# MECHANISM AND SIGNIFICANCE OF THE MAMMALIAN PATHWAY FOR ELIMINATION OF D-GLUTAMATE; INHIBITION OF GLUTATHIONE SYNTHESIS BY D-GLUTAMATE

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SUMMARY: Administration of a tracer dose of L-[$^{14}$C] glutamate to rats led to rapid labeling of tissue 5-oxoproline, a finding in accord with the γ-glutamyl cycle. After giving the same dose of D-[$^{14}$C] glutamate, the labeling of kidney 5-oxoproline was about 400 times greater than found after giving L-[$^{14}$C] glutamate; this reflects the activity of D-glutamate cyclase, which catalyzes cyclization of D-glutamate to 5-oxo-D-proline. The mammalian pathway for detoxication of D-glutamate, which does not require energy or utilization of other metabolites, may have evolved to protect L-amino acid-specific systems against inhibition by D-glutamate; D-glutamate cyclase seems to account for the presence of 5-oxo-D-proline in normal blood plasma and urine. Administration of D-glutamate decreased the glutathione level in the kidney markedly, reflecting inhibition of γ-glutamylcysteine synthetase; D-glutamate may be useful in various studies as an inhibitor of glutathione synthesis.

Recent studies in this laboratory indicate that (a) 5-oxo-L-proline (pyroglutamate) is a significant metabolite of glutathione and γ-glutamyl compounds, (b) mammalian tissues have measurable steady-state concentrations of 5-oxo-L-proline, and (c) 5-oxo-L-proline is utilized for glutathione synthesis via the γ-glutamyl cycle (1-5). Such studies have required methods for determination of 5-oxo-L-proline in the presence of 5-oxo-D-proline, which is known to occur in mammals. Thus, about 10% of the total 5-oxoproline of normal human blood plasma is present as 5-oxo-D-proline (6) and the urinary excretion of 5-oxo-D-proline in man is much greater than that of 5-oxo-L-proline (7). As shown here, 5-oxo-D-proline (which is formed from D-glutamate by D-glutamate cyclase (7,8)), is formed after administration of trace or large amounts of D-glutamate. In the course of studies on the potential toxicity of D-glutamate, we found that D-glutamate inhibits γ-glutamylcysteine synthetase *in vitro* and glutathione synthesis *in vivo*.

MATERIALS: L-[U-$^{14}$C] Glutamic acid and DL-(1-$^{14}$C] glutamic acid were obtained from New England Nuclear Corp. The labeled L-glutamate preparation was purified from 5-oxoproline and D-glutamate contaminants by incubation with D-glutamate cyclase (9) followed by chromatography on Dowex 50 ($H^+$). D-[1-$^{14}$C] Glutamate was prepared by treating the racemate with L-glutamate decarboxylase (E. coli, Type II, Sigma) essentially as described (10). The labeled glutamate isomers were $\overline{>99}$.9% optically pure by enzymatic tests (7, 11). Bovine liver glutamate dehydrogenase was obtained from Boehringer-Mannheim Corp. D-Glutamate cyclase was purified from mouse kidney (9). 5-Oxo-L-prolinase was isolated from a bacterial source (12). Sheep brain γ-glutamyl cyclotransferase (13) and rat kidney γ-glutamylcysteine synthetase (14) were isolated as described. Sheep brain glutamine synthetase (15) was kindly provided by Dr. W.B. Rowe of this laboratory.

RESULTS: Significant amounts of 5-oxo-[$^{14}$C] proline were found in kidney and liver 1 min. after L-[$^{14}$C] glutamate was injected (Table I). The amount of [$^{14}$C] found in 5-

TABLE I Formation of 5-oxo-[$^{14}$C] proline after injection of L- and D-[$^{14}$C] glutamate*

| Compound Injected | 5-Oxo-[$^{14}$C] proline (c.p.m.) | | | | | |
|---|---|---|---|---|---|---|
| | Kidney | | | Liver | | |
| | 1 min. | 5 min. | 10 min. | 1 min. | 5 min. | 10 min. |
| L-[$^{14}$C] glutamate | 5,200 | 2,300 | 2,300 | 1,660 | 2,000 | 1,270 |
| D-[$^{14}$C] glutamate | 365,000 | 755,000 | 999,000 | | 7,200 | 5,500 |

