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Fate of 1-Aminoproline and Urinary Excretion of 1-Aminoprolyl Hydrazone of Pyridoxal in Rats

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1-Aminoproline-U-^{14}C was administered to rats by intraperitoneal injection. The radioactivity was distributed in all the tissues examined. Among them, kidney, lung, liver and spleen had high specific activity. The radioactivity in the tissues and blood decreased rapidly as a function of time, except in brain. About 80% of the radioactivity administered was excreted in urine within 24 hr. Besides intact 1-aminoproline, several radioactive compounds were detected in the urine sample, and one of them was identified as 1-aminopropyl hydrazone of pyridoxal.

1-Aminoproline was found in linseed meal as a natural vitamin B_6 antagonist for chicks by Klosterman *et al.*[1] In the previous paper,[2] the authors have reported that the L-isomer of this hydrazinoacid was more toxic than the D-isomer to rats, and that the toxic effect was completely prevented by the administration of pyridoxine. Striking changes in free amino acid patterns in various tissues, plasma and urine by the 1-aminoproline treatment were also reported.

In the present paper, the fate of 1-aminoproline was followed isotopically to know the behavior of this hydrazino acid in rats.

MATERIALS AND METHODS

Materials. 1-Amino-L-proline-U-^{14}C was synthesized from L-proline-U-^{14}C (10 mCi/mmole, Radiochemical Centre, Amersham, England) and diluted with unlabelled L-proline, according to the method of Klosterman *et al.*[1] Recrystallized 1-amino-L-proline-U-^{14}C (mp 155～156°C) had a specific activity of 44.1 μCi/mmole. Tests for unreacted proline and 1-nitrosoproline were negative (proline, with isatin[3]; 1-nitrosoproline, with the Griess reagent[4]). And the radiochemical purity checked by ion-exchange chromatography described below was 97.2%. This labelled hydrazino acid was used throughout this work without further dilution.

Abbreviations: APPL, 1-aminoprolyl hydrazone of pyridoxal; APPLP, 1-aminoprolyl hydrazone of pyridoxal 5′-phosphate.

APPL and APPLP were prepared as follows: 0.5 mmole of 1-amino-L-proline was incubated with 0.5 mmole of pyridoxal or pyridoxal 5′-phosphate in 4 ml of water, pH of which was adjusted to 7.0 with 1.6 M NH_4OH at 37°C (pyridoxal, for 4 hr; pyridoxal 5′-phosphate, for 1 hr). After incubation, three volumes of acetone were added to the reaction mixture containing APPL formed, or the solution was acidified to pH 2.0 with formic acid in the case of the reaction mixture containing APPLP formed. Both the solutions were kept at 0°C overnight to yield crystalline products. APPL was recrystallized from ethanol-water, and the yield was 34%. This compound had the following properties: mp 213～215°C, decompn. (Found: C, 52.53; H, 6.53; N, 13.78. Calcd. for $C_{13}H_{17}N_3O_4 \cdot H_2O$: C, 52.53; H, 6.40; N, 14.14%). The hydrazone with pyridoxal 5′-phosphate recrystallized from acetone-water, and the yield was 40%. The crystal gave: mp 226～229°C, decompn. (Found: C, 43.94; H, 4.96; N, 11.60; P, 8.30. Calcd. for $C_{13}H_{18}N_3O_7P$: C, 43.46; H, 5.01; N, 11.69; P, 8.62%).

Animals and diet. Male weanling rats of Wistar strain with an average body weight of about 60 g were caged individually and fed the normal diet described in the previous paper.[2]

Analyses. For the radioactivity measurement, tissues were homogenized with definite volumes of water in the Potter-Elvehjem glass homogenizer. Aliquots of the homogenates and blood were treated with Soluene-100, a sample solubilizer, according to the procedures recommended by the manufacturer (Packard Instrument Co., Ill. U.S.A.). Expired CO_2 was collected according to the method of Jeffay and Alvarez.[5] The radioactivity was measured in the scintillation fluid described by Bruno and Christian[6]

by an Aloka LSC-602 liquid scintillation spectrometer equipped with an automatic external standardization system.

