ-glutamylcysteine by hydrolytic cleavage of the COOH-terminal glycine moiety (reaction 6) is probably not a quantitatively significant pathway.

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