*Male Sprague-Dawley rats (250 g) were anaesthetized by intraperitoneal injection of Inactin (100 mg per kg) and injected via the inferior vena cava with 1 μmole of [$^{14}$C] glutamate; ($2 \times 10^7$ cpm). After sacrifice, the right kidney and a liver sample (1 g) were homogenized in 4 ml of 1 % picric acid at 0°. After centrifugation, the supernatant was applied to a column (1.5 ml) of Dowex 2 ($Cl^-$), which was washed with 4 ml of 0.02 N HCl; the effluent (total fraction) was applied to a column (1.5 ml) of Dowex 50 ($H^+$). This column was washed with 4 ml of water, and an aliquot of the effluent (acidic fraction), was hydrolyzed in 2.5 N HCl at 100° for 90 min. After flash evaporation to dryness, the residue was dissolved in water and applied to a column (1.5 ml) of Dowex 50 ($H^+$) which was washed with 4 ml of water and then eluted with 4 ml of 3 N $NH_4OH$ (5-oxo-proline formation).

oxoproline was 1-3% of the total fraction (see footnote, Table I) and was 3-12% of the acidic fraction, which contains other metabolites of L-glutamate. After injection of D-[$^{14}$C] glutamate, the kidney showed very high levels of 5-oxo-[$^{14}$C] proline; about 50% of the [$^{14}$C] of the total fraction was recovered as 5-oxo-[$^{14}$C] proline. Although relative-

ly less [$^{14}$C] was found in the liver 5-oxoproline, the amount was much higher than that found after injection of L-[$^{14}$C] glutamate. After injection of D-[$^{14}$C] glutamate, about 15% of the [$^{14}$C] of the total fraction in liver was accounted for as 5-oxoproline after 10 min. The 5-oxoproline recovery from the acidic fraction was 70-80% in the kidney and 80-100% in the liver, suggesting that D-glutamate is mainly (if not entirely) converted to 5-oxoproline. Comparable studies were done with a commercial sample of "L-[$^{14}$C] glutamate," which we found by analysis with D-glutamate cyclase (7) to contain 1% of the D-isomer. After injection of this sample of "99% optically pure" L-[$^{14}$C] glutamate, 15,000 cpm were found in the kidney 5-oxoproline fraction 5 min. after injection. Analysis of this 5-oxoproline fraction by the 5-oxo-L-prolinase procedure (2), showed that only 2,180 cpm (or $\sim$ 15% of the total) were associated with 5-oxo-L-proline; this value agrees closely with that given in Table I for the experiment in which optically pure L-[$^{14}$C] glutamate was injected.

The effects of injecting mice with D-glutamate were determined on the kidney concentrations of L-glutamate, D-glutamate, 5-oxo-D-proline, glutamine, and glutathione (Fig. 1). After injection of D-glutamate, there was a substantial decrease in the level of glutathione which reached a minimum after about 1 hour*. The concentration of glutamine was decreased 15 min. after injection and rose to about the control level after 90 min. The administration of D-glutamate was followed by a rapid rise in the D-glutamate level and by substantial formation of 5-oxo-D-proline. There was no significant change in the level of L-glutamate.

D-Glutamate is a substrate of both glutamine synthetase (16,17) and $\gamma$-glutamyl-

*In studies in this laboratory on rats (under conditions previously described (29), the kidney glutathione level decreased by 51%, 1 hour after intraperitoneal injection of 1.67 mmole per kg of D-glutamate (Palekar, A.G., unpublished data). In the earlier work (29) kidney glutathione levels were depressed after giving L-methionine-SR-sulfoximine, L-a-aminobutyrate, and glycylglycine, but not after administration of L-glutamate, L-glutamine, L-cysteine, L-methionine, or glycine.

