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COLLAGEN METABOLISM

II. ENZYMIC FORMATION OF PYRROLE-2-CARBOXYLIC ACID FROM L-HYDROXYPROLINE IN RAT

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SUMMARY

The mechanism of pyrrole-2-carboxylic acid formation from L-hydroxyproline was investigated using rat tissues. After a single administration of 200 mg L-hydroxyproline, the urinary excretion of pyrrole-2-carboxylic acid remained strikingly elevated for 24 h. Pyrrole-2-carboxylic acid formation rates by rat kidney were studied *in vitro*. In this tissue, pyrrole-2-carboxylic acid formation was observed only under aerobic conditions. The pyrrole-2-carboxylic acid forming activity was primarily localized in the supernatant fraction obtained by centrifugation at 105 000 × *g*. In the purification steps used to obtain L-amino acid oxidase from the rat kidney, the activity of pyrrole-2-carboxylic acid formation directly increased as the L-amino acid oxidase activity increased. The ratio of the two activities was constant in each step of the purification of L-amino acid oxidase. From these results, it was concluded that L-hydroxyproline was oxidized to Δ'-pyrroline-4-hydroxy-2-carboxylic acid by L-amino acid oxidase and then converted to pyrrole-2-carboxylic acid spontaneously.

INTRODUCTION

Previously we reported that the measurement of urinary pyrrole-2-carboxylic acid (> Glu) excretion rates is a useful research tool for investigating collagen metabolism[1]. These studies on patients with hyperthyroidism and thyroxine-treated rats showed that the urinary excretion of > Glu was directly proportional to the increase in hydroxyproline excretion.

The existence of radioactive > Glu in rat urine was described by Wolf *et al.*[2,3] in studies on the metabolism of DL-[$^{14}C_2$]hydroxyproline and L-[$^{14}C_2$]hydroxyproline. Later, Gerber *et al.*[4] have shown the excretion of radioactive > Glu after the injection of [U-^{14}C]proline to rats. The formation of > Glu from D-hydroxyproline by D-amino

Abbreviation: >Glu, pyrrole-2-carboxylic acid.

acid oxidase *in vitro*, was reported by Radhakrishnan *et al.*[5] and Adams[6]. However, in mammals the mechanism of > Glu formation from L-hydroxyproline has not been established. Therefore, the transformation of L-hydroxyproline to > Glu in rat kidney was studied by *in vivo* and *in vitro* methods.

MATERIALS AND METHODS

Chemicals

> Glu was synthesized according to McCay and Schmidt[7] and was recrystallized from water; yield 2.5 g (15%), m.p. 177 °C (decompn). Calculated, for $C_5H_5NO_2$:C, 54.1; H, 4.5; N, 12.6%; found: C, 53.8; H, 4.5; N, 12.8%. L-Hydroxyproline was purchased from Tanabe Amino Acid Foundation (Osaka, Japan). A solution of 2.5% dimethylaminobenzaldehyde in 95% ethanol was used as Ehrlich's reagent.

Assay of urinary > Glu

Eight male Wistar strain rats (200 g) were fed a synthetic diet[1] and water *ad libitum*. Four rats were injected subcutaneously with 1 ml of a solution containing 200 mg of L-hydroxyproline. Four other control rats were similarly injected with 1 ml of 0.9% saline solution. Before and after these injections, a 24-h urine sample was collected from each rat in bottles containing toluene. The free hydroxyproline in the rat urine was assayed colorimetrically by a modification of Neuman and Logan[8]. > Glu was determined by the method of Yamanishi *et al.*[1].

Homogenization of rat kidney and fractionation by differential centrifugation

Rats were killed by decapitation. The kidneys were removed quickly, blotted on filter paper, weighed and homogenized in a Waring blender with 9 vol. of 0.25 M sucrose. All operations were conducted at 5 °C. The differential centrifugation of the homogenates was performed using the method of Schneider and Hogeboom[9] (Table II).

