

CONVERSION OF HYDROXYPROLINE TO PYRROLE-2-CARBOXYLIC ACID*

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Although studies on the degradative metabolism of hydroxyproline have revealed that this amino acid is converted at least in part to glutamic acid (1-6), knowledge of the intermediates in this conversion and of other products of hydroxyproline metabolism is not yet complete. In the course of experiments on the metabolism of hydroxyproline, it was observed that incubation of hydroxyproline with kidney preparations led to the formation of a compound which we have identified as pyrrole-2-carboxylic acid (PCA) (7).¹ Subsequent study revealed that kidney D-amino acid oxidase oxidizes hydroxy-D-proline and alhydroxy-D-proline to an intermediate compound which appears to be Δ^1 -pyrroline-4-hydroxy-2-carboxylic acid. The intermediate is spontaneously converted (at pH 8.3) to PCA; this conversion occurs much more rapidly in acid solution. PCA formation was also observed as a product of transamination of γ -hydroxyornithine with glyoxylate, pyruvate, or α -ketoglutarate. The evidence suggests that such transamination, in contrast to that of ornithine (8, 9), involves the α -amino group rather than the δ -amino group.

This report describes experiments on the formation of PCA by enzymatic oxidation, by enzymatic and non-enzymatic transamination, and also by non-enzymatic oxidation of hydroxyproline by hydrogen peroxide. Some implications of these findings in terms of the metabolism of hydroxyproline are discussed.

EXPERIMENTAL

Materials and Methods

D-Amino acid oxidase was prepared from sheep kidney according to Negelein and Brömel (10), and the designations of the enzyme fractions used in the present work are those of the above investigators. Crystalline

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¹ This finding has been confirmed by G. Letelier and L. P. Bouthillier (personal communication).

beef liver catalase was obtained from the Worthington Biochemical Corporation, and flavin adenine dinucleotide (FAD) was purchased from the Sigma Chemical Company. Allohydroxy-D-proline was purchased from the Nutritional Biochemicals Corporation. Samples of hydroxy-D-proline, γ -hydroxyornithine (70 per cent hydroxy-L-ornithine plus 30 per cent allohydroxy-D-ornithine), and β -hydroxy- γ -aminobutyric acid were generously provided by Dr. Jesse P. Greenstein, Dr. Bernhard Witkop, and Dr. George Wolf, respectively.

TABLE I
Paper Chromatographic Systems and R_F Values

Solvent No.	Composition	R_F values ($\times 100$)			
		Hydroxy-proline	Pyrrole-2-carboxylic acid	X*	β -Hydroxy- γ -amino-butyric acid
1	<i>n</i> -Butanol-acetic acid-water (4:1:1)	16	86		27
2	Formic acid- <i>tert</i> -butanol-water (15:75:15)	40	87		50
3	Pyridine-water (80:20)	51	88	39	25
4	Phenol (saturated with 10% Na_2CO_3)	62	66		
5	Methyl ethyl ketone- <i>tert</i> -butanol-formic acid-water (16:16:0.1:3.9)	7	95		17
6	Lutidine-water-ethanol-diethylamine (55:25:20:1)	24	62	35	19
7	Pyridine-water-methanol (4:20:80)	48	87		48
8	Ethyl alcohol-water (77:23)	44	74	55	33
9	<i>n</i> -Propanol-water (80:20)	20	81	40	17
10	Ethanol-water (90:10)	10	6	62	

* X = intermediate in pyrrole-2-carboxylic acid formation (see the text).

Ascending paper chromatography on Whatman No. 1 paper was carried out with the solvent systems listed in Table I. The spray reagent used to reveal the position of PCA was prepared by dissolving 10 gm. of *p*-dimethylaminobenzaldehyde in 100 ml. of concentrated hydrochloric acid. This solution was diluted with 9 volumes of acetone immediately prior to use. Hydroxyproline was detected by spraying the paper with the combined isatin reagent of Jepson and Smith and then with the *p*-dimethylaminobenzaldehyde reagent (11).

