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Accumulation of 5-Oxoproline in Mouse Tissues After Inhibition of 5-Oxoprolinase and Administration of Amino Acids: Evidence for Function of the γ-Glutamyl Cycle*

(pyroglutamate/pyrrolidone carboxylate/glutathione/amino-acid transport)

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ABSTRACT **5-Oxoprolinase catalyzes the conversion of 5-oxo-L-proline (L-pyroglutamate, L-2-pyrrolidone-5-carboxylate) to L-glutamate with concomitant stoichiometric cleavage of ATP to ADP and inorganic orthophosphate. In this reaction, a step in the γ-glutamyl cycle, 5-oxoproline (formed by the action of γ-glutamylcyclotransferase on γ-glutamyl amino acids, which are in turn formed by transpeptidation of amino acids with glutathione), is made available for glutathione synthesis. When mice are injected with L-2-imidazolidone-4-carboxylate, a competitive inhibitor of 5-oxoprolinase, they accumulate 5-oxoproline in their tissues (kidney, liver, brain, and eye) and excrete it in the urine. Mice given the inhibitor together with one of several L-amino acids accumulate and excrete much more 5-oxoproline than when they are given the inhibitor alone. Such augmentation of 5-oxoproline accumulation offers evidence for the function of the γ-glutamyl cycle *in vivo* and supports the view that 5-oxoproline is a quantitatively significant metabolite.**

The conversion of 5-oxo-L-proline† to L-glutamate is catalyzed by a widely distributed enzyme (5-oxoprolinase) in an ATP-dependent reaction in which ATP is cleaved to ADP and inorganic phosphate (2). This is a reaction in the γ-glutamyl cycle (2, 5, 6) in which 5-oxoproline, produced by the action of γ-glutamylcyclotransferase on γ-glutamyl amino acids, is made available as glutamate for the synthesis of glutathione. 5-Oxoprolinase has been partially purified from rat kidney, and also from a pseudomonad isolated by enrichment culture (1, 3). Previous work in this laboratory showed that L-2-imidazolidone-4-carboxylate, an analog of 5-oxoproline in which the 4-CH_2 moiety of 5-oxoproline is replaced by NH, competitively inhibits the conversion of 5-oxo-L-proline to L-glutamate catalyzed by the purified kidney enzyme (4). It was also found that L-2-imidazolidone-4-carboxylate decreases the rate of metabolism of 5-oxo-L-[^{14}C]proline to $^{14}CO_2$ by rat-kidney slices, but has little effect on the metabolism of L-[^{14}C]glutamate. Mice injected with the inhibitor exhibit greatly reduced ability to metabolize 5-oxo-L-proline, and excrete 5-oxo-L-proline in amounts that are substantially greater than the trace amounts found in the urine of untreated mice.

The present communication presents data which show that administration of L-2-imidazolidone-4-carboxylate to mice leads to accumulation of 5-oxoproline in their tissues. The findings also indicate that mice given the inhibitor together with one of several L-amino acids accumulate significantly more 5-oxoproline in their tissues than is found when the inhibitor is given alone.

EXPERIMENTAL

Materials. Sodium phosphoenolpyruvate, pyruvate kinase, and dithiothreitol were obtained from Sigma. The amino acids were obtained from commercial sources: L-methionine and L-proline (Nutritional Biochemicals), L-glutamate and α-aminoisobutyric acid (Calbiochem), L-valine and L-lysine·HCl (Schwarz), L-glutamic acid (Fisher), and glycine (Aldrich).

Bacterial 5-oxoprolinase was isolated from a pseudomonad isolated from soil by enrichment culture (3); this enzyme acts only on the L-isomer of 5-oxoproline, and is much less sensitive to inhibition by L-2-imidazolidone carboxylate than is rat-kidney 5-oxoprolinase.

2-Imidazolidone-4-carboxylic acid was first prepared by Karrer and associates (7, 8), who obtained it in low yields by the Hofmann reaction of *N*-acetyl-L-asparagine with bromine in barium hydroxide. Later it was prepared from *N*-carbobenzoxy-L-asparagine by Schneider (9). This method was improved by Shiba *et al.* (10). Dittmer *et al.* (11) prepared racemic 2-imidazolidone-4-carboxylic acid by catalytic (Raney nickel) hydrogenation at high pressure and temperature of 2-imidazolidone-4-carboxylic acid. In the present work, L-2-imidazolidone-4-carboxylic acid was prepared in 23% yield by the Hofmann reaction on *N*-carbobenzoxy-L-asparagine with the conditions described by Shiba *et al.* (10). The white crystalline product melted at 186–190°; published values are 179.5° (15) and 190–191° (14).

