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Stimulation of the Hydrolytic Activity and Decrease of the Transpeptidase Activity of γ-Glutamyl Transpeptidase by Maleate; Identity of a Rat Kidney Maleate-Stimulated Glutaminase and γ-Glutamyl Transpeptidase

(amino-acid transport/ammoniagenesis/γ-glutamyl cycle/glutathione/glutamine)

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ABSTRACT **γ-Glutamyl transpeptidase catalyzes transfer of the γ-glutamyl moiety of glutathione (and other γ-glutamyl compounds) to amino acid and peptide acceptors; this reaction probably involves (*a*) formation of a γ-glutamyl enzyme and (*b*) reaction of the γ-glutamyl-enzyme with an acceptor. Maleate decreases the latter reaction and markedly increases hydrolysis of the γ-glutamyl donor, apparently by affecting the enzyme so as to facilitate reaction of the γ-glutamyl enzyme with water. Transpeptidase catalyzes γ-glutamyl hydroxamate formation from many γ-glutamyl compounds and hydroxylamine; this reaction is stimulated 4- to 5-fold by maleate. Glutamine, a poor substrate for transpeptidation as compared to glutathione, is slowly hydrolyzed and converted to γ-glutamyl-glutamine by the transpeptidase; in the presence of maleate, hydrolysis of glutamine is markedly (>10-fold) increased, as is also its conversion to γ-glutamyl hydroxamate in the presence of hydroxylamine. The findings suggest that the previously described "maleate-stimulated phosphate-independent glutaminase" is a catalytic function of γ-glutamyl transpeptidase. Transpeptidase-catalyzed glutaminase activity may play a role in renal ammoniagenesis. The ability of maleate to decrease transpeptidation of γ-glutamyl compounds (and to increase their hydrolysis to glutamate), when considered in the light of earlier findings that treatment of animals with maleate produces aminoaciduria, is consistent with function of transpeptidase and the γ-glutamyl cycle in amino-acid transport.**

Previous studies in this laboratory have led to isolation of apparently homogeneous preparations of rat kidney γ-glutamyl transpeptidase and to studies on the interaction of this enzyme with a variety of γ-glutamyl donors and amino-acid and peptide acceptors (1). These and other studies on this enzyme are consistent with the hypothesis that it functions in amino-acid (or peptide) transport by way of the γ-glutamyl cycle (2–5). The transpeptidase is membrane-bound and is abundant in kidney, where it is localized in the brush border of the proximal convoluted tubules (6–10). Kinetic data were obtained which are consistent with a 2-step reaction in which a γ-glutamyl enzyme is formed (1). Additional evidence has now been obtained for this idea, and also that maleate dissociates the catalytic steps. Although glutamine is a poor substrate of the transpeptidase, it is highly reactive in the presence of maleate. Evidence presented here indicates that "maleate-stimulated-phosphate-independent glutaminase" (11, 12) is an activity catalyzed by γ-glutamyl transpeptidase.

EXPERIMENTAL

Materials. *S*-Methylglutathione and L-methionyl-glycine were purchased from Sigma. L-[*methyl*-^{14}C]methionine and L-[*U*-^{14}C]glutamine were obtained from New England Nuclear Corp. The γ-glutamyl amino acids were synthesized (13–15) by Dr. Ralph Stephani of this laboratory. Rat kidney γ-glutamyl transpeptidase (form A) (1) was used; identical results were obtained with form B.

Methods. The assay methods used here have been described (1). The several enzymatic products were determined with a Durrum model D-500 amino-acid analyzer. γ-Glutamyl-glutamine, γ-glutamyl-γ-glutamyl-α-aminobutyrate, and *S*-methylcysteinyl-glycine were separated from the other reactants; however, the γ-glutamyl-methionine and glutamate peaks, and the *S*-methylglutathione and γ-glutamyl-*S*-methylglutathione peaks overlapped to some extent. In studies with ^{14}C-labeled glutamine and γ-glutamyl-α-aminobutyrate, the products were separated by chromatography on Whatman 3 MM paper by means of a solvent consisting of 1-butanol, pyridine, and water (1:1:1; v/v). γ-Glutamyl-glutamine and γ-glutamyl-methionine were identified by comparison with authentic samples; other products were analyzed for constituent amino acids after hydrolysis with 6 N HCl. The spots were cut out from the chromatograms and the radioactivity was determined with a Nuclear Chicago model 181 A gas-flow counter. When unlabeled γ-glutamyl derivatives and [^{14}C]methionine were used, the γ-glutamyl-[^{14}C]methionine formed was determined after separation by paper electrophoresis (1). γ-Glutamyl hydroxamate was determined as described (16). The action of transpeptidase on *S*-acetophenone-glutathione was monitored spectrophotometrically (1).