Enzymatic Oxidation of Hydroxyproline to Pyrrole-2-carboxylic Acid—Initial studies revealed that, when crude hog kidney preparations were shaken in air with allohydroxy-D-proline or hydroxy-D-proline, a new compound was formed which moved more rapidly than did hydroxyproline in

several paper chromatographic systems. This compound could be revealed on chromatograms sprayed with Ehrlich's reagent, but did not yield color when treated with ninhydrin. Further work indicated that the formation of the new compound was catalyzed by D-amino acid oxidase in the presence of catalase, and subsequent studies were therefore carried out with preparations of purified oxidase.

Isolation of the product of oxidation of allohydroxy-D-proline was accomplished as follows. A mixture consisting of allohydroxy-D-proline (1 gm.), D-amino acid oxidase (Fraction II, 200 ml.), FAD (600 γ), crystalline catalase (15,000 units), and sodium pyrophosphate (0.023 mole) in a final volume of 230 ml. was placed in a water bath at 37°, and a stream of oxygen was bubbled continuously into the solution; foaming was controlled with a few drops of capryl alcohol. The initial pH was 8.3. After 3 hours, additional quantities of oxidase (160 ml.), FAD (600 γ in 20 ml. of water), and catalase (15,000 units in 10 ml. of water) were added, and the stream of oxygen was continued for 18 hours at 37°; only traces of hydroxyproline could then be detected. The reaction mixture was acidified to Congo red paper, and the precipitated protein was removed by centrifugation. The supernatant solution was extracted five times with an equal volume of ether, and the combined ether extracts were dried over sodium sulfate. Upon evaporation, a yellow crystalline residue weighing 590 mg. was obtained which, after decolorization with Norit, was recrystallized from ethanol-petroleum ether. The yield of recrystallized material was 510 mg. (60 per cent). Calculated, for $C_5H_5O_2N$, C 54.1, H 4.5, N 12.6; found, C 54.0, H 4.5, N 12.6.

The elemental analyses were consistent with the empirical formula of PCA, and further characterization supported the belief that the isolated material was indeed this substance. The melting point was 189° (dec.), and a mixed melting point carried out with a synthetic sample of PCA (obtained through the courtesy of Dr. Bernhard Witkop) gave no depression. Paper chromatography with several solvent systems (Table I) revealed identical R_F values for the isolated and synthetic samples. The infrared (3 to 15 μ ; in potassium bromide pellets) and ultraviolet absorption curves (Fig. 1) of the isolated and synthetic compounds were identical. The molar extinction coefficients of PCA in pyrophosphate buffer and in 0.1 N hydrochloric acid are 12,700 (λ_{max}^{255}) and 15,100 (λ_{max}^{266}), respectively.

Catalytic reduction of the isolated material and of an authentic sample of PCA gave proline as the major product. For this purpose, the compound (10 mg.) was dissolved in 0.5 ml. of water, aqueous ethanol, or absolute ethanol, and 10 mg. of platinum oxide catalyst were added. Reduction was carried out for 18 hours in a Parr hydrogenator at 40 pounds pressure at 26°. Paper chromatographic study then revealed the forma-

tion of considerable quantities of proline, smaller amounts of α -aminovaleric acid, and three other ninhydrin-reacting substances which were present in trace amounts and were not further investigated. The formation of α -aminovaleric acid was confirmed by chromatography in a variety of solvents, and it was also found that proline² itself gave α -aminovaleric acid when hydrogenated under these conditions. The reduction of PCA proceeded to about 30 per cent of completion; considerable quantities of unchanged PCA remained after hydrogenation, even when very large quantities of catalyst were added. Reduction of PCA to proline was also observed after hydrogenation at ordinary pressure with palladium catalyst.