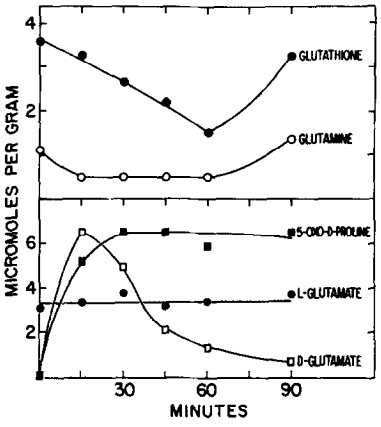


Fig. 1. Effect of D-glutamate on the levels of glutathione and other compounds in the kidney. Male strain CF-1 mice (25-35 g) fed ad libitum were injected intraperitoneally with Na D-[1-$^{14}$C] glutamate (4.0 μmoles/g; 5.7 x $10^6$ cpm/mmole), and the animals were sacrificed at intervals. The kidneys were homogenized with trichloroacetic acid and worked up as described (3). Glutathione (as its S-acetamido derivative), glutamate, and glutamine were determined by automated amino acid analysis using a Li citrate buffer system (3). [$^{14}$C] was determined with a flow-through scintillation counter coupled to the amino acid analyzer. The values for total glutamate (ninhydrin) and those for D-glutamate [$^{14}$C] were used to calculate values for L-glutamate. 5-Oxo-D-proline was estimated from the [$^{14}$C] that did not bind to Dowex 50 ($H^+$). The values are averages of determinations on 3-4 animals.

cysteine synthetase (18). Studies on brain glutamine synthetase showed that the apparent Km for D-glutamate is about 3.5 mM when hydroxylamine is the acceptor, but when ammonia is the acceptor substrate, the apparent Km for D-glutamate is much higher (14 mM [19]); the apparent Km for L-glutamate with ammonia is about 0.18 mM. The effect of D-glutamate on glutamine synthetase was studied in reaction mixtures containing the enzyme (0.2 unit), ATP (10 mM), $MgCl_2$ (20 mM), $NH_4Cl$ (10 mM), imidazole-HCl buffer (pH 7.2; 25 mM), Na EDTA (0.5 mM), 2-mercaptoethanol (2.5 mM), L-[$^{14}$C] glutamate (5 mM; 5.7 x $10^5$ cpm/μmole) and D-glutamate; after incubation at 37° for 15 min.; the formation of [$^{14}$C] glutamine was determined. D-Glutamate, at a concentration of 5 mM, inhibited L-glutamine synthesis by only 15%, suggesting that the decrease in kidney glutamine found after giving D-glutamate may not be produced solely by inhibition of glu-

tamine synthesis. Substantial utilization of glutamine may occur as a result of acidosis produced by 5-oxoproline formation; thus, after D-glutamate was given, we found about a 3-fold increase in urinary ammonia as compared to controls given sodium chloride.

D-Glutamate was found to be a potent inhibitor of γ-glutamylcysteine synthetase. Thus, 2 mM D-glutamate produced about 50% inhibition in reaction mixtures containing 2 mM L-glutamate. Inhibition of γ-glutamylcysteine synthetase by D-glutamate is competitive (Fig. 2). The apparent Ki for D-glutamate is 0.8 mM; the Km for L-glutamate is 1.8 mM. Glutathione synthetase is not significantly inhibited by D-glutamate (20).

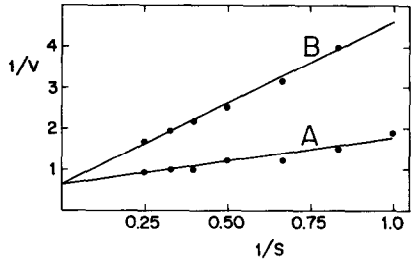


Fig. 2. Inhibition of γ-glutamylcysteine synthetase by D-glutamate. The reactions (final vol., 0.25 ml) contained Tris-HCl buffer (pH 8.2; 0.25 M), $MgCl_2$ (0.0125 M), ATP (5 mM), EDTA (5 mM), L-α-aminobutyrate (25 mM), γ-glutamyl cyclotransferase (2.5 units), γ-glutamylcysteine synthetase (0.25 unit), and L-[U-$^{14}$C] glutamate (1-4 mM; 7.2 x $10^5$ cpm/μmole); 37° (A). In B, D-glutamate (2 mM) was also present. Ordinate: v, initial velocity [c.p.m. 5-oxoproline formed/5 min. produced by the action of γ-glutamyl cyclotransferase (present in excess) on the synthesized dipeptide].