RESULTS

Effect of Maleate on Transpeptidation and Hydrolysis of S-Methylglutathione and γ-Glutamyl-α-aminobutyrate. When *S*-methylglutathione was incubated with the enzyme, glutamate and γ-glutamyl-*S*-methylglutathione were formed at low rates (Table 1) indicating occurrence of both hydrolysis and transpeptidation. When maleate was added there was little if any transpeptidation and extensive hydrolysis to glutamate. In comparable studies with *S*-methylglutathione and methionine, the presence of maleate also led to marked decrease in transpeptidation (as judged by γ-glutamyl methionine and γ-glutamyl-*S*-methylglutathione formation) and again, an increase in hydrolysis (glutamate formation). In studies with γ-glutamyl-α-aminobutyrate (Table 2, *Exps. 1* and *2*), the total utilization of substrate was about the same in the presence as in the absence of maleate. However, in both Exps. 1

TABLE 1. *Effect of maleate on the interaction of γ-glutamyl transpeptidase with S-methylglutathione and methionine*[a]

Substrates	Maleate present: + absent: 0	Products, nmoles formed: Glutamate[b]	γ-Glu-S-methyl-glutathione[c]	S-methyl-Cys-Gly[d]	γ-Glu-Met[e]
S-Methylglutathione	0	84	100	183	
S-Methylglutathione	+	655	<20	638	
S-Methylglutathione + methionine	0	(40)	10	372	335
S-Methylglutathione + methionine	+	625	0	735	161

[a] The reaction mixtures (final volume, 0.1 ml) contained Tris·HCl buffer (50 mM; pH 8.0), enzyme (2 μg), S-methylglutathione (10 mM), and, as indicated, L-[^{14}C]methionine (10 mM), and sodium maleate (50 mM). After incubation at 37° for 20 min, 10 μl of 50% sulfosalicylic acid was added and, after centrifugation, aliquots were taken for amino-acid analysis.

[b] Determined with the amino-acid analyzer; the value in parentheses is approximate since there was some overlap between the peaks for glutamate and γ-glutamyl-methionine.

[c] γ-Glutamyl-S-methylglutathione emerged just ahead of S-methylglutathione and there was overlap between these peaks; these values are therefore approximate. The findings were qualitatively confirmed by paper chromatography.

[d] S-Methyl-cysteinyl-glycine appears on amino-acid analysis in the tyrosine area.

[e] γ-Glutamyl-[^{14}C]methionine was determined after paper electrophoretic separation as described (1).

and 2 the formation of glutamate was increased by maleate; in Exp. 2, γ-glutamyl-methionine formation was decreased in the presence of maleate, as was the formation of $(\gamma\text{-glutamyl})_2$-α-aminobutyrate in both Exps. 1 and 2. There was an apparent increase in the formation of the transpeptidation product tentatively considered to be $(\gamma\text{-glutamyl})_3$-α-aminobutyrate in both experiments.

The S-acetophenone derivative of glutathione is very active in transpeptidation (1), but in contrast to glutathione and other γ-glutamyl compounds, S-acetophenone-glutathione does not disappear to a significant extent when incubated with the enzyme in the absence of an acceptor. However, S-acetophenone-glutathione is readily hydrolyzed in the absence of an added acceptor when maleate is added. Thus, hydrolysis of this substrate is substantial even at low maleate concentrations (Fig. 1).

Effect of Maleate on Hydrolysis and Transpeptidation of Glutamine. Incubation of L-glutamine with the enzyme (Table 2, *Exp. 3*) led to slow formation of both γ-glutamyl-glutamine and glutamate; in the presence of maleate much more substrate was utilized, and most of it (68%) was converted to glutamate. However, there was increased formation of γ-glutamyl-glutamine and additional transpeptidation products. Similar results were obtained when glutamine was incubated with the enzyme in the presence of methionine (Table 1, *Exp. 4*) and methionyl-glycine (*Exp. 5*). In these studies conversion of substrate to glutamate was substantially increased

TABLE 2. *Effect of maleate on the reactions of glutamine and γ-glutamyl-α-aminobutyrate catalyzed by γ-glutamyl transpeptidase*[a]

