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Evidence that the γ-glutamyl cycle functions *in vivo* using intracellular glutathione: Effects of amino acids and selective inhibition of enzymes

(γ-glutamyl transpeptidase/5-oxoproline/prothionine sulfoximine/amino acid transport/β-aminoglutaryl-α-aminobutyrate)

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ABSTRACT **The function of the γ-glutamyl cycle was explored in *in vivo* studies in which amino acids and specific inhibitors of cycle enzymes (γ-glutamyl transpeptidase, γ-glutamyl cyclotransferase, γ-glutamylcysteine synthetase, and 5-oxoprolinase) were administered to mice. The findings, which show that the γ-glutamyl cycle functions *in vivo*, support the conclusion that γ-glutamyl amino acids formed by γ-glutamyl transpeptidase from externally supplied amino acids and intracellular glutathione are translocated into the cell and thus indicate that there is a significant physiological connection between the metabolism of glutathione and the transport of amino acids.**

Glutathione occurs intracellularly in millimolar concentrations whereas the level of extracellular glutathione (e.g., blood plasma) is in the micromolar range. The steady-state level of glutathione in a tissue such as kidney is a function of the γ-glutamyl cycle and thus reflects a balance between the synthesis of glutathione (catalyzed by γ-glutamylcysteine and glutathione synthetases) and the utilization of glutathione (catalyzed by γ-glutamyl transpeptidase) (1). When glutathione synthesis is inhibited, the glutathione level falls rapidly (2, 3), reflecting the substantial normal rate of glutathione utilization. Glutathione levels also decrease when there is an increase in transpeptidation. Thus, administration to rats of glycylglycine, a good transpeptidase acceptor substrate, leads to marked decreases in the levels of glutathione in the kidney and liver (2). Such a decrease in glutathione did not occur after equivalent doses of glycine or other amino acids (2), presumably because after small doses of amino acids the amount of glutathione used for transpeptidation can be rapidly regenerated by synthesis. However, the very rapid transpeptidation reaction that occurs after glycylglycine administration uses glutathione at a much faster rate than that of its resynthesis, and the glutathione level therefore decreases.

We have now found that administration of a large amount of amino acid to mice also decreases renal glutathione and that this effect (which is accompanied by increased 5-oxoproline formation) is markedly diminished by administration of an inhibitor of γ-glutamyl transpeptidase. Inhibition of transpeptidase *in vivo* also (*i*) decreases the rate of glutathione disappearance that occurs after inhibition of glutathione synthesis, and (*ii*) leads to a moderate increase in kidney glutathione levels in otherwise untreated animals. Previous studies showed that *in vivo* inhibition of 5-oxoprolinase led to 5-oxoproline accumulation and that such accumulation was greater after amino acid administration (4). In the present work we found that *in vivo* inhibition of γ-glutamylcyclotransferase decreased the accumulation of 5-oxoproline found after inhibition of 5-oxoprolinase and also decreased the normal steady-state levels of 5-oxoproline. This indicates that γ-glutamyl cyclotransferase and 5-oxoprolinase are major *in vivo* catalysts for the formation and utilization, respectively, of 5-oxoproline.

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EXPERIMENTAL

Materials. Amino acids were obtained from Sigma. L-2-Imidazolidone-4-carboxylate was obtained from Bachem (Torrance, CA). β-Aminoglutaryl-L-α-amino[^{14}C]butyrate was prepared enzymatically (5). NCS strain male mice, 6–7 weeks old (25–30 g), were obtained from The Rockefeller University.

Methods. Mice, fasted 24 hr with free access to water, were injected intraperitoneally (two-thirds of dose) and subcutaneously on the back (one-third of dose). The doses, 32 mmol/kg, were given as 0.4 M solutions; lower doses were proportionately diluted. Multicompound injections were made with mixed solutions. Animals were killed by decapitation and the kidneys were removed and homogenized in 5 vol of 1% picric acid. After centrifugation, an aliquot was treated with 2-vinylpyridine and analyzed for amino acids and glutathione (6). Tissue 5-oxoproline was isolated (7) and then hydrolyzed and quantitated by amino acid analysis (4, 6). Data given are average values based on 4–20 separate determinations.