Methods. In the experiments described in Table 1, mice of the BALB/c strain (25–30 g, male, fasted 1 day) were injected intraperitoneally with 0.8 ml of a solution containing 400 μmol of either sodium chloride or sodium L-4-imidazolidone-4-carboxylate, and 0.7 ml of a solution containing 175 μmol of the amino acid. In the experiments described in Fig. 1, mice of the CF-1 strain (25–30 g, male, fasted 1 day) were used. They were injected with either sodium chloride or L-2-imidazolidone-4-carboxylate and amino acid as described above. The amino acids were administered orally through a

* These findings were presented in part at the meeting of the American Society of Biological Chemists (April 17, 1973: Atlantic City, N.J.) (ref. 1).

† L-Pyroglutamic acid, L-2-pyrrolidone-5-carboxylic acid, L-5-oxopyrrolidine-2-carboxylic acid.

TABLE 1. *5-Oxo-L-proline concentrations of mouse tissues and urine after administration of L-2-imidazolidone-4-carboxylate (ICA) and L-methionine**

Treatment	5-Oxo-L-proline (nmol/g) Kidney	Brain	Liver	Eye	5-Oxo-L-proline† Urine
Control	37	21	27	29	1.0
L-Methionine	37	18	31	32	0.6
ICA	76	69	68	78	2.2
ICA + L-methionine	121	105	137	131	3.2

* Mice were given NaCl (control), L-methionine, and L-2-imidazolidone carboxylate (*ICA*) as described in the *text;* after 60 min the tissue concentration of 5-oxo-L-proline was determined by a procedure involving use of bacterial 5-oxo-L-prolinase (see *text*).

† μmol of 5-oxo-L-proline per mg of creatinine.

stomach tube in a single dose (250 μmol; 0.8 ml) or two doses of 125 μmol each; the first dose was given 30 min before administration of L-2-imidazolidone-4-carboxylate and the second dose was given at the same time that the inhibitor was given. No significant differences in 5-oxoproline content were observed in comparable experiments in which single intraperitoneal or single or double oral doses of amino acids were given. [The tissue concentrations of the administered amino acids (determined on a Durrum model 500 amino-acid analyzer) were 3–8 times higher than those found in control animals.] The animals were killed 1 hr after administration of inhibitor, and the tissues (200–500 mg) were rapidly removed, weighed, and homogenized at 0° in 2 ml of 1% picric acid for 1 min in a Potter–Elvehjem apparatus. The homogenates were centrifuged for 20 min at 2000 × *g* at 4°, and the supernatant solutions were applied to small columns (volume, 1 ml) of Dowex 2 (chloride form). The columns were eluted with 3 ml of 0.2 N hydrochloric acid, and the eluates were applied to the top of small columns (volume, 1 ml) of Dowex 50 (H^+) that were eluted with 3 ml of water. The eluates were lyophilized. 5-Oxoproline was then determined by enzymatic or nonenzymatic conversion to glutamate followed by amino-acid analysis. Enzymatic determinations of 5-oxo-L-proline were carried out as follows. The lyophilized residues were dissolved in 0.5 ml of 0.5 M Tris·HCl buffer (pH 7.8). Aliquots of 0.2 ml were incubated at 26° in reaction mixtures (final volume, 1 ml) containing Tris·HCl buffer (pH 7.8; 150 μmol), bacterial 5-oxo-L-prolinase (10 units), sodium ATP (4 μmol), magnesium chloride (8 μmol), potassium chloride (80 μmol), dithiothreitol (4 μmol), phosphoenolpyruvate (10 μmol), and pyruvate kinase (2 units). After incubation for 6 hr, the reaction mixtures were lyophilized and the dried residues were suspended in 0.3 ml of 5% sulfosalicylic acid. The insoluble material was removed by centrifugation in a Beckman Microfuge and aliquots (20 μl) of the clear supernatant solution were taken for analysis on a Durrum model 500 amino-acid analyzer. Samples prepared for analysis by nonenzymatic hydrolysis were processed as follows. The eluate from the Dowex 50 column was lyophilized; the residue was dissolved in 1 ml of 5 N hydrocyloric acid and placed at 100° for 90 min. The solution was then flash evaporated and the dried residue was suspended in 0.5 ml of sodium citrate buffer (86 mM; pH 2.2). After centrifugation in a Beckman Microfuge, aliquots (20-μl) were analyzed on a Durrum analyzer. The recovery of