Initial Product of Hydroxyproline Oxidation—The observation that PCA exhibits a high characteristic absorption in the ultraviolet region (Fig. 1) made it possible to follow the reaction spectrophotometrically. Experiments were carried out in which mixtures consisting of purified oxidase, crystalline catalase, and substrate were shaken in the Warburg apparatus. The oxygen uptake was recorded manometrically, and samples of the reaction mixtures were diluted with buffer and examined in a Cary recording spectrophotometer. These studies revealed that only a small fraction of the oxidized hydroxyproline could be accounted for as PCA. However, spectrophotometric studies after acidification of the reaction mixtures indicated that the formation of PCA and the oxygen consumption were stoichiometrically related. In the experiment in Fig. 2, after 1 hour of incubation, the reaction mixture was diluted with 0.1 N hydrochloric acid. The absorption immediately after dilution with acid (Curve 2) revealed evidence consistent with the presence of some PCA. Upon standing at 26°, the absorption of the acidic solution increased progressively to a maximum in 5 hours. Calculation of PCA from the maximal absorption observed (Curve 5) showed the presence of 4.3 μ moles of PCA, in good agreement with the data for oxygen uptake (Fig. 2). These results suggest the formation of an intermediate compound which was converted to PCA upon acidification.

Further evidence for the formation of an intermediate compound was obtained by a paper chromatographic study of the reaction mixtures at neutral or alkaline values of pH. Thus, chromatography in Solvents 3, 6, 8, 9, and 10 (Table I) provided evidence for a new compound which was demonstrated by spraying the paper with Ehrlich's reagent. Since this reagent is dissolved in concentrated hydrochloric acid, it is probable that

² Commercial preparations of L-proline were found to contain traces of hydroxyproline which were removed by cellulose column chromatography. The column was prepared with cellulose powder (Whatman No. 1) and developed with a solvent containing butanol, acetic acid, and water (4:1:1). The first fractions of the eluate contained only proline; these were combined and evaporated, and the proline was recrystallized from ethanol-petroleum ether.

the actual color-producing material is PCA formed on the paper at the time of spraying. The chromatographic studies carried out at neutral or alkaline pH showed some PCA, a finding consistent with the ultraviolet

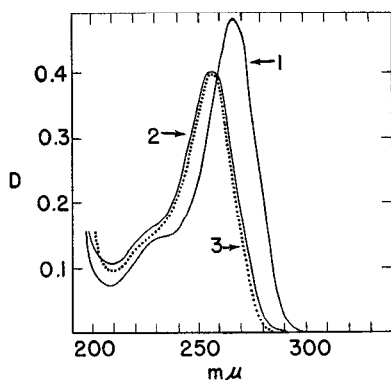


FIG. 1

FIG. 1. Ultraviolet absorption curves of pyrrole-2-carboxylic acid (32.1×10^{-6} M). Curve 1, in 0.1 N hydrochloric acid; Curve 2, in 0.1 M sodium pyrophosphate buffer (pH 8.3); Curve 3, in water.