Spectrophotometric determination of 1-aminoproline was performed according to the method of Klosterman *et al.*,[1] with slight modification. 1-Amino-L-proline (below 1.0 μmole) was incubated with 70 μmole of pyridoxal 5'-phosphate in 6 ml of 0.1 M sodium phosphate buffer (pH 7.0) at 37°C for 1 hr. The pH of the reaction mixture was adjusted to 1.0 with 1 M HCl, and the absorbance at 370 nm was measured. By this modified method, the effect of unreacted pyridoxal 5'-phosphate on the measurement could be eliminated. Below pH 2.0, the absorbance maximum of APPLP was at 370 nm and the molar extinction coefficient at 370 nm was 2.8×10^4. APPL also gave the same value. The measurement was carried out by a Hitachi 323 recording spectrophotometer.

Radio active compounds in urine samples were fractionated by ion-exchange chromatography. An urine sample was applied to a 0.9×10 cm column of Dowex 50W-X8 (100~200 mesh, H^+ form). The column was washed with 0.01 M HCl (30 ml) and compounds were eluted with 0.5 M HCl (100 ml) and then with pyridine-acetic acid-ethanol-water (10: 24: 200: 766, by vol., 60 ml). The fractions of radioactive peaks separated on the Dowex 50W column were evaporated to dryness under reduced pressure below 35°C. Each residue was dissolved in 0.01 M HCl and was applied to a 0.9×10 cm column of Dowex 2-X8 (100~200 mesh, acetate form). The column was washed with 0.01 M HCl (20 ml) and the compounds adsorbed were eluted with formic acid (linear gradient, 0~3.33 M of formic acid).

For paper chromatographic analyses, the following solvent systems were used: system I, *n*-butanol-acetic acid-water (4: 1: 1, by vol.); system II, *n*-propanol-water (4: 1, v/v). The filter paper used was Tôyô No. 51A. Radioactive compounds were detected by radioautography. 1-Aminoproline was visualized by Tollen's reagent.[1] APPL and APPLP were located under ultraviolet light.

RESULTS AND DISCUSSION

Distribution of the radioactivity of 1-aminoproline-U-^{14}C in tissues

Table I shows the distribution of radioactivity in rat tissues. At 1 hr after the injection of 1-aminoproline-U-^{14}C, radioactivity was distributed in all the tissues examined. Kidney had the highest specific activity, followed by lung, liver and spleen. At 7 hr after the injection, the radioactivity in most tissues decreased to less than 60% of that at 1 hr.

TABLE I. DISTRIBUTION OF RADIOACTIVITY IN RAT TISSUES

Six rats fed the normal diet (average body weight, 105 g) were starved for 13 hr and then were injected intraperitoneally with 26 mg of 1-aminoproline-U-^{14}C per kg body weight (LD_{50} for young male rats fed the normal diet[2]). Water was given *ad libitum*. At the time indicated, three rats alive were exsanguinated by heart puncture with heparinized syringes after anesthesia with ether and the chests were opened. The tissues were quickly removed, rinsed with ice-cold physiological saline, weighed and treated as described in MATERIALS AND METHODS.

Tissue	Specific activity ($dpm\times10^{-3}$/g wet weight)	
	1 hr	7 hr
Brain	2.0±0.2	1.8±0.6[a]
Spinal Cord	2.0±0.2	1.3±1.0[a]
Eyeball	7.8±2.0	2.8±1.0
Thyroid	11.0±0.9	2.2±1.1
Salivary Gland	9.4±1.3	2.0±1.0
Thymus	12.1±0.7	3.2±1.0
Heart	14.5±0.7	4.7±0.6
Lung	19.9±1.6	7.5±1.0
Liver	17.7±0.3	6.2±1.4
Kidney	172.1±5.5	24.0±6.1
Spleen	15.5±2.0	5.1±1.6
Pancreas	9.0±1.5	1.9±0.3
Stomach	9.4±0.7	3.7±1.1
Duodenum	8.3±1.4	3.9±1.4
Small Intestine	8.2±0.6	4.1±1.3
Caecum	7.9±0.8	2.1±0.3
Colon	8.3±0.5	2.6±1.4
Mesentery	5.1±0.3	1.6±0.4
Testis	6.6±0.6	4.2±0.7
Adipose Tissue (abdominal)	3.5±0.8	0.6±0.5
Muscle (gastrocnemius)	12.4±4.2	2.9±0.8
Skin (pectoral)	13.7±1.8	1.7±1.4
Bone (femur)	8.0±1.1	3.7±0.6

The values are the means±S.D. ($n=3$).

Differences from respective 1 hr values: [a] not significant ($p>0.10$). All other differences are significant, at least with $p<0.01$.