Identification of the reaction product

For identification of > Glu formation, incubation was carried out as follows; 50 ml of reaction mixture containing 1 mmole of sodium pyrophosphate buffer (pH 8.3), 1 mmole of L-hydroxyproline and 20 ml of homogenate were incubated at 37 °C with constant shaking. After 2 h, the reaction mixture was acidified to pH 2.0–2.5 with HCl and the precipitate was removed by centrifugation. The deproteinized supernatant was extracted with ether and the ether phase was evaporated to dryness *in vacuo*. The residue was dissolved in a small volume of water or 0.1 M HCl and subjected to thin-layer chromatography and to determination of the ultraviolet absorption spectrum. The details of the thin-layer chromatography have already been described[1]. The chromatogram was sprayed with Ehrlich's reagent. The > Glu formed was identified by comparison of its chromatographic behavior with that of an authentic sample. The ultraviolet spectrum was determined with a Hitachi Model EPS type 3 recording spectrophotometer.

Assay of > Glu formation

For assay of > Glu formation and for the study of intracellular distribution

of this activity, incubation was carried out as shown in Table II, unless otherwise indicated. The > Glu formed in the deproteinized supernatant was determined by the colorimetric method mentioned above[1]. Blanks were prepared by the addition of inactivated enzyme which was heated at 80 °C for 5 min.

Assay of L-*amino acid oxidase activity*

The incubation was carried out at 37 °C with constant shaking for 30 min. Details are shown in Table II and III. The α-ketoisocaproic acid formed in this reaction was determined according to the method described by Wada and Snell[10].

Protein estimation

The protein content of enzyme solution was estimated by the quantitative biuret method[11].

Purification of L-*amino acid oxidase*

L-Amino acid oxidase was purified from acetone powder of rat kidney according to the method of Blanchard *et al.*[12] in order to compare the change of specific activities between L-amino acid oxidase and > Glu formation during the preparation.

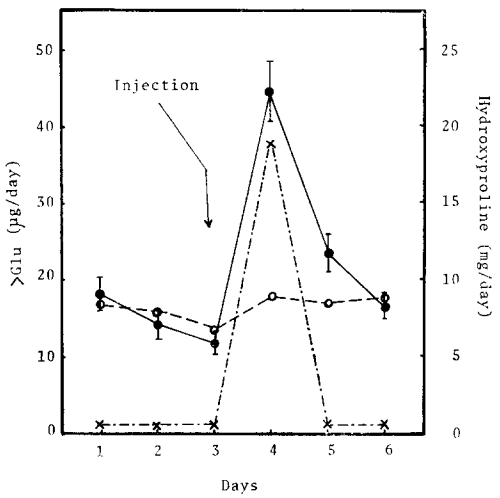

Fig. 1. Urinary excretion of hydroxyproline and pyrrole-2-carboxylic acid. Male rats of the Wistar strain were given a 1-ml solution of 200 mg of L-hydroxyproline by intraperitoneal injection. ×, hydroxyproline; open circles, >Glu of controls; filled circles, >Glu of injected rats.

RESULTS

Change of urinary excretion of > Glu after L-*hydroxyproline injection to intact rats*

As shown in Fig. 1, it was found that when L-hydroxyproline was administered to intact rats, urinary excretion of free hydroxyproline and > Glu were strikingly elevated during the first 24 h and rapidly returned to control level within 48 h. One can infer, therefore, that the injected L-hydroxyproline was partially converted to > Glu in these rats.

TABLE I

COMPARISON OF THE R_F VALUES OF THE EHRLICH'S REAGENT-POSITIVE METABOLITE AND PYRROLE-2-CARBOXYLIC ACID ON THIN-LAYER CHROMATOGRAPHY

The reaction mixture consisted of 20 ml of kidney homogenate, 1 mmole of L-hydroxyproline and 1 mmole of $Na_4P_2O_7$ buffer (pH 8.3) in a total volume of 50 ml.

Solvent system		*R_F values*	
Components	*Ratio (by vol.)*	*Metabolite*	*Authentic > Glu*
n-Butanol–acetic acid–water	4 : 1 : 1	0.80	0.80
Ethylacetate–isopropanol–ammonia water	9 : 6 : 4	0.60	0.60
Chloroform–Isopropanol methanol–ammonia water	9 : 6 : 6 : 4	0.18	0.18
Chloroform–acetic acid–methanol	15 : 4 : 2	0.95	0.94
Ethylacetate–isopropanol–water	9 : 6 : 4	0.93	0.94

The formation of > Glu from L-*hydroxyproline by the homogenate of rat kidney*

L-Hydroxyproline was incubated with the homogenate of rat kidney and the reaction product was analyzed as described under Materials and Methods. The compound formed had R_F values identical with those of > Glu in the solvent systems employed (Table I). Spots of the reaction product and a synthetic sample of > Glu gave an identical color reaction with Ehrlich's reagent. The ultraviolet absorption spectrum of the reaction product was identical with that of authentic > Glu in 0.1 M HCl (Fig. 2). Heating the kidney homogenate or stopping the reaction with acidification at zero time inhibited the reaction completely.