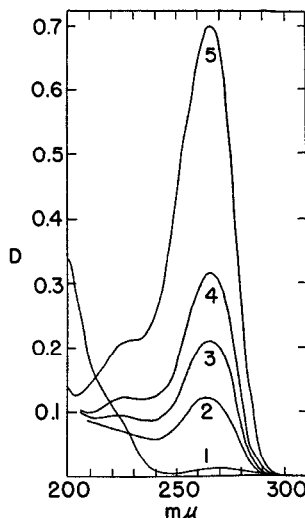


FIG. 2

FIG. 2. Spectrophotometric evidence for the conversion of an intermediate to pyrrole-2-carboxylic acid. The reaction mixture consisted of purified enzyme (Fraction II, 0.25 ml.), crystalline catalase (75 units), FAD (35 γ), sodium pyrophosphate buffer of pH 8.3 (0.8 ml. of 0.1 M), aldehydo-D-proline (25 μ moles) in a final volume of 1.15 ml. The mixture was shaken in a Warburg vessel at 37° for 1 hour; at this time 53.6 μ l. of oxygen had been absorbed, corresponding to the oxidation of 4.8 μ moles of substrate. The absorption curves were obtained at various intervals after dilution with 79 volumes of 0.1 N hydrochloric acid. Curve 1, initial absorption of the mixture prior to enzymatic oxidation; Curve 2, initial absorption after enzymatic reaction; Curve 3, absorption 30 minutes after dilution with hydrochloric acid; Curve 4, absorption 1 hour after dilution with acid; Curve 5, absorption 5 hours after dilution with acid (maximal absorption).

absorption data. These observations indicate that the intermediate is slowly converted to PCA at neutral or slightly alkaline pH.

Catalytic reduction of reaction mixtures (see Fig. 1, legend) containing the intermediate resulted in formation of hydroxyproline. In these studies, 0.05 ml. of the reaction mixture (at pH 8.3) and 10 mg. of catalyst (platinum oxide or palladium on charcoal) were hydrogenated for 30 minutes at 20 pounds pressure and at 26°. After removal of catalyst, paper chroma-

tography revealed the presence of hydroxyproline; no intermediate was detected after hydrogenation. Evidence that the same intermediate arises in the course of enzymatic and non-enzymatic transamination of γ -hydroxyornithine and in the non-enzymatic oxidation of hydroxyproline by hydrogen peroxide is presented below.

Conversion of Hydroxyproline to β -Hydroxy- γ -aminobutyric Acid—In studies with highly purified D-amino acid oxidase possessing very low catalase activity, it was found that oxidation of hydroxyproline gave only traces of PCA. When crystalline catalase was added to such oxidase preparations, oxidation of hydroxyproline gave equimolar quantities of PCA. Such experiments also revealed that twice as much oxygen was consumed in the absence of catalase as in its presence. The observation that 1 mole of carbon dioxide was formed for each mole of substrate oxidized in the absence of catalase was consistent with observations on the oxidation of other amino acids, which indicate that the resulting α -keto acid is decarboxylated by peroxide to yield the next lower homologous acid. Thus, enzymatic oxidation of L-glutamine in the absence of catalase has been employed for the preparation of succinamic acid (12). The formation of β -hydroxy- γ -aminobutyric acid by oxidation of allohydroxy-D-proline in the absence of catalase was demonstrated by paper chromatography in the solvent systems listed in Table I; in each case the enzymatically formed compound gave a ninhydrin-positive spot whose R_F value was identical with that of an authentic sample of β -hydroxy- γ -aminobutyric acid. Similarly, oxidation of proline by D-amino acid oxidase preparations low in catalase activity gave γ -aminobutyric acid.

Non-Enzymatic Formation of Pyrrole-2-carboxylic Acid by Oxidation of Hydroxyproline with Hydrogen Peroxide—The finding that PCA is the major product of enzymatic oxidation of hydroxyproline suggested an examination of the product of the oxidation of hydroxyproline by hydrogen peroxide in the presence of alkaline copper sulfate. This reaction forms the basis of a method for the quantitative determination of hydroxyproline (13, 14). When hydroxyproline was oxidized under these conditions, spectrophotometric evidence for the formation of PCA was obtained after acidification. Chromatographic evidence was obtained for the formation of an intermediate possessing the same R_F value as that observed for the intermediate in enzymatic oxidation. Catalytic reduction of this intermediate, carried out as described above for the enzymatically formed compound, also gave hydroxyproline. Upon acidification, the intermediate product was rapidly converted to PCA. PCA was isolated from the oxidation mixture as follows: A reaction mixture consisting of hydroxy-L-proline (1 gm.), copper sulfate pentahydrate (1.25 gm.), sodium hydroxide (50 gm.), and hydrogen peroxide (100 ml. of a 3 per cent solution) in a final volume of 400 ml. was shaken for 5 minutes at 26°. Ferrous sulfate solution (20 ml. containing