Especially the decreases in kidney and skin were remarkable. In brain, accumulation of radioactivity was low and the decrease as a function of time was relatively small as compared with the other tissues. In this connection, it should be noted that the amino acid pattern in brain was not so markedly disturbed as in the other tissues, as described in the previous paper.[2]

The measurement of the radioactivity in blood was carried out at 10 min after the injection and the radioactivity decreased steadily, as shown in Fig. 1A.

Figure 1B shows the cumulative excretion of radioactivity in urine. Twenty seven % of the radioactivity administered was excreted at 2 hr after the injection, 57% at 5 hr and 83% within 24 hr. At 24 hr, about 62% of the radioactivity excreted was in unchanged 1-aminoproline. In this connection, it is worth noting that 90% of the radioactivity of labelled isonicotinic acid hydrazide, a vitamin B_6 antagonist, was excreted at 6 hr after the intravenous injection.[9] Rapid excretion of 5'-deoxypyridoxine and 4'-deoxypyridoxine was also reported by Shane and Snell[10] and Coburn *et al.*,[11] in rat urine.

Less than 2% of the radioactivity administered was found in CO_2 during 24 hr.

The above results indicate that by intraperitoneal injection, the radioactivity of 1-aminoproline-U-^{14}C was distribued widely in rat body and that the major portion was excreted in urine within 24 hr.

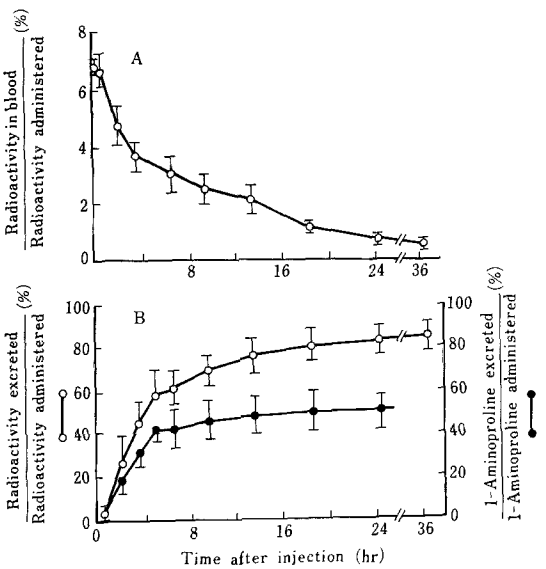

FIG. 1. Radioactivity in Blood and Urine.

Urinary bladders of eight rats were connected with catheters of silicone rubber[7] (inside diameter, 0.5 mm) by a surgical operation. The growth was restored 4 days after the operation, and the rats (average body weight, 103 g) were fasted for 13 hr and were injected with 26 mg of 1-aminoproline-U-^{14}C per kg body weight. Each rat was placed in a glass metabolic cage. Water was given *ad libitum*. At the time indicated, 20 μl of blood was withdrawn from the tail vein by a heparinized syringe and was treated as described in MATERIALS AND METHODS. The total volume of blood in the rat was calculated by the use of the value proposed by Hall *et al.*[8] (7.47 ml/100 g body weight). The urine samples were obtained by syringes through the catheters. The urinary bladders were washed three times with physiological saline. The urine was combined with the washings and was diluted to 10 ml with 0.01 M HCl. 1-Aminoproline was determined spectrophotometrically (see MATERIALS AND METHODS) In this experiment, four rats were alive and the points in the figure represent the means±S.D. of these four rats.

A, blood; B, urine.

Urinary excretion of APPL

Radioactive compounds in the urine sample were separated by chromatography on Dowex 50W column and three main radioactive peaks were observed (Fig. 2). The fractions of these radioactive peaks were further chromatoragphed on Dowex 2 column, and at least nine peaks were separated (Fig. 3).

The peak 5 fraction in Fig. 3 contained unchanged 1-aminoproline. And, when this

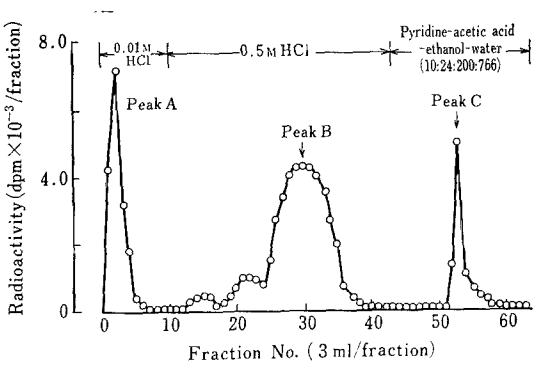

FIG. 2. Chromatographic Separation of Radioactive Compounds in Urine (Dowex 50W).