Exp. no.	Substrates	Maleate present: + absent: 0	Disappearance of γ-glutamyl substrate nmoles	Formation of $(\gamma\text{-Glu})_2$-α-ab[b] or γ-Glu-Gln nmoles	Formation of $(\gamma\text{-Glu})_3$-α-ab[b] or $(\gamma\text{-Glu})_2$-Gln[b] nmoles	Formation of $(\gamma\text{-Glu})_3$-Gln[b] nmoles	Formation of γ-Glu-Met or γ-Glu-Met-Gly nmoles	Formation of glutamate nmoles (%)[c]
1	γ-Glutamyl-α-aminobutyrate	0	569	260	3			41 (7.2)
1M	γ-Glutamyl-α-aminobutyrate	+	578	126	13			287 (50)
2	γ-Glutamyl-α-aminobutyrate + methionine	0	673	110	4		316 (333)[d]	128 (19)
2M	γ-Glutamyl-α-aminobutyrate + methionine	+	597	78	10		156 (180)[d]	257 (43)
3	Glutamine	0	128	21	0	0		33 (26)
3M	Glutamine	+	596	79	17	6		407 (68)
4	Glutamine + methionine	0	93	16	0	0	25	26 (28)
4M	Glutamine + methionine	+	577	65	11	4	97	322 (56)
5	Glutamine + Met-Gly	0	146	14	0	0	72	26 (18)
5M	Glutamine + Met-Gly	+	447	51	0	0	185	159 (36)

[a] The reaction mixtures (final volume, 0.1 ml) contained Tris·HCl buffer (50 mM; pH 8.0), enzyme (2 μg) and, as indicated, sodium maleate (50 m*M*), L-[^{14}C]glutamine (10 mM), L-methionine (10 mM), L-methionyl-glycine (10 mM), and L-γ-[^{14}C]glutamyl-L-α-aminobutyrate (γ-Glu-α-ab) (10 mM). After incubation at 37° for 20 min (*Exps. 1* and *2*) or 60 min (*Exps. 3–5*), 10 μl of 50% sulfosalicylic acid was added and after centrifugation the products were separated by paper chromatography and quantitated.

[b] Tentative identification; see *Methods*.

[c] % of γ-glutamyl substrate utilized found as glutamate.

[d] Data from a parallel experiment with unlabeled γ-Glu-α-ab and [^{14}C]methionine; γ-Glu-Met formation was determined after paper electrophoretic separation (1).

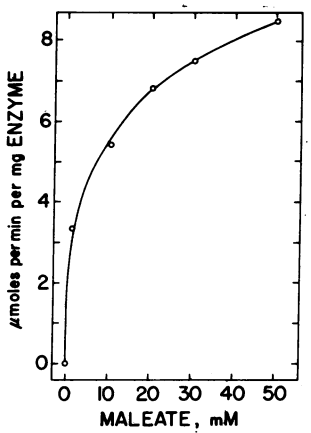

FIG. 1. Hydrolysis of *S*-acetophenone-glutathione by γ-glutamyl transpeptidase in the presence of maleate. The reaction mixtures (final volume, 1.0 ml) contained *S*-acetophenone-glutathione (2.5 mM), Tris·HCl buffer (50 mM, pH 8.0), enzyme (2 μg) and sodium maleate, as indicated. The formation of product was determined spectrophotometrically (1).

in the presence of maleate; although the absolute amounts of substrate converted to transpeptidation products increased, the percent of substrate utilized by the transpeptidase pathway decreased. In contrast, the percent of substrate utilized that was hydrolyzed to glutamate increased 2-fold in both of these experiments.

Fig. 2*A* shows the striking effect of maleate on the conversion of glutamine to glutamate catalyzed by the enzyme at various values of pH; the apparent pH optimum shifts from about 6.5 to 7.0 in the presence of maleate. A similar but less dramatic augmentation of γ-glutamyl-glutamine formation was found in the presence of maleate (Fig. 2*B*). The pH-dependence of this reaction, clearly different from that for hydrolysis of glutamine, resembles that found (1) for transpeptidation of glutathione with methionine.