13.9 gm.) was then added, and the pH was adjusted to 3.9 by addition of glacial acetic acid; the pH was further adjusted to approximately 2 by cautious addition of concentrated hydrochloric acid. The mixture was extracted with several portions of ether, and the combined ether extracts were evaporated *in vacuo* to give 560 mg. of a crude product which was homogeneous on paper chromatograms in several solvents. After decolorization with Norit, the product was recrystallized from ethanol-petroleum ether; yield, 520 mg. (61 per cent). The ultraviolet absorption curves, chromatographic behavior, and melting point of the isolated material were identical with those of an authentic sample of PCA.

When oxidation of hydroxyproline with peroxide, carried out as described above, was followed by acidification in the presence of hydrogen peroxide (*i.e.*, ferrous sulfate treatment was omitted), considerable quantities of β -hydroxy- γ -aminobutyric acid were formed as determined by paper chromatography.

The reported lack of reproducibility of the hydrogen peroxide oxidation method for hydroxyproline (14) may be related to the instability of PCA in strong mineral acids. Although PCA is relatively stable in 0.1 N HCl (less than 2 per cent is destroyed per hour), it is rapidly decomposed in more concentrated acid solution. The present findings may provide a useful basis for establishing optimal conditions for the quantitative determination of hydroxyproline by the Neuman and Logan (13) colorimetric procedure.

Transamination of γ -Hydroxyornithine with α -Keto Acids—Non-enzymatic transamination between γ -hydroxyornithine and glyoxylic acid was carried out in the presence of 0.001 M aluminum ion at pH 4.9 and 100°, by the general procedure of Metzler and Snell (15); paper chromatographic study revealed formation of both PCA and glycine. Similar experiments were carried out with γ -hydroxyornithine and pyridoxal; formation of pyridoxamine and PCA was demonstrated. An analogous enzymatic transamination reaction has also been observed (Table II). In these studies, a rat liver preparation was incubated with γ -hydroxyornithine and α -ketoglutarate, pyruvate, or glyoxylate. In each instance, the corresponding amino acid was formed, and the presence of PCA was demonstrated chromatographically and the amount determined by spectrophotometric measurement.³

Transamination involving the α -amino group of γ -hydroxyornithine would be expected to yield initially Δ^1 -pyrroline-4-hydroxy-2-carboxylic acid, *i.e.* the same intermediate formed in the course of enzymatic oxidation of hydroxyproline. On the other hand, transamination involving the δ -amino group of γ -hydroxyornithine would yield γ -hydroxyglutamic- γ -

³ Preliminary studies of transamination between γ -hydroxyornithine and glyoxylate were reported (16).

semialdehyde. Evidence for the formation of the latter compound has been obtained by Taggart and Krakaur (17) and by Lang and Mayer (18). These workers observed the formation of a compound with the properties of γ -hydroxyglutamic- γ -semialdehyde after oxidation of hydroxy-L-proline by liver and kidney particle preparations. We have carried out similar experiments with rat kidney, dog liver, and rabbit kidney preparations. After incubation, the reaction mixtures were acidified and chromatographed; no evidence for the presence of PCA was obtained.

The reaction mixtures were also treated (at neutral pH) with a solution of *o*-aminobenzaldehyde, and the resulting highly colored yellow compounds

TABLE II
*Enzymatic Transamination of γ -Hydroxyornithine**

α -Keto acid	Pyrrole-2-carboxylic acid formation	Amino acid formation†
	<i>μmole</i>	
None.....	0	None
α -Ketoglutaric.....	0.35	Glutamic acid
Pyruvic.....	0.37	Alanine
Glyoxylic.....	0.48	Glycine

* The reaction mixtures consisted of 5 μ moles of γ -hydroxyornithine, 5 μ moles of the sodium salt of the α -keto acid, and 0.1 ml. of enzyme preparation (rat liver homogenate; 1 gm. of fresh tissue per 3 ml. of 0.1 M potassium phosphate buffer, pH 7.4) in a final volume of 0.25 ml. of potassium phosphate buffer (0.1 M, pH 7.4). The mixtures were incubated for 2 hours at 37°. No PCA was formed with α -ketoglutarate and pyruvate in the absence of enzyme. PCA formation with glyoxylate and γ -hydroxyornithine occurred in the absence of enzyme at about 10 per cent of the rate observed with the enzyme.