RESULTS

When methionine or certain amino acids were given to mice, there was a substantial decrease in the level of glutathione in the kidney (Table 1). At 30 min after L-methionine was given at a dose of 32 mmol/kg, the glutathione level decreased to about half of the control (Table 1; Fig. 1), and this effect was accompanied by a marked increase in the level of 5-oxoproline. Increases in 5-oxoproline levels were found after administration of serine or alanine but not glycylglycine, the γ-glutamyl derivative of which is not a substrate of γ-glutamylcyclotransferase. Because administration of methionine was accompanied by decreases in the levels of glutamate, glycine, and cysteine (Table 2), a study was done in which glutamate, glycine, and cysteine were also given. Administration of the amino acid constituents of glutathione increased the glutathione level somewhat (Exp. 3) but, when these amino acids and methionine were given together, the net decrease of the glutathione levels [3.7 (Exp. 3) minus 2.1 (Exp. 4) = 1.6] was about the same as found when methionine was given alone [3.0 (Exp. 1) minus 1.24 (Exp. 2) = 1.76].

The effects of giving a transpeptidase inhibitor [serine plus borate (8, 9)] on the phenomena described above are given in Table 3. Injection of serine and borate led to a moderate increase in the glutathione level, whereas borate had no effect. O-Methylserine [which does not serve in place of serine in inhibiting transpeptidase (unpublished data)] did not replace

Table 1. Effect of amino acid administration on kidney levels of glutathione and 5-oxo-L-proline*

Amino acid	Dose, mmol/kg	Glutathione μmol/g	Glutathione % of control	5-Oxoproline nmol/g	5-Oxoproline % of control
None	—	3.0 ± 0.3	[100]	36 ± 5	[100]
L-Methionine	16	1.8 ± 0.2	61	49 ± 4	136
L-Methionine	32	1.6 ± 0.2	53	59 ± 4	164
L-Methionine†	32	1.2 ± 0.1	41	69 ± 5	191
L-Glutamine	32	2.0 ± 0.1	67		
L-Serine	32	2.3 ± 0.3	77	109 ± 3	303
L-Alanine	32	2.0 ± 0.2	67	65 ± 2	181
Glycylglycine	3.3	2.3 ± 0.2	77	33 ± 3	97

* Mice were killed 30 min after injection. The data are shown as mean ± SD.
† These mice were killed 2 hr after injection.

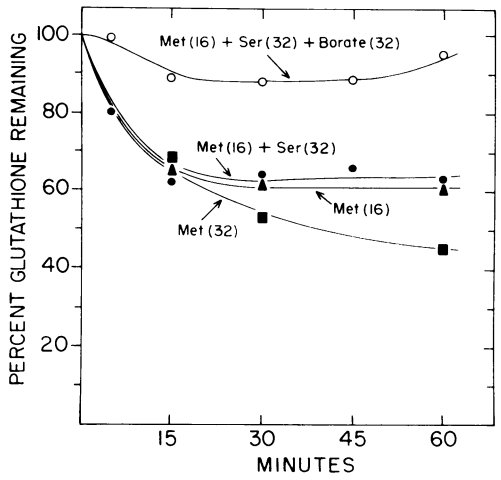

FIG. 1. Inhibition of methionine-induced depletion of kidney glutathione by serine and borate; values in parentheses indicate doses (mmol/kg).

serine in preventing the amino acid-dependent decline in glutathione level. When serine and borate were given with methionine, the glutathione level was not significantly affected (Table 3; Fig. 1); similar findings were obtained when glycylglycine was substituted for methionine. These findings indicate that γ-glutamyl transpeptidase activity is a major factor in producing the decline of the glutathione level found after administration of methionine and glycylglycine. A note of caution seems relevant, however, because it is evident that administration of a large dose of amino acid could well perturb other aspects of metabolism, which might affect glutathione levels. Changes in electrolytes and other solutes between plasma and tissues are possible, and various amino acid competitive effects need to be considered. Thus, after administration of α-aminoisobutyrate, which is not a substrate of γ-glutamyl transpeptidase, we found decreased levels of renal glutathione and increased levels of plasma amino acids. The effect of α-aminoisobutyrate on glutathione levels was only partially eliminated by simultaneous administration of serine and borate, suggesting that α-aminoisobutyrate influences glutathione levels by making other amino acids more available for transpeptidation and also by another mechanism, which needs to be elucidated. α-Aminoisobutyrate was found not to affect the activities of γ-glutamylcysteine and glutathione synthetases *in vitro*.

