SOURCE OF PYRROLE-2-CARBOXYLATE IN MAMMALIAN URINE

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Received November 27,1972

Summary: Pyrrole-2-carboxylate, earlier reported in human urine and labeled in rat urine after administration of radioactive proline, arises more directly from labeled hydroxyproline. Antibiotic treatment appeared to exclude epimerization of administered hydroxy-L-proline to a D-epimer by intestinal bacteria. A likely reaction for the in vivo conversion is hydroxy-L-proline oxidation by the L-amino acid oxidase of rat kidney, demonstrable with purified enzyme. Crystalline D-amino acid oxidase also catalyzes a slow oxidation of hydroxy-L-proline. These two reactions are adequate to account for the normal excretion of pyrrole-2-carboxylate by a number of species.

As shown in Figure 1, pyrrole-2-carboxylate arises by non-enzymatic dehydration of the pyrroline product formed from the D-isomers of hydroxyproline by D-amino acid oxidase (1). Pyrrole-2-carboxylate has been identified in human and rat urine after administering the D-isomers of hydroxyproline, either alone or in racemic mixtures (2). While this latter finding has a clear enzymatic explanation, no enzymatic route has been established to explain the subsequent report (3) that excretion of labeled pyrrole-2-carboxylate followed the administration of ^{14}C-L-proline. This finding was interpreted to result from initial conversion of L-proline to collagen hydroxy-L-proline (3), although a pathway from hydroxy-L-proline to pyrrole carboxylate is unknown in animals (4).

We decided to investigate three alternative routes to pyrrole-2-carboxylate from labeled L-proline: a direct pathway, independent of hydroxyproline, which might proceed via 3,4-dehydro-L-proline (5); conversion of hydroxy-L-proline to a D-epimer by intestinal bacteria (4); or direct oxidation of hydroxy-L-proline to the ketimine product (Fig 1), by mammalian enzymes.

MATERIALS AND METHODS

Male Wistar rats (75-150 gm) were injected intraperitoneally with the appropriate isotopic compounds and housed in metabolic cages with free access to food and water. L-Proline-(U)-^{14}C and hydroxy-L-proline-5-^{3}H were purchased from New England Nuclear Corp; DL-proline-5-^{3}H was prepared by treating DL-Δ^{1}-pyrroline-5-carboxylate with $NaB^{3}H_{4}$, and was a gift of Dr. L. Frank; hydroxy-L-proline-(U)-^{14}C was isolated from chick-embryo collagen after incubating minced tissue with (U)^{14}C-L-proline under the conditions used for the preparation of proline hydroxylase substrate (6), except for the omission of α,α'-dipyridyl so as to permit proline hydroxylation. Antibiotic-treated rats were given an oral dose of neomycin sulfate (50 mg), phthalylsulfathiazole (75 mg), chloramphenicol (25 mg), and chlortetracycline (25 mg), twice daily by stomach tube for 4 days. Effectiveness of antibiotic treatment was demonstrated by plating fecal samples on McConkey's agar; treatment produced a marked drop in fecal colonies, to less than 10 per mg (wet weight) of feces. Rat urine was collected for a 24-hour period in flasks containing 1 ml of toluene and 2 ml of 2.5 N NaOH to protect pyrrole-2-carboxylate from acid-catalyzed decomposition; carrier pyrrole-2-carboxylate (5 mg) was added initially to each urine collection flask.

Radioactive pyrrole-2-carboxylate was recovered from acidified urine by extraction into ether, passage of the redissolved dried ether extract through Dowex-50 (H^{+}) columns to absorb materials interfering with a colorimetric pyrrole determination (7), and further purification by paper electrophoresis at pH 2. Specific activity of pyrrole carboxylate after paper electrophoresis was usually unchanged from that of the Dowex-50 effluent; radioactivity in the excreted pyrrole carboxylate was corrected for losses during purification by recovery of the originally added carrier.

Kidney L-amino acid oxidase was purified to Step 6 (Table II, (8)) by the procedures described (8), using tissue from Wistar rats or rat kidneys

Table 1. Urinary excretion of labeled pyrrole-2-carboxylate after administration of labeled proline or hydroxyproline. See text for details.

Number of Rats Per Group	Compound Administered[a] (Radioactivity per group)	Fraction of Dose Recovered as Pyrrole-2-carboxylate
		%
8	L-Proline-(U)-^{14}C (3.9×10^6 dpm)	0.025
4	L-Proline-(U)-^{14}C (1.7×10^6 dpm)	0.015
	DL-Proline-5-^{3}H (2.4×10^8 dpm)	0.013[b]
2	Hydroxy-L-proline-5-^{3}H (1.3×10^8 dpm)	0.12
	Hydroxy-L-proline-(U)-^{14}C (2.1×10^5 dpm)	0.24
4	Hydroxy-L-proline-5-^{3}H (2.7×10^8 dpm)	0.11
3	Hydroxy-L-proline-5-^{3}H (1.8×10^8 dpm)	0.15[c]

[a] Specific activity of all administered compounds ranged from 3×10^8 to 5×10^8 dpm/μmole, except for U-^{14}C-hydroxy-L-proline whose specific activity was 1.4×10^6 dpm/μmole.

[b] Relative to radioactivity in L-proline.

[c] Isotopic compound administered by stomach tube.

obtained from Pel-Freez (Rogers, Arkansas). D-Amino acid oxidase was the crystalline Sigma product.