† Observed by paper chromatography.

(presumably the dihydroquinazolinium derivative formed by condensation of *o*-aminobenzaldehyde and the Δ^1 -pyrroline compound (19, 20)) were chromatographed in several solvents together with the intensely colored yellow compound formed by the reaction of *o*-aminobenzaldehyde with the intermediate formed in the oxidation of hydroxyproline by D-amino acid oxidase. The R_F values of these *o*-aminobenzaldehyde derivatives were sufficiently different to justify the conclusion that the products of these reactions were not identical.⁴ The absence of PCA formation in these experiments is also consistent with this conclusion.

⁴ The R_F values of the *o*-aminobenzaldehyde derivative obtained in the oxidation of hydroxy-L-proline by kidney particle preparations were, respectively, 0.42, 0.24, and 0.03, with Solvents 8, 9, and 1 (Table I). The corresponding values for the derivative of the compound formed in the oxidation of aldehydoxy-D-proline by D-amino oxidase were, respectively, 0.60, 0.47, and 0.22.

Enzymatic Conversion of Hydroxy-L-proline to Pyrrole-2-carboxylic Acid—These experiments were carried out with a soil bacterium, provisionally considered to be a *Pseudomonas*, which was selected by growth on hydroxy-L-proline as the sole organic substrate by Dr. Elijah Adams.⁵ It has been reported by Adams (5) that resting cells of this organism as well as extracts of such cells are capable of utilizing either hydroxy-L-proline or allohydroxy-D-proline. Such utilization is associated with the formation of glutamate; glutamate formation, however, corresponds to only about one-half of the hydroxyproline which disappears. In our experiments, cells of the Adams

TABLE III
*Formation of Pyrrole-2-carboxylic Acid from Hydroxy-L-proline and Allohydroxy-D-proline by Soil Bacterium**

Enzyme preparation	Pyrrole-2-carboxylic acid formation	
	Hydroxy-L-proline	Allohydroxy-D-proline
	μmoles	μmoles
Medium 1, cell suspension	0.32	0.80
“ 1, broken cell suspension	1.02	1.94
“ 2, cell suspension	0.92	1.60
“ 2, broken cell suspension	1.60	2.40

* The reaction mixtures consisted of hydroxy-L-proline or allohydroxy-D-proline (20 μmoles); enzyme preparation 0.3 ml.; buffer (potassium phosphate 0.1 M, pH 8.0) in a final volume of 0.8 ml. The mixtures were incubated at 37° for 4 hours. The cells were grown for 20 hours at 37° with shaking; the cells were harvested by centrifugation and thoroughly washed with ice-cold water and suspended in about 15 ml. of water. Broken cell suspensions were obtained by treatment for 10 minutes in a sonic oscillator (Raytheon). Medium 1 was that of Davis and Mingioli (21); Medium 2 (Adams) consisted of dipotassium hydrogen phosphate, 1.5 gm.; potassium dihydrogen phosphate, 0.5 gm.; magnesium sulfate, 0.2 gm.; yeast extract, 0.5 gm.; hydroxy-L-proline, 1 gm.; final volume 1 liter.