Formation of γ-Glutamyl Hydroxamate From Hydroxylamine and Various γ-Glutamyl Compounds. γ-Glutamyl transpeptidase catalyzes the formation of γ-glutamyl hydroxamate from a number of γ-glutamyl compounds at relative rates which closely approximate those found (1) for transpeptidation reactions with amino acids (Table 3). When maleate was added, the formation of γ-glutamyl hydroxamate was increased about 4- to 5-fold. When glutamine was incubated with hydroxylamine, the formation of γ-glutamyl hydroxamate was about 15% of that found with γ-glutamyl-methionine (Table 4); addition of maleate led to about a 14-fold increase in hydroxamate formation. Both the maleate-stimulated hydrolysis of glutamine and the conversion of glutamine to γ-glutamyl hydroxamate were inhibited by borate and L-serine (17). Although moderate increases in hydrolysis and hydroxamate formation were found with several compounds, the striking effect of maleate on these γ-glutamyl-transpeptidase-catalyzed reactions of glutamine is evident from the data.

DISCUSSION

The findings support the view that the transpeptidation reaction involves intermediate formation of a γ-glutamyl enzyme (1) (Fig. 3). That the enzyme catalyzes γ-glutamyl hydroxamate formation from γ-glutamyl compounds (Table 3) is consistent with this concept. Interaction of maleate with the enzyme favors hydrolysis of the γ-glutamyl donor and decreases transpeptidation, suggesting that maleate may modulate the enzyme so as to facilitate access of water to the active site, i.e., to the enzyme-bound γ-glutamyl moiety; thus, ability of the enzyme to transfer the γ-glutamyl group to an amino-acid or peptide acceptor is reduced. Maleate thus appears to uncouple or dissociate the enzyme's transpeptidation

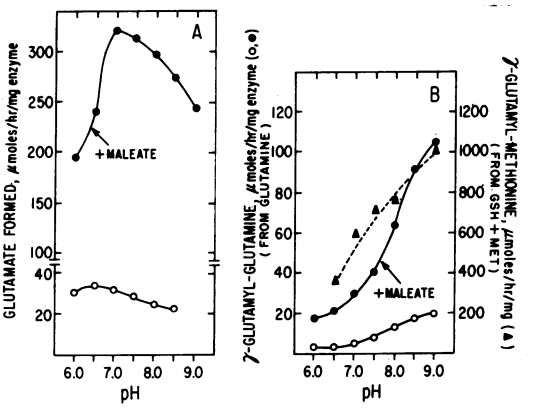

FIG. 2. The effect of pH and maleate on the formation of glutamate from glutamine and the formation of γ-glutamyl-methionine from glutamine and methionine. Imidazole·HCl buffers (50 mM) (pH 6–7.5) and Tris·HCl buffers (50 mM) (pH 8.0–9.0) were used. In the studies with maleate (final concentration, 50 mM), the pH was adjusted by addition of Tris. The reaction mixtures (final volume, 0.1 ml) contained L-glutamine (10 mM), transpeptidase (4 μg), buffer, and, as indicated, maleate. After incubation at 37° for 60 min (without maleate) or 10 min (with maleate), 10 μl of 50% sulfosalicylic acid was added and, following centrifugation, aliquots were analyzed. (*A*) Formation of glutamate. (*B*) Formation of γ-glutamyl-glutamine. The *interrupted curve* (*right-hand ordinate*) gives comparable data on transpeptidation between glutathione (*GSH*) and L-methionine; taken from (1).

TABLE 3. *Effect of maleate on the formation of γ-glutamyl hydroxamate from various γ-glutamyl compounds and hydroxylamine*[a]

L-γ-Glutamyl compound	Activity[b] Without maleate (A)	With maleate (B)	Ratio B/A
S-Methylglutathione	50.9	227	4.5
γ-Glutamyl-L-methionine	46.2	227 (100)	4.9
γ-Glutamyl-L-α-aminobutyrate	41.4	199 (87.6)	4.8
γ-Glutamyl-L-serine	32.0	157 (69.1)	4.9
γ-Glutamyl-glycine	25.4	125 (54.9)	4.9
γ-Glutamyl-L-valine	7.8	36.6 (16.1)	4.7
γ-Glutamyl-L-phenylalanine	24.4	120 (52.7)	4.9
γ-Glutamyl-L-lysine	24.1	120 (52.7)	5.0
γ-Glutamyl-L-proline	1.5	6.1 (2.7)	4.1
γ-Glutamyl-D-methionine	4.6	19.3 (8.5)	4.2

[a] The reaction mixtures (final volume, 0.5 ml) contained Tris·HCl buffer (80 mM; pH 8.0), γ-glutamyl compound (10 mM), and hydroxylamine hydrochloride (250 mM; adjusted with NaOH to pH 8.0). After addition of 0.75 ml of ferric chloride reagent (16) and centrifugation the formation of hydroxamate was determined spectrophotometrically.