RESULTS AND DISCUSSION

Proline and Hydroxyproline as Sources of Urinary Pyrrole-2-carboxylate - The data of Table 1 confirm earlier findings (3) in that either ^{14}C- or ^{3}H-L-proline labels urinary pyrrole-2-carboxylate. In extension of these findings, however, it appears that hydroxy-L-proline, whether fed or injected, is a considerably better source of pyrrole-2-carboxylate than is proline. This finding makes it unnecessary to consider a direct pathway from free

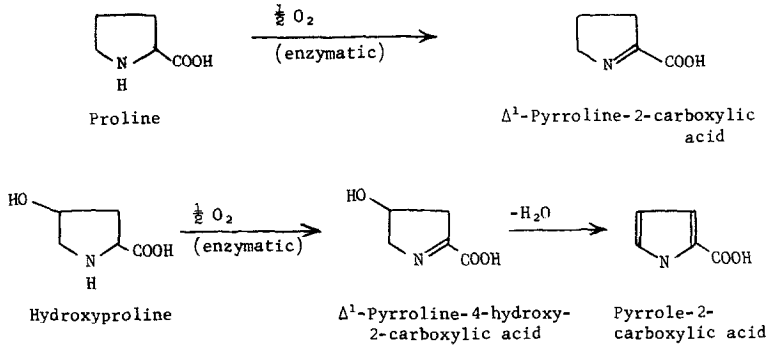

Fig. 1 Oxidation of proline and hydroxyproline to the cyclic ketimines. The reactions shown are catalyzed by D-amino acid oxidase acting on D-isomers of proline or hydroxyproline (or allo hydroxyproline). The ketimine product of hydroxyproline is unstable and readily loses water to yield pyrrole-2-carboxylate.

proline as the major basis of the present and earlier observations of pyrrole-2-carboxylate labeling from proline. An earlier report that feeding labeled hydroxy-L-proline led to excretion of 0.3% of the dose as urinary pyrrole-2-carboxylate (9) is difficult to interpret because of the later recognition that rat intestinal bacteria are capable of epimerizing hydroxy-L-proline to the D-allo epimer (4).

Possible Role of Intestinal Bacteria - A comparison of the efficiency of conversion of hydroxy-L-proline to pyrrole carboxylate after feeding or injecting hydroxyproline (Table 1), gave no support to the possibility that this conversion requires epimerization of hydroxyproline in the intestine.

More conclusive evidence on this point was sought. Feeding hydroxy-L-proline to rats in divided doses (total of 8-9 mmoles/100g) resulted in a 10-20-fold increase in the daily excretion of pyrrole-2-carboxylate (from 0.03-0.05 μmoles/100g to 0.4-0.9 μmoles/100g). Rats whose feces were rendered almost sterile by antibiotic treatment showed no reduction in the excretion of pyrrole carboxylate after feeding hydroxy-L-proline on the same schedule as controls.

Oxidation of Hydroxy-L-Proline to the Pyrroline Ketimine by Mammalian Enzymes - With no evidence for direct conversion of proline to pyrrole-2-carboxylate or for initial epimerization of hydroxy-L-proline to the D-epimer, we considered mammalian enzymes which might account for the oxidation of hydroxy-L-proline to the cyclic ketimine, as in Fig 1. A plausible candidate is the L-amino acid oxidase of kidney, first described by Blanchard et al (10) and more recently crystallized by Nakano and Danowski (8). Although L-proline was reported as a substrate for this enzyme in its initial description (10), we could find no report of trials of hydroxy-L-proline as a substrate. The enzyme, purified 80-fold from rat kidney by the procedures described (8), acted both on L-proline and hydroxy-L-proline to form the expected ketimine products (Fig 1). Over an enzyme purification range of 33-fold, the rate of oxidation of L-leucine (one of the best amino acid substrates) maintained a constant ratio with that of hydroxy-L-proline; furthermore, both activities were eluted from the major protein band seen on electrophoresis of purified enzyme in polyacrylamide gels. The reaction rate with hydroxy-L-proline, while small (V_{max} about one-fifth that for leucine), is consistent with the small fraction of administered counts in hydroxy-L-proline excreted as pyrrole-2-carboxylate by the intact animal (Table 1).

Another possible enzymatic reaction leading to pyrrole-2-carboxylate from hydroxy-L-proline is that catalyzed by D-amino acid oxidase. In our findings, the crystalline hog-kidney enzyme catalyzes a slow rate of oxidation of hydroxy-L-proline (about 0.07 μmoles/mg enzyme/hour) to yield the cyclic ketimine, from which pyrrole-2-carboxylate is formed, in extension of earlier reports that L-proline is a substrate for D-amino acid oxidase (5). Our control experiments appeared to rule out both contamination of the enzyme with L-amino acid oxidase and contamination of the substrate with traces of the D-epimers of hydroxyproline.

The above findings support the conclusion that urinary pyrrole-2-carboxylate is formed by the direct oxidation of hydroxy-L-proline through

the action of mammalian enzymes. Although this pathway is not the major oxidative route for free hydroxy-L-proline (4), it would appear of interest to examine pyrrole-2-carboxylate excretion as an index of collagen degradation.

Only fragmentary information on pyrrole-2-carboxylate excretion by man has been reported: an earlier study of several subjects (11) recorded 24-hour values ranging from 1.1 to 3.5 μmoles. In a recent report (12), which appeared after the completion of our studies, the mean 24-hour excretion for 13 healthy adults was 2.2 μmoles. Our own data for 24-hour excretion by 22 healthy adults give a mean of 2.5 μmoles with a standard deviation of 1.6.

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