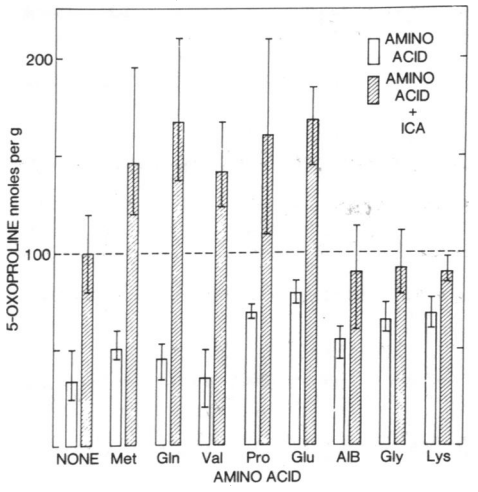

FIG. 1. Concentration of 5-oxoproline in kidney after administration of L-2-imidazolidone-4-carboxylate (*ICA*) and various amino acids (*AIB* = α-aminoisobutyrate). The mice were given the inhibitor and amino acids as indicated, and the concentration of 5-oxoproline in the kidney was then determined as glutamate after acid hydrolysis, as described in the *text*. The *bars* represent the average values of three to five experiments; the ranges of the values obtained are indicated.

added 5-oxoproline by these procedures was 90–100%. Close agreement was obtained between the values obtained for kidney 5-oxoproline after enzymatic hydrolysis and those found after nonenzymatic hydrolysis. This result indicates that virtually all of the 5-oxoproline is of the L-configuration. This point is of significance in relation to earlier work which showed that mouse kidney contains an enzyme capable of converting D-glutamate to 5-oxo-D-proline (12)‡.

RESULTS

Accumulation of Tissue 5-Oxoproline after Inhibition of 5-Oxoprolinase and Administration of Amino Acids. The data given in Table 1 indicate that the 5-oxoproline concentrations of the kidney, brain, liver, eye, and urine increase substantially after administration of the 5-oxoprolinase inhibitor, L-2-imidazolidone-4-carboxylate. The increases ranged from 2 to 3 times those of the respective control values. Administration of L-methionine alone did not significantly affect the concentration of 5-oxoproline in these tissues or in urine. However, when both the inhibitor and L-methionine were given together, the concentrations of tissue and urinary 5-oxoproline were very substantially increased over the values obtained with the inhibitor alone. Similar results were obtained in several separate experiments.

In the studies summarized in Fig. 1, the concentration of 5-oxoproline in the kidney was determined after administration of the inhibitor and various amino acids. The data indicate that marked increases in 5-oxoproline levels were found after administration of inhibitor alone (as found in the experiments

‡ The existence of this enzyme (present also in rat and human kidney) seems to explain the conversion of administered D-glutamate to urinary 5-oxo-D-proline (13). Human urine (freshly voided) contains very little 5-oxoproline, and about 90% of this (50–400 μmol/day) is 5-oxo-D-proline, probably derived from bacterial or dietary sources (14).

given in Table 1). Values significantly higher than those found with the inhibitor alone were observed after administration of the inhibitor plus L-methionine, L-glutamine, L-valine, L-glutamate, or L-proline. No such increased values were observed when the inhibitor was given together with glycine, α-aminoisobutyric acid, or L-lysine.

DISCUSSION

The results are consistent with the interpretation that administration of amino acids leads to increased transpeptidation of amino acids with glutathione to form γ-glutamyl amino acids and that there is increased conversion of the latter to 5-oxoproline, which accumulates in the presence of an inhibitor of 5-oxoprolinase. The findings that animals treated with a 5-oxoprolinase inhibitor accumulate 5-oxoproline in brain, liver, and the eye provide additional evidence that the γ-glutamyl cycle functions in several mammalian tissues; accumulation of 5-oxoproline in the brain under these conditions is in accord with studies that have demonstrated the presence of the enzymes of the γ-glutamyl cycle in the brain, especially the choroid plexus (15). The accumulation of 5-oxoproline in the eye is in accord with considerations discussed elsewhere (16), which suggest that the γ-glutamyl cycle may function in the transport of amino acids across the ciliary body into the aqueous humor and from the latter into the ocular lens.