[b] Micromoles of γ-glutamyl hydroxamate formed per hr/mg of enzyme; the values in parentheses are activities relative to L-γ-glutamyl-L-methionine.

TABLE 4. *Effect of various compounds on activities of γ-glutamyl transpeptidase with glutamine*

Compound added	Hydrolysis to glutamate[a]		Formation of γ-glutamyl hydroxamate[c]	
	Specific activity[b]	Relative activity	Specific activity[d]	Relative activity
None (control)	24	(100)	7.1	(100)
Maleate	297	(1250)	96.6	(1360)
Maleate, L-serine (5 mM), borate (5 mM)	49	(205)	7.4	(104)
L-Serine (5 mM), borate (5 mM)			1.3	(18)
L-Serine (5 mM)			7.1	(100)
Borate (5 mM)			7.1	(100)
Fumarate	35	(146)	7.8	(110)
Citrate	29	(121)	7.4	(104)
Succinate	39	(163)	14.5	(204)
Acetate	41	(171)	17.9	(252)
$NaHCO_3$			7.4	(104)
Potassium phosphate			6.4	(90)
Ethylenediaminetetraacetate (40 mM)			12.8	(180)
Pyruvate (40 mM)	39	(163)	12.8	(180)
α-Ketoglutarate (40 mM)	43	(179)	20.6	(290)
N-Acetyl-DL-methionine (40 mM)			10.3	(145)

[a] The reaction mixtures (final volume, 0.1 ml) contained Tris·HCl buffer (50 mM; pH 8.0), L-glutamine (10 mM), added compound (adjusted to pH 8.0 with NaOH; 50 mM except as noted) and the enzyme (2 μg). After incubation at 37° for 10–60 min., 10 μl of 50% sulfosalicylic acid was added; after centrifugation the protein-free solution was analyzed for glutamate.

[b] Micromoles of glutamate formed per hr/mg of enzyme.

[c] The reaction mixtures (final volume, 0.5 ml) contained Tris·HCl buffer (80 mM; pH 8.0), L-glutamine (10 mM), added compound (as above), hydroxylamine hydrochloride (adjusted to pH 8.0 with NaOH; 250 mM), and enzyme (2 μg). After incubation at 37° for 1 hr, 0.75 ml of ferric chloride reagent was added and the formation of hydroxamate was determined.

[d] Micromoles of γ-glutamyl hydroxamate formed per hr/mg of enzyme.

function from its function in reacting with γ-glutamyl compounds to form a γ-glutamyl enzyme. It is probable that the acceptor binds to an enzyme site different from that which binds the γ-glutamyl moiety (1), and that hydroxylamine acts directly from solution rather than from an enzyme site. A separate acceptor site is supported by the data on hydroxamate formation (Table 3). The amino acid attached to the γ-glutamyl moiety has considerable influence on the rate of utilization of substrate.

This uncoupling phenomenon resembles that found after treatment of glutamine-dependent carbamyl phosphate synthetase with sulfhydryl reagents (refs. 18 and 19; V. P. Wellner and A. Meister, in preparation); such treatment increases the enzyme's glutaminase activity as much as 100-fold and decreases utilization of glutamine for carbamyl phosphate synthesis. Similar uncoupling was observed with glutamine-dependent asparagine synthetase (20).

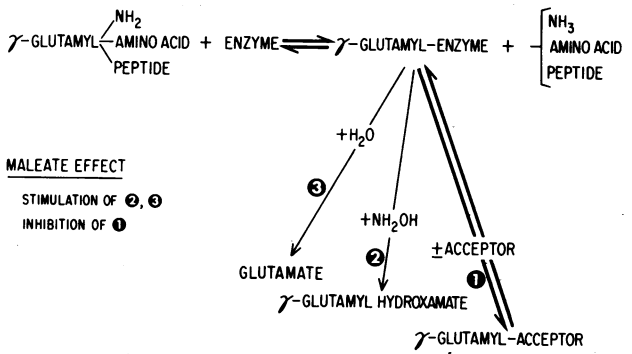

FIG. 3. Reactions catalyzed by γ-glutamyl transpeptidase (see the *text*).

