Metabolic Fate of Glycyl- ^{14}C -prolylhydroxyproline in Young Rats

Hideyuki TANAKA, Mikio ARAI and Hiroshi NISHI

*Department of Agricultural Chemistry, Faculty of Agriculture,
Utsunomiya University, Mine-350, Utsunomiya 321, Japan*

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To examine the origin of urinary hydroxyproline peptides, the metabolism of the radioactive tripeptide, glycyl- ^{14}C -prolylhydroxyproline, was investigated in normal young rats *in vivo*. The radioactive tripeptide was synthesized from glycine, L-(U- ^{14}C)proline and hydroxy-L-proline in our laboratory. The distributions of the radioactivity in body protein, lipid and soluble fractions were 23.7, 1.8 and 0.12% of the injected dose, respectively, 56 hr after the intraperitoneal injection of the ^{14}C -tripeptide. The excretions of the radioactivity into expired carbon dioxide and urine were 29.6 and 34.2% of the injected dose, respectively, and large proportions of both the ^{14}C excretions occurred during the first 12 hr.

The results suggest that not a small amount of the glycylprolylhydroxyproline peptide injected is hydrolyzed in tissues of animals and the free proline derived is used for protein synthesis and/or further degraded to expired carbon dioxide.

Hydroxyproline, the characteristic amino acid residue of collagen, is excreted normally in urine, mainly as peptides, and the urinary hydroxyproline level is taken as an indication of endogenous collagen metabolism. Pro-Hyp is usually a predominant hydroxyproline peptide in urine,^{1~3)} and this peptide linkage seems to originate from the sequence Gly-Pro-Hyp in the collagen molecule that escapes complete proteolysis.⁴⁾ The presence of Gly-Pro-Hyp in urine was demonstrated first by Meilman *et al.*,¹⁾ about 2 to 4% of the excreted hydroxyproline being present in this tripeptide. Kibrick and Milhorat,⁴⁾ who have measured the concentration of Gly-Pro-Hyp in urine and blood serum, have proposed that much of the urinary Pro-Hyp comes from serum Gly-Pro-Hyp.

In this experiment, to examine whether Gly-Pro-Hyp is degraded to Pro-Hyp or further to the constituent amino acids, the metabolic fate of Gly- ^{14}C -Pro-Hyp was investigated in young rats, by measuring the distribution of the radioactivity in the expired carbon dioxide, urine and body components such as protein, lipid and soluble fractions.

MATERIALS AND METHODS

L-(U- ^{14}C)Proline was purchased from The Radiochemical Centre, Amersham and L-prolyl-hydroxy-L-proline was from Sigma.

Synthesis of Gly- ^{14}C -Pro-Hyp.

Carbobenzoxy(Z)-Gly-O-succinimide (-ONSu). To a solution of Z-glycine (2 mmol) and N-hydroxysuccinimide (3 mmol) in 5 ml of dimethyl formamide was added dicyclohexylcarbodiimide (DCC, 3 mmol) dissolved in 3 ml of the same solvent at 0°C. The mixture was stirred for 2 hr at 0°C and overnight at room temperature. After 0.7 ml of glacial acetic acid was added, a white precipitate (DC urea) was filtered off, the filtrate was evaporated *in vacuo*, and the residue was taken up in dichloromethane. The solution was washed with 5% sodium bicarbonate and water. After drying over sodium sulfate, the filtered solution was evaporated *in vacuo*. The residue was dissolved in dichloromethane, then petroleum ether was added slowly. The obtained precipitate was recrystallized by the same solvent system. Beautiful silky crystals were obtained: yield 63%; mp 86~88°C.

Z-Gly-L-(U- ^{14}C)Pro. Z-Gly-ONSu (0.5 mmol) and triethylamine (0.2 mmol) were added to a solution of L-(U- ^{14}C)proline (0.5 mmol, 250 μCi) in a 10 ml mixture of water-dioxane (1:1, v/v). The mixture was stirred overnight at room temperature, and evaporated to dryness. The residue was redissolved in 4 ml of water, extracted twice with 5 ml of ethyl acetate, and then the ethyl acetate layer was washed twice with water. After drying over

FIG. 1. Chromatography of Synthesized Gly- ^{14}C -Pro-Hyp in an Amino Acid Analyzer.