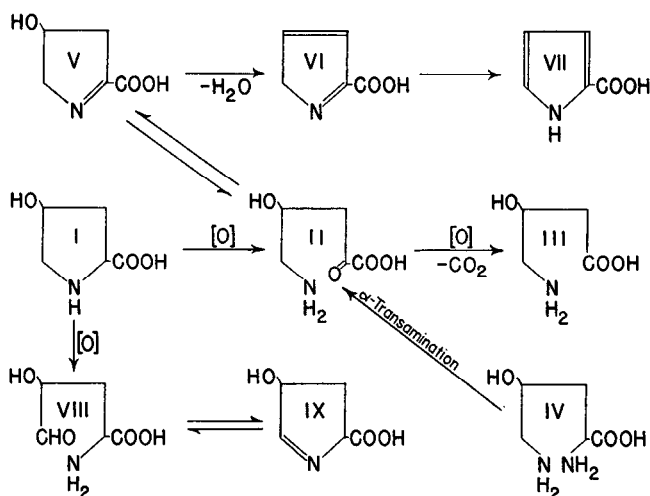
strain were grown on a hydroxy-L-proline-enriched medium and also on the medium of Davis and Mingioli (21). Experiments were carried out with resting cells which had been thoroughly washed by centrifugation with ice-cold water and also with preparations of such cells after disruption of the cell walls by sonic oscillation. The results of several representative experiments are described in Table III. Incubation of resting cells or of broken cell suspensions with hydroxy-L-proline or allohydroxy-D-proline led to the formation of PCA. This reaction was somewhat more rapid with cells grown on the hydroxyproline-enriched medium, and was considerably more rapid with the broken cell preparations than with the sus-

⁵ The authors thank Dr. Elijah Adams for generously providing a culture of this microorganism.

pensions of resting cells. In similar experiments carried out with hydroxy-DL-proline-2-C¹⁴,⁶ the formation of a compound chromatographically identical with PCA was observed on radioautograms. Under our conditions, the major quantitatively significant radioactive areas were those corresponding to hydroxyproline and PCA. Several radioactive spots of low intensity were also observed but were not further investigated. PCA was also detected in the culture medium when the cells were grown in the presence of hydroxy-L-proline. Investigation of the enzyme system which catalyzes formation of PCA from hydroxy-L-proline is necessary. It is possible that PCA is formed directly from hydroxy-L-proline or that the latter compound is converted to a hydroxy-D-proline isomer before conversion to PCA.

DISCUSSION

The accompanying scheme summarizes some of the available information concerning the biochemical transformation of hydroxyproline (I). The



present studies indicate that alhydroxy-D-proline and hydroxy-D-proline are converted by D-amino acid oxidase to an intermediate which promptly yields PCA (VII) upon acidification. Evidence for the existence of such an intermediate is based on spectrophotometric and paper chromatographic study. The existence of this intermediate is also consistent with studies carried out in the absence of catalase in which decarboxylation leading to β-hydroxy-γ-aminobutyric acid (III) occurred. The formation of PCA, of the intermediate, and of β-hydroxy-γ-aminobutyric acid in the D-amino acid oxidase reaction has also been confirmed by employing hydroxy-DL-

⁶ We thank Dr. George Wolf for the labeled hydroxyproline.

proline-2-C¹⁴. No other spots could be detected on the radioautograms. In analogy with Δ^1 -pyrroline-2-carboxylic acid and Δ^1 -piperidine-2-carboxylic acid, which exist in solution in equilibrium with the corresponding α -keto acids (22), it may be postulated that Δ^1 -pyrroline-4-hydroxy-2-carboxylic acid (V) is in equilibrium with α -keto- γ -hydroxy- δ -aminovaleric acid (II). Structure II would be expected to be susceptible to decarboxylation by peroxide to yield Structure III, while Structure V could undergo dehydration to Structure VI and rearrangement to PCA (VII).