All of the amino acids used in these studies, except proline and α-aminoisobutyrate, are known to be substrates of γ-glutamyltranspeptidase preparations obtained from various animal tissues. In general, glutamine, glutamate, alanine, and methionine have been found to be somewhat more active than glycine and a number of the other amino acids (see, for example, ref. 15). The present findings do not reflect exactly the relative activity values that have been found in various studies *in vitro;* however, there is evidence indicating species differences, and there are as yet no detailed studies available on the kinetics and specificity of mouse-kidney γ-glutamyltranspeptidase and γ-glutamylcyclotransferase. The effects of the relatively high tissue concentrations of amino acids achieved here on various relevant enzyme activities are not yet known, nor has the interconversion of administered amino acid to other amino acids been thoroughly studied. Nevertheless, the substantial increase in 5-oxoproline accumulation found after administration of the inhibitor plus L-methionine, L-glutamine, or L-glutamate is consistent with observations that indicate that these amino acids are good substrates for γ-glutamyltranspeptidase. Although L-valine is known to be active in transpeptidation with glutathione, presently available data indicate that γ-glutamylvaline is not a good substrate for γ-glutamylcyclotransferase. While it is possible that *in vivo*, γ-glutamylcyclotransferase exhibits activity toward γ-glutamylvaline, alternative pathways for conversion of γ-glutamylvaline to 5-oxoproline and valine involving intermediate formation of di-γ-glutamylvaline and γ-glutamylglutamine (6) must be considered.

The accumulation of 5-oxoproline observed after administration of proline is of interest since presently available data indicate that proline is not significantly active as a substrate for γ-glutamyltranspeptidase. The findings with this imino acid might possibly be explained by the existence of a transpeptidase activity, not yet demonstrated *in vitro*, that acts on proline or a proline derivative. On the other hand, administration of proline is probably accompanied by substantial conversion of this imino acid to glutamate and glutamine; the presence of sufficient concentrations of these or other amino acids could thus explain the accumulation of 5-oxoproline after administration of proline. This possibility was examined in several experiments by performing amino-acid analyses on the kidneys of animals given proline. Relatively high glutamate concentrations (2.34–3.56 mM) were found, compared to those (1.05–1.41 mM) found in the kidneys of animals given other amino acids (e.g., glycine and lysine). These observations suggest that the accumulation of 5-oxoproline observed after administration of proline might be associated with the conversion of proline to other amino acids; however, the available data do not settle this point and the possibility that proline itself participates in the reactions of the γ-glutamyl cycle must still be considered. We have also considered the possibility that there are competitive interactions between proline and 5-oxoproline in metabolism or transport; this might explain the finding of an elevation of blood proline in 5-oxoprolinuria (17, 18).

The findings on the experimental system described here are in certain respects analogous to those that have been reported in studies on a patient with 5-oxoprolinuria (18). Thus, when the patient's serum concentration of amino acids was increased greatly by intravenous administration of amino acids, the urinary excretion of 5-oxoproline increased about 2-fold. In both the animal treated with 5-oxoprolinase inhibitor and the patient with 5-oxoprolinuria, the increase in 5-oxoproline accumulation and excretion can be explained in terms of the function of the γ-glutamyl cycle. Thus, in both systems increased concentrations of amino acids can lead to increased formation of 5-oxoproline by way of the γ-glutamyl transpeptidase–γ-glutamyl cyclotransferase pathway. The block in the experimental animal system is evidently at the 5-oxoprolinase step of the cycle. The block in patients with 5-oxoprolinuria may also be at this step, but conclusive evidence for this is still needed and therefore alternative explanations including, for example, the possibility of overproduction of 5-oxoproline (6) must be considered. Although both of the patients with 5-oxoprolinuria that have thus far been reported excrete very large amounts of urinary 5-oxoproline, studies on tissue-cultured fibroblasts of one patient (19) and of the peripheral leukocytes of the other (20) have failed to indicate a decrease in 5-oxoprolinase activity compared to controls. While these findings do not exclude the possibility that these patients have decreased 5-oxoprolinase activity in other organs (e.g., kidney, liver, and brain), they again raise the question as to whether the enzymatic lesion in 5-oxoprolinuria may be at another step of the γ-glutamyl cycle. Recently, studies have been carried out in our laboratory by Dr. Vaira P. Wellner (unpublished) on the erythrocytes of 2 patients§ with 5-oxoprolinuria and on the placenta obtained at the delivery of one of these patients; these materials were kindly provided by Dr. Agne Larsson, Department of Pediatrics, St. Göran's Children's Hospital, Karolinska Institutet, Stockholm, Sweden. These studies showed that the placenta and the erythrocytes from both patients were markedly deficient in glutathione synthetase but that substantial γ-glutamyl cysteine synthetase activity was present. The findings, which indicate that these patients with 5-oxoprolinuria