Previous studies showed that inhibition of glutathione synthesis in mice by administration of prothionine sulfoximine was accompanied by a rapid decrease in the level of kidney glutathione (3). In the present work we found that *in vivo* inhibition of γ-glutamyl transpeptidase by administration of L-serine and borate decreased the rate of glutathione disappearance in the kidneys of mice treated with prothionine sulfoximine. Injection of L-serine into prothionine sulfoximine-treated mice (Table 4) increased the rate of glutathione disappearance by about 20%, presumably because serine stimulates utilization of glutathione by transpeptidation. Injection of borate did not affect glutathione disappearance but, when serine and borate were given together, the rate of glutathione disappearance was decreased significantly—i.e., by about 30% compared to the value found with serine.

Administration of imidazolidone carboxylate, a competitive inhibitor of 5-oxoprolinase (10), leads to increased tissue 5-oxoproline levels (4) (Table 5). When β-aminoglutaryl-α-aminobutyrate, a competitive inhibitor of γ-glutamyl cyclotransferase (11), was given together with imidazolidone carboxylate there was a marked decrease in 5-oxoproline accumulation. It is significant that, when β-aminoglutaryl-α-aminobutyrate was given to otherwise untreated mice, there was a substantial decrease in the steady-state level of 5-oxoproline. Administration of β-aminoglutaryl-α-aminobutyrate was not accompanied by detectable (>0.5 μmol/kg) accumulation of γ-glutamyl amino acids, suggesting that the cyclotransferase may not have been completely inhibited or that the expected γ-glutamyl amino acids were destroyed by transpeptidase, or both. It is notable the 5-oxoproline accumulation occurred after administration of imidazolidone carboxylate to prothionine sulfoximine-treated mice, indicating that formation of 5-oxoproline by the combined activities of γ-glutamylcysteine synthetase and γ-glutamyl cyclotransferase [as occurs in 5-oxoprolinuria (12, 13)] does not play a major role in the normal formation of 5-oxoproline.

DISCUSSION

Glutathione is found almost exclusively intracellularly, whereas γ-glutamyl transpeptidase is bound to cell membranes. It has been concluded that the transpeptidase is bound to the outer surface of the cell membrane because it is accessible to externally supplied substrates (1, 14–16) and because some of the enzyme is readily released from the membrane by treatment with proteolytic enzymes (17, 18). However, the data do not exclude the possibility that the transpeptidase may also be lo-

Table 2. Methionine-induced glutathione depletion: effect of glutamate, cysteine, and glycine*

Exp.	Amino acids administered (mmol/kg)	Level in kidney, μmol/g Glutathione	Glutamate	Cysteine	Glycine
1	None	3.0 ± 0.3	3.3 ± 0.5	0.20	2.9 ± 0.4
2	Methionine (32)	1.24 ± 0.06	1.7 ± 0.1	0.17	0.9 ± 0.1
3	Glutamate (10) + cysteine (3.2) + glycine (3.2)	3.7 ± 0.1	5.2 ± 1.3	0.33	2.6 ± 0.2
4	Methionine (32) + glutamate (10) + cysteine (3.2) + glycine (3.2)	2.1 ± 0.2	3.8 ± 1.0	0.53	3.3 ± 0.1

* Mice were killed 60 min after injection. Data are shown as mean ± SD. Aliquots from four to six kidney homogenates were pooled for the cysteine determinations (2-vinyl pyridine derivative).