Enzymic properties of rat kidney preparation

The activity of > Glu formation was measured with $Na_4P_2O_7$ and borate buffer between pH 7.0 and 10.5. Optimal activity was found between pH 8.0 and 9.0 (Fig. 3). The effect of enzyme concentration on the rate of > Glu formation was determined as shown in Fig. 4. A linear relationship between the activity and the enzyme concentration was obtained with up to 40 mg of protein. No formation of > Glu could be detected under anaerobic conditions (Fig. 5).

These data indicated that an aerobic enzyme reaction is required for > Glu formation from L-hydroxyproline in rat kidney homogenates.

Intracellular distribution of L-*amino acid oxidase and > Glu formation in rat kidney*

Since it was thought that > Glu formation from L-hydroxyproline may be

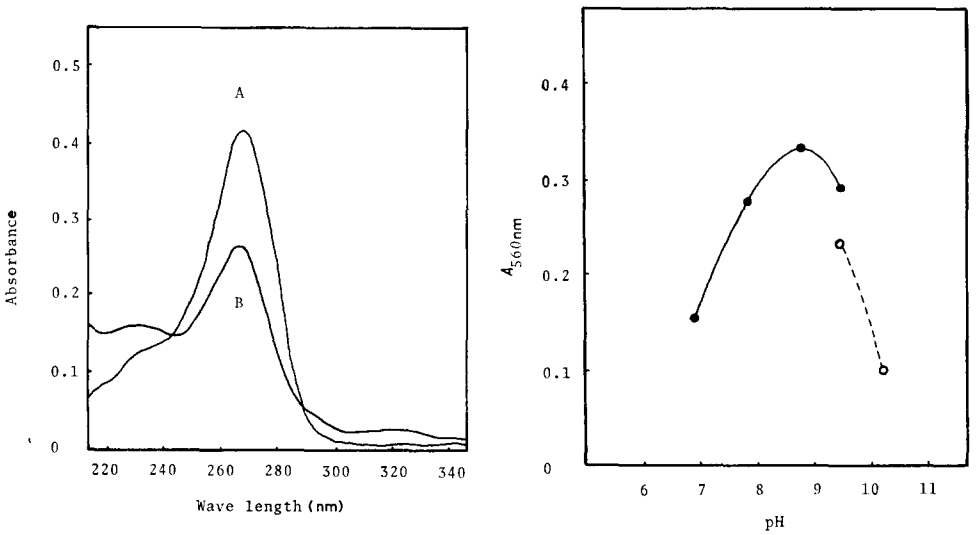

Fig. 2. Ultraviolet absorption spectrum of pyrrole-2-carboxylic acid and reaction product. The reaction mixture was the same as that for Table I. Curve A, authentic pyrrole-2-carboxylic acid ($27.2 \cdot 10^{-6}$ M) in 0.1 M HCl; Curve B, difference spectrum obtained by reading a final time against a zero time after dilution with 0.1 M HCl.

Fig. 3. Effect of pH on pyrrole-2-carboxylic acid formation. The reaction mixture consisted of 1 mmole of L-hydroxyproline, 5 ml of kidney homogenate and 1 mmole of $Na_4P_2O_7$ buffer (filled circles) or 200 μmoles of borate buffer (open circles) in a total volume of 15 ml. Incubation was at 37 °C for 2 h with shaking.