Although maleate did not affect the extent of utilization of γ-glutamyl-α-amino-butyrate, it increased substantially the utilization of glutamine (Tables 2 and 3). Glutamine is a poor substrate for transpeptidation (1) and its affinity for the enzyme is evidently low as compared to that of glutathione and other γ-glutamyl compounds. Thus, while the enzyme is essentially saturated by 10 mM γ-glutamyl-α-aminobutyrate (Table 2), 10 mM glutamine probably does not saturate. Although additional studies are needed to fully explain the increased utilization of glutamine in the presence of maleate, it would appear that maleate may act by increasing the affinity of the enzyme for glutamine as well as by facilitating hydrolysis of the γ-glutamyl enzyme. Maleate may also increase the affinity for γ-glutamyl-α-aminobutyrate, an effect that might become apparent at lower concentrations of the latter substrate.

Maleate has previously been found to activate a glutaminase activity found in kidney and other tissues which is not activated by phosphate (11, 12, 21). Both this activity and the phosphate-activated glutaminase were observed many years ago by Greenstein *et al.** The present work shows that highly purified γ-glutamyl transpeptidase exhibits low glutaminase activity that is markedly stimulated by maleate; such activ-

* Greenstein *et al.* (22–27) discovered several glutaminase activities in mammalian tissue: (*a*) "Glutaminase I" is a true glutaminase and is activated by phosphate, arsenate, and sulfate. (*b*) "Glutaminase II" is actually not a glutaminase, and requires α-keto acids; this pathway for the deamidation of glutamine involves two reactions which are catalyzed, respectively, by glutamine-α-keto acid transaminase and ω-amidase (28–30). (*c*) Glutaminase activity was found in the sedimentable fraction of rat liver and kidney which was active in the absence of added phosphate.

ity is inhibited by L-serine plus borate (Table 4), a reagent combination that has long been known to inhibit γ-glutamyl transpeptidase (17). The maleate-stimulated phosphate-independent glutaminase activity studied by Katunuma *et al.* (11, 21) may reflect a catalytic activity of γ-glutamyl transpeptidase†. It is possible that transpeptidase plays a role in renal ammoniagenesis. γ-Glutamyl transpeptidase is localized in brush borders of the renal proximal convoluted tubules, which may be one of the sites of ammonia formation; "phosphate-independent glutaminase" has been found to have a relatively similar localization (12). The pH optimum of the glutaminase activity of transpeptidase is about 6.5; thus, the enzyme's role in renal ammonia formation may be significant in acidosis in which there is increased excretion of ammonia (32).

The maleate-induced dissociation of the catalytic functions of γ-glutamyl transpeptidase leads to impaired ability of the enzyme to use amino-acid or peptide acceptors; thus, the γ-glutamyl moiety is converted to glutamate rather than to a γ-glutamyl amino acid. Such an effect, if operative *in vivo*, would be expected to interfere with the proposed function of the γ-glutamyl cycle in amino-acid transport. It is thus of interest that administration of maleate to animals leads to extensive aminoaciduria (33, 34). Other studies on aminoacid transport *in vivo* using a microinjection technique led to the suggestion that maleic acid produces aminoaciduria by increasing efflux of amino acid, leading to or secondary to a loss of cellular amino-acid accumulation (35). These *in vivo* studies, when considered in the light of the present enzymatic findings, appear to be consistent with function of the transpeptidase and of the γ-glutamyl cycle in amino-acid transport. It must be noted, however, that administration of maleate also leads to increased excretion of phosphate and glucose (33, 36). There is evidence that maleate may also interfere with other enzymes (37), and that this reagent can react with glutathione as well as other sulfhydryl compounds (38). Further studies are needed to determine the mechanism of action of maleate on transpeptidase; such work may lead to a more specific inhibitor of this enzyme.

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† Katunuma *et al.* (21) concluded that the phosphate-independent glutaminase is not indentical to "γ-glutamyl transferase." On the other hand, N. Curthoys recently suggested that the maleate-stimulated phosphate-independent glutaminase might be identical or similar to γ-glutamyl transpeptidase (Third International Conference on Isozymes, 1974, New Haven, Conn.). It should be emphasized that "γ-glutamyl transferase" activity is a property of several enzymes; thus, glutamine synthetase, glutamine amidotransferases, and various glutaminases can catalyze γ-glutamyl hydroxamate formation. Some of the properties of the enzyme studied by Katunuma *et al.* (11, 12, 31), e.g., specific activity, pH optimum, insolubility, and extractability with deoxycholate, are similar to those of the transpeptidase. Furthermore, we have prepared the purified phosphate-independent glutaminase according to the directions of Katunuma *et. al.* (39), and have found that its catalytic properties are indeed identical to those of the γ-glutamyl transpeptidase preparation used here and in previous work in this laboratory (1).

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