Urine of the 1-aminoproline-U-^{14}C-treated rats (Fig. 1) was collected between 5 to 7 hr after the injection and was applied to the Dowex 50W column. The volume of urine used corresponded to one-half of that excreted by one rat. The chromatographic conditions are described in MATERIALS AND METHODS.

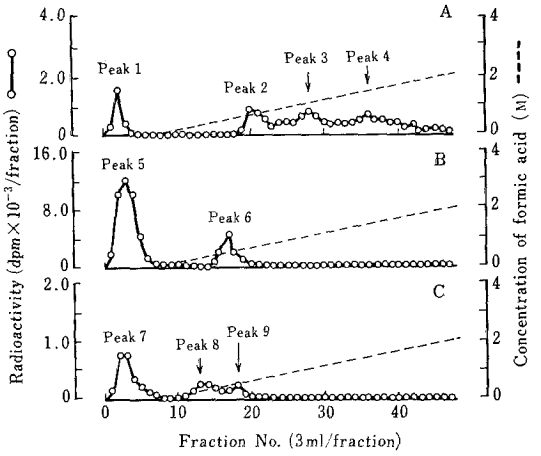

FIG. 3. Chromatographic Separation of Radioactive Compounds in Urine (Dowex 2).

Each fraction of the radioactive peaks separated on the Dowex 50W column described in Fig. 2 was evaporated to dryness. The peak B fraction was combined with the fractions of two minor peaks emerging in front of it. The residues was dissolved in 0.01 M HCl and was applied to the Dowex 2 column. The conditions are described in MATERIALS AND METHODS. A, B and C in this figure represent the elution patterns of the fractions of peaks A, B and C in Fig. 2, respectively.

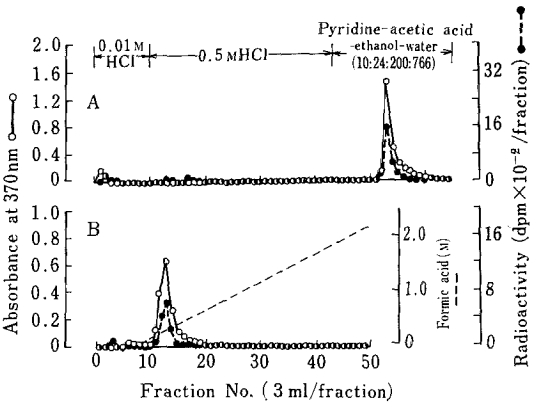

FIG. 4. Co-chromatography with Authentic APPL.

The peak 8 fraction pooled by repeated chromatography was evaporated to dryness under reduced pressure below 35°C and the residue was dissolved in 0.01 M HCl. To an aliquot of this solution containing the compound corresponding to 23.1 nmoles of APPL (as calculated from the radioactivity), 290 nmoles of authentic unlabelled APPL were added, and the solution was applied to the Dowex 50W column. The fraction of the radioactive peak obtained was then applied to the Dowex 2 column as described in MATERIALS AND METHODS.

A, elution pattern on the Dowex 50W column; B, elution pattern on the Dowex 2 column.

fraction was re-chromatographed on the Dowex 2 column after removal of 1-aminoproline by the hydrazone-formation with pyridoxal 5'-phosphate, unidentified radioactive compound(s) remained in the position of peak 5. The amount of the compound(s) was about one-third of that of 1-aminoproline in the peak 5 fraction.

The position of peaks C and 8 on the elution patterns in Figs. 2 and 3 corresponded to those of APPL. Therefore, the peak 8 fraction was pooled by repeated chromatography. An aliquot of the pooled sample was co-chromatographed with the authentic unlabelled hydrazone and the radioactivity coincided with the absorbance at 370 nm on both the chromatograms as shown in Fig. 4. The identity was further confirmed by radioautography of the two-dimensional paper chromatogram. The main radioactive spot absorbed ultraviolet light on the chromatogram. The *Rf* values of

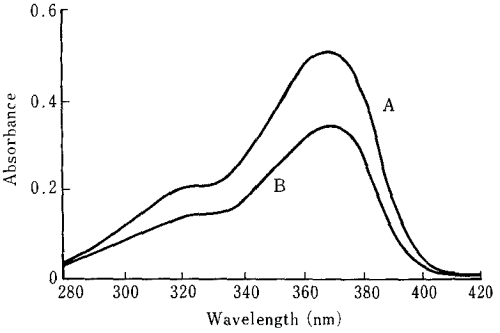

FIG. 5. Absorption Spectra of the Isolated Compound and Authentic APPL.