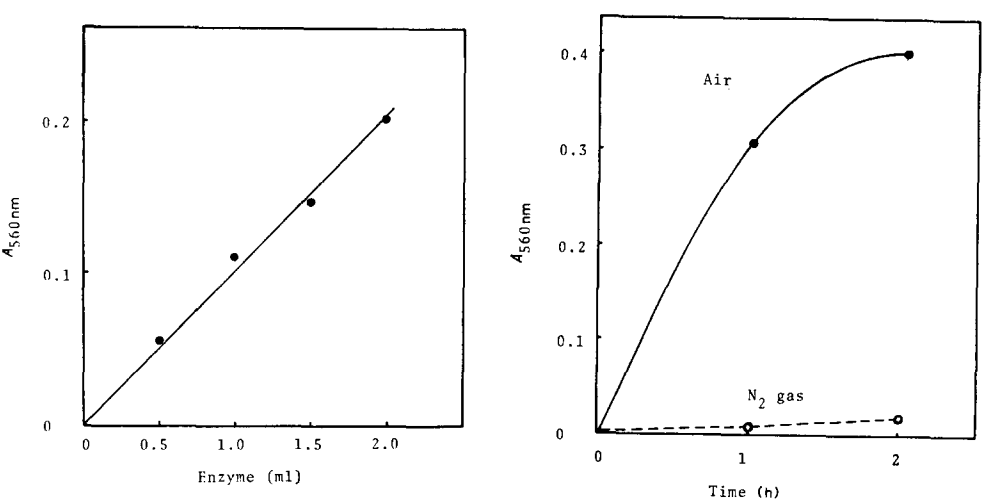

Fig. 4. Relationship between enzyme concentration and pyrrole-2-carboxylic acid formation. The reaction mixture consisted of 500 μmoles of $Na_4P_2O_7$ buffer (pH 8.3), 1 mmole of L-hydroxyproline and the indicated amount of enzyme (20.4 mg/ml) in a total volume of 10 ml. Incubation was at 37 °C for 2 h with shaking.

Fig. 5. Requirement of oxygen. The reaction mixture consisted of 500 μmoles of $Na_4P_2O_7$ buffer (pH 8.3), 2 mmoles of L-hydroxyproline and 10 ml of kidney homogenate in a total volume of 17 ml. Filled circle, incubation was at 37 °C in air with shaking; open circle, incubation was at 37 °C in N_2 gas phase using a Thunberg tube.

TABLE II

INTRACELLULAR DISTRIBUTION OF L-AMINO ACID OXIDASE AND ACTIVITY OF PYRROLE-2-CARBOXYLIC ACID FORMATION

The reaction mixture consisted of 300 μmoles of $Na_4P_2O_7$ buffer (pH 8.3), 1 ml of each cell fraction and 1 mmole of L-hydroxyproline or 10 μmoles of L-leucine in a total volume of 10 ml. Incubation was at 37 °C for 30 min with leucine and for 2 h with hydroxyproline.

Fraction	L-*Amino acid oxidase activity* ($A_{440\ nm}$/*mg protein per h*·10^3)	>*Glu formation* (*μg/mg protein per h*)
Homogenate	8.1	1.9
Supernatant	11.9	3.6
Microsome	3.3	0.9
Mitochondria	2.0	1.5
Nuclei	4.2	1.0

catalyzed by L-amino acid oxidase, the oxidase activity of each centrifugation fraction was measured to determine if they had a similar intracellular distribution. > Glu-forming activity was primarily localized in the supernatant fraction (Table II).

Purification of L-*amino acid oxidase*

The activities of both L-amino acid oxidase and > Glu formation were assayed at each step of the purification from acetone powder of rat kidney. During the course of purification of L-amino acid oxidase, the activity of > Glu formation increased with each increase in L-amino acid oxidase activity. The ratio between the two activities was found to be constant throughout the purification procedure (Table III).

TABLE III

PURIFICATION OF L-AMINO ACID OXIDASE

The reaction mixture consisted of 150 μmoles of $Na_4P_2O_7$ buffer (pH 8.3), 1 mmole of L-hydroxyproline or 10 μmoles of L-leucine and 0.5 ml of enzyme in a total volume of 5.0 ml. Incubation was at 37 °C for 1 h with shaking.

Step	*Activity*		*Protein* (*mg/ml*)	*Specific activity**		
	Leucine ($A_{440\,nm}$/*ml*)	*Hydroxyproline* (*μg/ml*)		*Leucine*	*Hydroxyproline*	*Leucine/Hydroxyproline*
1. Acetone powder aqueous	0.162	16.5	12.7	12.8	1.3	9.9
2. Na_2SO_4 (15%) fraction with heating (57 °C)	0.406	29.9	6.0	67.7	5.0	13.5
3. 2nd Na_2SO_4 (15%) fraction	0.592	43.2	4.4	134.5	9.8	13.7
4. Dialyzed supernatant	0.372	31.0	2.7	143.1	11.5	12.4

* Specific activity: leucine as substrate, $A_{440\ nm}$/mg protein per h·10^3; hydroxyproline as substrate, μg >Glu/mg protein per h.