The oxidation of hydroxy-L-proline to γ -hydroxyglutamic- γ -semialdehyde (VIII) is suggested by the studies of Taggart and Krakaur (17) and of Lang and Mayer (18), who reported the isolation of the 2,4-dinitrophenyl-osazone of this compound after incubating liver and kidney preparations with hydroxyproline. Our studies, which failed to reveal formation of PCA in these systems, indicate that Structure V undergoes conversion to Structure VII more readily than does either Structure VIII or IX, and also that Structures V and IX are not readily interconvertible. Evidence that Δ^1 -pyrroline-2-carboxylic acid and Δ^1 -pyrroline-5-carboxylic acid are not spontaneously interconvertible was previously reported (22). In view of these considerations, the formation of PCA in the non-enzymatic and enzymatic transamination reactions of γ -hydroxyornithine suggests that the α -amino group rather than the δ -amino group is involved; transamination involving the δ -amino group of γ -hydroxyornithine would be expected to yield γ -hydroxyglutamic- γ -semialdehyde (VIII), presumably in equilibrium with Δ^1 -pyrroline-3-hydroxy-5-carboxylic acid (IX).

The metabolic significance of PCA requires further study. It is of interest that PCA has been isolated as a product of the degradation of mucoproteins and of sialic acid (23, 24). Preliminary experiments by paper chromatographic procedures suggest that PCA is present in low concentration in human urine. It is possible that PCA represents a non-enzymatic degradation product of hydroxyproline metabolism and that its precursor (Δ^1 -pyrroline-4-hydroxy-2-carboxylic acid) may undergo other enzymatic transformations. The present studies provide evidence for the formation of PCA from the D isomers of hydroxyproline by kidney D-amino acid oxidase and also from hydroxy-L-proline by the Adams strain of *Pseudomonas*. The further metabolism of PCA and the possibility that its precursor may participate in other metabolic reactions are being investigated.

SUMMARY

1. Enzymatic oxidation of hydroxy-D-proline or allohydroxy-D-proline by kidney preparations (in the presence of catalase) yielded an intermediate compound which was non-enzymatically transformed to pyrrole-2-

carboxylic acid. Pyrrole-2-carboxylic acid was isolated in good yield and characterized by comparison of its infrared and ultraviolet spectra, chromatographic behavior, melting point, and behavior on catalytic hydrogenation with a synthetic preparation of pyrrole-2-carboxylic acid.

2. Formation of the intermediate compound was shown by spectrophotometric and chromatographic methods. That the intermediate is Δ^1 -pyrroline-4-hydroxy-2-carboxylic acid (in equilibrium with α -keto- γ -hydroxy- δ -aminovaleric acid) was indicated by its conversion upon catalytic hydrogenation to hydroxyproline and by its oxidative decarboxylation to β -hydroxy- γ -aminobutyric acid. In the absence of catalase, enzymatic oxidation of hydroxyproline yielded β -hydroxy- γ -aminobutyric acid; under these conditions proline gave γ -aminobutyric acid.

3. Non-enzymatic oxidation of hydroxyproline by hydrogen peroxide in the presence of alkaline copper sulfate also gave pyrrole-2-carboxylic acid as the major product. An intermediate was formed in this reaction which appeared to be identical with that present during the enzymatic oxidation of hydroxyproline.

4. Pyrrole-2-carboxylic acid was shown to be formed during enzymatic transamination between γ -hydroxyornithine and pyruvate, α -ketoglutarate, or glyoxylate. The evidence suggests that the α -amino group rather than the δ -amino group of γ -hydroxyornithine participates in this reaction. Non-enzymatic transamination between glyoxylate or pyridoxal and γ -hydroxyornithine to yield glycine or pyridoxamine and pyrrole-2-carboxylic acid, respectively, was also observed.

5. Evidence is presented that Δ^1 -pyrroline-4-hydroxy-2-carboxylic acid is probably not in equilibrium with Δ^1 -pyrroline-3-hydroxy-5-carboxylic acid (γ -hydroxyglutamic- γ -semialdehyde).

6. Enzymatic conversion of hydroxy-L-proline to pyrrole-2-carboxylic acid by intact and broken cell suspensions of a soil bacterium was observed. Δ^1 -Pyrroline-4-hydroxy-2-carboxylic acid also appears to be an intermediate in this reaction.

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