§ One of these was previously studied (20) and the other is a sibling of this patient.

have a block of glutathione synthetase, together with earlier observations (20) that are consistent with the view that these patients have a considerable capacity to utilize 5-oxoproline, indicate that there is in this disease an overproduction of 5-oxoproline produced by reactions of the γ-glutamyl cycle.

We have reported previously (1) that, although conversion of 5-oxoproline to glutamate by 5-oxoprolinase is inhibited by L-2 imidazolidone-4-carboxylate, incubation of the inhibitor with enzyme leads to ATP cleavage without appearance of a new product. Similar results were observed with dihydroorotic acid. It thus appears that these inhibitors of 5-oxoprolinase act by uncoupling the decyclization of substrate from ATP utilization. It has also been found that bacterial 5-oxoprolinase is only slightly inhibited by L-2-imidazolidone-4-carboxylate; with this enzyme, the apparent K_i value is 30 mM, compared to 0.12 mM for the rat-kidney enzyme (3). It is conceivable that some patients with 5-oxoprolinuria might have a 5-oxoprolinase, which is genetically altered in such a way as to render it more sensitive to inhibition (or uncoupling) than is the normal enzyme. Although 5-oxoprolinuria might result from inhibition of 5-oxoprolinase or from a deficiency of this enzyme activity, the findings cited above indicate that the condition in two patients is associated with deficiency of glutathione synthetase. The possibility exists that, as in certain other errors of metabolism (e.g., gout, glucose-6-phosphate dehydrogenase deficiency), there may be heterogeneity in the enzyme defects associated with 5-oxoprolinuria.

It is remarkable that the concentration of 5-oxoproline in the skin of various animals including humans (21) is very much greater than that found in kidney, liver, and brain. Studies in this laboratory have shown, for example, that the concentration of 5-oxoproline in mouse skin (all layers) is about 0.3 mM; after administration of L-2-imidazolidone-4-carboxylate, the concentration of 5-oxoproline in mouse skin was found to increase about 3-fold. The relatively high concentration of 5-oxoproline in skin may be associated with the relatively high activity of γ-glutamylcyclotransferase in skin (22). Further studies on the enzymes of the γ-glutamyl cycle in the several layers of skin would be of interest.

In the present work we found values for the 5-oxoproline content of kidney, brain, and liver that are in the range 0.02–0.05 mM. However, in view of the possibility that γ-glutamyl compounds (including glutamine) present in tissues may cyclize nonenzymatically to form 5-oxoproline during preparation of the tissues for analysis, we are inclined to believe that the true normal tissue concentrations of 5-oxoproline may be lower than found here. Further work on the analytical procedures for 5-oxoproline determination seems desirable; in addition to methods involving enzymatic or nonenzymatic conversion of 5-oxoproline to glutamate (as used here), procedures in which gas–liquid chromatography of esters of 5-oxoproline have also been used (23, 24, 17–20). It is of interest to note that the chemically related compound 2-hydroxy-5-oxoproline has been found to be present in kidney and several other rat tissues in concentrations of about 0.01 mM (Duffy, T. E., Cooper, A. J. L. & Meister, A., in preparation).

It should be emphasized that the conclusion that 5-oxoproline is a metabolite does not rest crucially on the finding of this compound in tissues or body fluids. Many metabolites are known to be present in biological materials in very low concentrations. For example, while there is much evidence that ammonia is a metabolite, it has been difficult to establish exactly its concentration in tissues and body fluids. Although the concentration of ammonia in most tissues and body fluids is very low, there may be a substantial concentration of ammonia in portal blood. Presently available data indicate that the concentration of 5-oxoproline in many tissues and body fluids is also very low; skin is a notable exception, as stated above, but further studies on other mammalian tissues are clearly needed. The evidence now available concerning the formation and utilization of 5-oxoproline as well as the consequences of a metabolic block of its utilization offer strong support for its existence as an intermediate in the metabolism of glutathione by way of the γ-glutamyl cycle.

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