Table 3. Kidney levels of glutathione after inhibition of γ-glutamyl transpeptidase by serine and borate*

Compounds given (mmol/kg)	Glutathione μmol/g	Glutathione % of control
None	3.0 ± 0.2	100
L-Serine (32) + borate (32)	3.5 ± 0.1	117
L-Serine (32)	2.3 ± 0.3	77
Borate (32)	3.0 ± 0.1	100
O-Methyl-DL-serine (32) + borate (32)	2.4 ± 0.2	81
O-Methyl-DL-serine (32)	2.1 ± 0.3	69
L-Methionine (16)	1.6 ± 0.2	53
L-Methionine (16) + L-serine (32)	1.6 ± 0.3	53
L-Methionine (16) + L-serine (32) + borate (32)	2.7 ± 0.2	90
GlyGly (16)	1.6 ± 0.2	53
GlyGly (16) + L-serine (32)	1.7 ± 0.3	57
GlyGly (16) + L-serine (32) + borate (32)	2.4 ± 0.3	80

* Mice were killed 30 min after injection. Data are shown as mean ± SD.

cated in the membrane at regions other than the external surface. Although it is possible that the enzyme acts on extracellular γ-glutamyl substrate under physiological conditions, the present data indicate clearly that the transpeptidase interacts effectively with intracellular glutathione. Thus, it appears that intracellular glutathione must be accessible to γ-glutamyl transpeptidase, to account for the significant turnover of intracellular glutathione (19). If the enzyme is located only on the outer surface of the membrane, it would seem that there must be a channel or carrier system of some sort to transport intracellular glutathione to the transpeptidase.

That intracellular glutathione is transported to membrane-bound transpeptidase is consistent with findings on a patient with γ-glutamyl transpeptidase deficiency who exhibited substantial glutathionemia and glutathionuria, which were attributed to secretion or leakage of glutathione into the plasma and glomerular filtrate (20). The leakage of intracellular glutathione to the plasma in this patient may reflect an aspect of a process that occurs normally to provide substrate to the membrane-bound enzyme. Although renal transpeptidase may also act on glutathione that enters the systemic circulation as a consequence of cell destruction, it is not clear whether such glutathione could account for as much as 850 mg/day, the amount excreted in the urine by this patient. Furthermore, glutathione is readily converted in blood plasma to glutathione disulfide (20), which is a poor substrate of the transpeptidase (21).

The dramatic decrease in kidney glutathione levels found after administration of amino acids seems to be due to increased transpeptidation that utilizes intracellular glutathione more rapidly than it can be synthesized. Such utilization of glutathione would be expected to lead to increased intracellular levels of γ-glutamyl amino acids; that this does in fact occur is reflected by the finding that the 5-oxoproline levels increased substantially. That the amount of 5-oxoproline found in the kidney is less than the amount of glutathione that disappears is to be expected because 5-oxoproline would be converted to glutamate by 5-oxoprolinase and would diffuse from the kidney into the general circulation. Some glutathione might disappear without formation of 5-oxoproline if γ-glutamyl amino acids or glutathione were hydrolyzed by transpeptidase.

Table 4. Effect of serine and borate on the rate of glutathione utilization during inhibition of glutathione synthesis by prothionine sulfoximine

Compound given (mmol/kg)*	Disappearance of glutathione, μmol/g†
None	1.69 ± 0.17
Borate (32)	1.60 ± 0.15
L-Serine (32)	2.04 ± 0.13
L-Serine (32) + borate (32)	1.45 ± 0.12

* Injected [subcutaneously (hindquarter, one third of dose) and intraperitoneally (two-thirds of dose)] 10 min before subcutaneous (upper back) administration of DL-prothionine-*SR*-sulfoximine (4 mmol/kg); animals were killed 15 min later.
† Shown as mean ± SD.