DISCUSSION

It has been demonstrated that *Pseudomonas* extracts[6] catalyze the conversion of L-hydroxyproline (I) to D-*allo*-hydroxyproline (II) and Δ'-pyrroline-4-hydroxy-2-carboxylic acid (IV). This reaction may involve two enzymes; epimerase and D-amino acid oxidase. Radhakrishnan and Meister[5] have shown that D-amino acid oxidase prepared from sheep kidney oxidizes D-hydroxyproline (III) and D-*allo*-hydroxyproline to Δ'-pyrroline-4-hydroxy-2-carboxylic acid which is converted to > Glu (V) spontaneously. Moreover, evidence for the conversion of δ-hydroxy-ornithine (VII) to > Glu was obtained by them[5] using rat liver homogenates. In this pathway, δ-hydroxyornithine is first converted to α-keto-γ-hydroxy-δ-amino-valeric acid (VI) by transamination and then compound IV is formed by dehydration and ring closing. The proposed routes of > Glu formation and related pathway are summarized in Fig. 6. However, little or no information about > Glu formation from L-hydroxyproline in mammals is available.

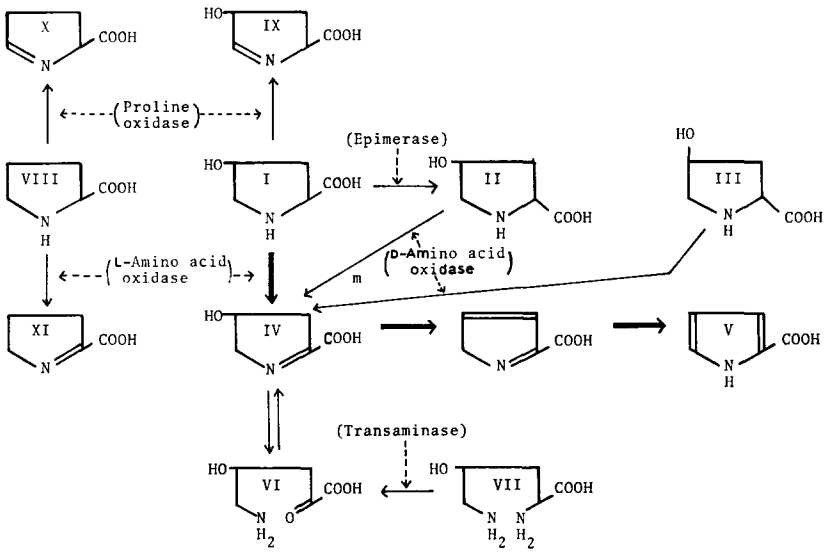

Fig. 6. Pathway of pyrrole-2-carboxylic acid synthesis.

On the other hand, using proline oxidase prepared from rat kidney and liver, Lang *et al.*[13,14] have demonstrated that L-proline (VIII) and L-hydroxyproline were oxidized to Δ'-pyrroline-5-carboxylic acid (X) and Δ'-pyrroline-3-hydroxy-5-carboxylic acid (IX), respectively. They have also proved that L-proline is oxidized to Δ'-pyrroline-2-carboxylic acid (XI) by L-amino acid oxidase. The data summarized here suggest that L-hydroxyproline may be converted to > Glu through Δ'-pyrroline-4-hydroxy-2-carboxylic acid (IV) by L-amino acid oxidase as shown in Fig. 6.

In the present study, it was demonstrated that urinary excretion of > Glu is strikingly increased for 24 h after L-hydroxyproline injection into rats. This may mean that L-hydroxyproline is converted to > Glu *in vivo*. Further, rat kidney homogenate was found to catalyze the conversion of L-hydroxyproline to > Glu. The formation of > Glu in the reaction mixture was confirmed by thin-layer chro-

matography and by the ultraviolet absorption spectrum. The activity of > Glu formation was chiefly found in the supernatant fraction obtained by centrifugation at 105 000 × *g*. During the L-amino acid oxidase purification from acetone powder extracts of rat kidney, the activity of > Glu formation increased with each increase in L-amino acid oxidase activity in the same preparation. It was also found that the ratio between the two enzyme activities is constant at each stage of purification. From the above results, it is evident that > Glu formation from L-hydroxyproline may be catalyzed by L-amino acid oxidase in the rat.

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