In agreement with earlier results (21), we found that D-glutamate inhibits beef liver glutamate dehydrogenase competitively. The apparent Km for α-ketoglutarate was 0.43 mM and the apparent Ki for D-glutamate was 0.17 mM. Inhibition was also observed in the direction of L-glutamate deamination; thus, about 50% inhibition was observed with 1 mM each of D- and L-glutamate.

DISCUSSION: Ratner gave DL-glutamate labeled with $^{15}$N and deuterium to rats and found that they excreted 5-oxo-D-proline which had an unaltered isotope ratio; almost

all of the D-glutamate appeared as urinary 5-oxo-D-proline (22). Similar observations were made later (23-25). Rat liver and kidney slices catalyze conversion of D-glutamate to 5-oxoproline (25); the enzyme that catalyzes the cyclization was demonstrated in the kidney and liver of several species including man (7,8) and was purified from mouse kidney (8,9,26). The equilibrium of the reaction catalyzed by D-glutamate cyclase, which requires $Mn^{++}$ or $Mg^{++}$, greatly favors cyclization (7,8).

Our data show that trace amounts as well as large amounts of D-glutamate are efficiently cyclized in vivo. The study with "99% optically pure" L-[$^{14}$C] glutamate seems to explain an earlier report (27) in which administration of commercial L-[$^{14}$C] glutamate to rats led to substantial (68% of the non-glutamate radioactivity) formation of 5-oxoproline in kidney. The effective cyclization of D-glutamate is consistent with our inability to produce mortality in mice with doses of D-glutamate as high as 0.03 mole per kilo.

The existence of a special pathway for D-glutamate elimination in mammals raises the question as to its physiological significance. One cannot exclude the possibility that the enzyme has another catalytic activity and that its active site fortuitously also interacts with D-glutamate, but it seems notable that D-glutamate cyclase, like D-amino acid oxidase, is highly concentrated in the kidney and that D-amino acid oxidase exhibits virtually no activity toward D-glutamate. Urinary excretion of 5-oxo-D-proline in man is not large, suggesting only moderate exposure to D-glutamate, which may arise from the bacterial flora or from the diet. Nevertheless, in the absence of the normal pathway for D-glutamate elimination, D-glutamate might accumulate and produce toxicity. Patients with end-stage renal disease and anephric patients have plasma concentrations of 5-oxo-D-proline that are about 25 times greater than those found normally (6). The pathway for D-glutamate elimination might conceivably be more significant during development and infancy. The evolutionary development of L-amino acid-specific systems must have been accompanied by mechanisms that excluded or destroyed D-amino acids. Thus, D-gluta-

mate cyclase may have evolved to protect against the toxic effects of D-glutamate, which at an earlier time may have been a serious environmental hazard. It would be of interest to learn whether D-glutamate cyclase is present in lower animal forms and in bacteria.

The detoxication mechanism for D-glutamate is simple and efficient as compared to others (28). Thus, in contrast to the pathways for elimination of benzoate, phenylacetate, and various foreign compounds which are conjugated, hydroxylated, esterified, methylated, oxidized or reduced, which require energy and use various intracellular compounds, the intramolecular acylation reaction of D-glutamate does not require utilization of other metabolites and it is energetically favored.

These studies show that administered D-glutamate depresses the level of glutathione in the kidney; such an effect, which would be most readily seen in this organ because of its high turnover of glutathione (3), may also occur in other tissues. The data indicate that D-glutamate inhibits glutathione synthesis by interacting with γ-glutamylcysteine synthetase, which exhibits a somewhat higher affinity for D-glutamate than for L-glutamate. This effect of D-glutamate, which is much less toxic than methionine sulfoximine (29), may be useful in various studies on glutathione, especially in systems that do not have D-glutamate cyclase. The observed decrease in kidney glutamine is probably explained by acidosis associated with 5-oxo-D-proline formation. Kidney glutamine levels decrease in acidosis (30-32) and we found increased $NH_4^+$ excretion after giving D-glutamate. Increased 5-oxo-L-proline formation in patients with 5-oxoprolinuria, who have a marked deficiency of glutathione synthetase, leads to severe acidosis (33-36). Administration of large amounts of D-glutamate can thus produce, to some degree, two of the findings seen in this disease, i.e., acidosis and decreased glutathione levels, but the mechanisms involved are quite different.

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