Table 5. Effect of β-aminoglutaryl-α-aminobutyrate (β-Glu-αAba) on kidney levels of 5-oxoproline*

Compound given	5-Oxoproline, nmol/g	β-Glu-α-Aba, μmol/g
None (Control)	36 ± 5	—
β-Glu-α-Aba	18 ± 4	36 ± 10
ICA	110 ± 9	—
ICA + β-Glu-α-Aba	60 ± 20	46 ± 7

* β-Glu-α-[^{14}C]Aba (10 mmol/kg) and L-2-imidazolidone-4-carboxylate (ICA) (2.4 mmol/kg) were given intraperitoneally. After 30 min the doses were repeated subcutaneously; 30 min later the animals were killed. Data are shown as mean ± SD.

The effects of serine and borate [a presumptive transition state inhibition of γ-glutamyl transpeptidase (9)] in (*i*) inhibiting the amino acid-induced utilization of glutathione and the rate of glutathione disappearance after inhibition of glutathione synthesis and (*ii*) in increasing glutathione levels in the kidneys of otherwise untreated mice are in accord with the view that the transpeptidase is the major catalyst of glutathione utilization. Further study is needed to determine why the inhibition by serine and borate is not more effective *in vivo*. Possibly, the transpeptidase is protected against inhibition *in vivo* by virtue of being predominantly in the γ-glutamyl form. The latter may undergo hydrolysis, or, in the presence of high enough amino acid concentrations, participate in transpeptidation. Other studies in our laboratory have shown that γ-glutamyl transpeptidase may also be inhibited by γ-glutamyl hydrazones of α-keto acids (21), γ-glutamyl hydrazides, L-azaserine, and 6-diazo-5-oxo-L-norleucine (22). Studies in which mice were treated with 6-diazo-5-oxo-norleucine showed that this inhibitor, like serine plus borate (Table 4), decreased the rate of glutathione disappearance after inhibition of glutathione synthesis. Certain γ-glutamyl hydrazide inhibitors [e.g., γ-glutamyl-(*o*-carboxy)phenylhydrazide and 1-γ-glutamyl 2-(1-carboxyethyl)hydrazide] are very effective *in vivo*, and results similar to those described in Table 4 were also obtained with this type of inhibitor. It is particularly notable that a substantial glutathionuria occurs when there is virtually complete inhibition of γ-glutamyl transpeptidase. This is then a useful experimental animal model for the human disease (20).

Although the findings indicate a close relationship between increased extracellular amino acid concentration on the one hand and decreased intracellular glutathione levels and increased 5-oxoproline levels on the other, it cannot be excluded that the transpeptidase can act on intracellular amino acids that might be transported into the cell by a mechanism not involving the γ-glutamyl cycle. The physiological significance of such an "intracellular γ-glutamyl cycle" and its relationship to the characteristic localization of transpeptidase in the membranes of certain epithelial cells (e.g., jejunal villi, renal tubules, choroid plexus) at sites known to be involved in transport need to be considered.

That there is indeed a balance between glutathione synthesis and utilization is supported by the present findings with enzyme inhibitors. The level of glutathione evidently also can be influenced by the availability of its amino acid constituents; we found that fasting lowers glutathione levels and that administration of glycine, glutamate, and cysteine increases the level of glutathione (Table 2).

Direct evidence for membrane translocation of γ-glutamyl amino acids formed by the transpeptidase is not yet at hand. However, there is evidence that administered γ-glutamyl-α-aminobutyrate is transported into kidney and directly incorporated into ophthalmic acid (23). Studies on the uptake of methionine sulfoximine by the kidney have shown that more methionine sulfoximine is found in the kidney after administration of γ-glutamylmethionine sulfoximine to mice than after administration of methionine sulfoximine itself; it is particularly significant that intact γ-glutamylmethionine sulfoximine is found in the kidney (unpublished data). That γ-glutamylmethionine sulfoximine is well transported is consistent with the postulated transport role of the γ-glutamyl cycle. γ-Glutamylmethionine sulfoximine might be transported [by the mechanism previously proposed (24)] as γ-glutamyl-γ-glutamylmethionine sulfoximine, or it may "fit" into a channel that accomodates γ-glutamyl amino acids. Thus, the transpeptidase may function to form γ-glutamyl compounds that are then transported by a separate mechanism, or the action of the transpeptidase *in vivo* may itself involve translocation.

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