A, authentic APPL (44.6 nmoles in 2.5 ml of 0.1 M HCl). B, the compound further purified from the pooled sample of the peak 8 fraction by preparative paper chromatography with the solvent systems I and II (corresponding to 30.4 nmoles of APPL as calculated from the radioactivity; in 2.5 ml of 0.1 M HCl).

this compound with the solvent systems I and II were 0.72 and 0.64, respectively and these values were identical with those of authentic

APPL. Figure 5 shows the spectrum of the compound isolated from the pooled sample of the peak 8 fraction and that of authentic APPL. The above chromatographic and spectrophotometric evidence indicates the excretion of APPL in urine by the 1-aminoproline treatment. Though Biehl *et al.*[12] postulated the excretion of the hydrazone of isonicotinic acid hydrazide with pyridoxal in urine of patients receiving the hydrazide, Boone *et al.*[9] could not obtain evidence of the occurrence of this hydrazone in urine of the hydrazide-treated rats. McPhillips and Manthei[13] reported that the hydrazone of isonicotinic acid hydrazide with pyridoxal occurred at a detectable level in mouse plasma at 0.5 hr after the administration of the hydrazide, falling to a trace level at 1 hr after the administration, and that the hydrazone could not be detected in urine. In the present experiment, the level of APPL determined isotopically was $0.15\pm0.06\%$ of 1-aminoproline administered at 1 hr after the injection and was $0.07\pm0.04\%$ in the urine sample collected during the interval from 5 to 7 hr after the injection (means $\pm$ S.D., $n=3$). This is the first report on the urinary excretion of APPL. Evidence for the excretion of APPLP in urine could not be obtained in the present experiments. In our preliminary experiments (H. Tsuji, T. Ogawa and K. Sasaoka, unpublished), alkaline phosphatase (EC 3.1.3.1) obtained from calf intestinal mucosa (Type I, Sigma Chemical Co., Mo., U.S.A.) and a rat liver homogenate converted APPLP to APPL. These observations suggest that 1-aminoproline combines with pyridoxal 5′-phosphate and that APPLP formed is excreted as APPL after dephosphorylation in rat tissues.

REFERENCES

1) H. J. Klosterman, G. L. Lamouleux and J. L. Parsons, *Biochemistry*, **6**, 170 (1967).
2) K. Sasaoka, T. Ogawa, K. Moritoki and M. Kimoto, *Biochim. Biophys. Acta*, **428**, 396 (1976).
3) R. M. C. Dowson, D. C. Elliott, W. H. Elliott and K. M. Jones, "Data for Biochemical Research," 2nd ed, Oxford University Press, London, 1964, pp. 532.
4) F. Feigl, "Spot Test in Organic Analysis," 6th ed, Elsevier Publishing Co., Amsterdam, 1960, pp. 176.
5) H. Jeffay and J. Alvarez, *Anal. Chem.*, **33**, 612 (1961).
6) G. A. Bruno and J. E. Christian, *ibid.*, **33**, 1216 (1961).
7) S. Dimant, *Lancet*, **267**, 533 (1954).
8) C.E. Hall, J.B. Nash and O. Hall, *Am. J. Physiol.*, **190**, 327 (1957).
9) I. U. Boone, M. Magee and D. F. Turney, *J. Biol. Chem.*, **221**, 781 (1956).
10) B. Shane and E. E. Snell, *Biochem. Biophys. Res. Commun.*, **66**, 1294 (1975).
11) S. P. Coburn, J. D. Mahuren and S. I. Sallay, *J. Biol. Chem.*, **251**, 1646 (1976).
12) J. P. Biehl, R. W. Vilter, F. C. Beal and C. E. Kennedy, *Proc. Soc. Exp. Biol. Med.*, **85**, 389 (1954).
13) J. J. McPhillips and R. W. Manthei, *Fed. Proc.*, **18**, 421 (1959).