Chromatography was done in an amino acid analyzer using a lithium citrate buffer system. Fractions of 2 ml were collected from 0 to 10 hr. The chromatogram during 0 to 5 hr is shown, since nothing was detected for ninhydrin and the radioactivity from 5 to 10 hr.

.....: ninhydrin at E_{570} ; —○—: ^{14}C radioactivity.

sodium sulfate, Z-glycyl-(U- ^{14}C)proline was crystallized from ethyl acetate–petroleum ether: yield 86%.

Z-Gly-L-(U- ^{14}C)Pro-L-Hyp benzyl ester (-OBzl). Z-Gly- ^{14}C -Pro (0.43 mmol) and hydroxy-L-prolyl-OBzl Tos OH (0.43 mmol) were dissolved in 5 ml of dichloromethane. To the mixture was added a solution of DCC (0.43 mmol) and triethylamine (0.07 ml) in 5 ml of dichloromethane. The reaction mixture was stirred for 4 hr at 0°C and left overnight at room temperature. To the mixture was added 0.01 ml of acetic acid, and the resulting DC urea was removed by filtration. The filtered solution was evaporated and the residue was taken up in ethyl acetate. The solution was then washed successively with 3N HCl, water, 4% sodium bicarbonate (twice), and water. After drying over sodium sulfate, ethyl acetate was evaporated, and Z-Gly- ^{14}C -Pro-Hyp-OBzl was precipitated from ethyl acetate–petroleum ether: yield 46%.

Gly-L-(U- ^{14}C)Pro-L-Hyp. Z-Gly- ^{14}C -Pro-Hyp-OBzl dissolved in a 5 ml mixture of ethanol–acetic acid–water (4:1:1, v/v/v) was hydrogenated for 10 hr in the presence of Pd black. After removal of the catalyst, the solution was evaporated to dryness and Gly- ^{14}C -Pro-Hyp was precipitated from water–ethanol–diethyl ether as a hygroscopic white powder: yield 51%. The purity of the product was tested in an amino acid analyzer (JEOL-6AHS) using a lithium citrate buffer system. The effluent from the column of the amino acid analyzer was divided into two currents, the first one being used to detect the ninhydrin peaks and the second allowing the radioactivity to be measured. The preparation of Gly- ^{14}C -Pro-Hyp contained a significant amount of non-radioactive Gly-Hyp and trace amount of ^{14}C -labeled proline, Pro-Hyp, Gly-Pro and unknown ones (prob-

ably the diketopiperazines of ^{14}C -Pro-Hyp and Gly- ^{14}C -Pro, Fig. 1). The identification of peptides in the chromatogram was conducted with the authentic peptides and/or by quantitative amino acid assay before and after hydrolysis. Approximately 90% of the ^{14}C was accounted for in Gly- ^{14}C -Pro-Hyp (83.7%) and ^{14}C -Pro-Hyp (5.8%). This product was used without further purification.

Animals. Three young male rats of the Wistar strain were preliminarily fed with a 20% casein diet until the mean body weight reached about 170 g. The diet contained 200 g of casein, 100 g of corn oil, 40 g of salt mixture, 20 g of cellulose powder, 10 g of a vitamin mixture and enough corn starch to make 1 kg, as described previously.⁵⁾ The animals were maintained in a temperature controlled room (about 23°C) with a 12 hr light–12 hr dark cycle.

Isotope experiment. The radioactive tripeptide preparation (2.1 μCi , 3.8 μmol per rat) was administered by intraperitoneal injection. Immediately after the injection, each animal was placed in a glass metabolism apparatus with water and the 20% casein diet. The expired carbon dioxide was continuously collected in a mixture of monoethanolamine and ethyleneglycol monomethyl ether (1:2, v/v)⁶⁾ and the radioactivity was determined as described previously.⁷⁾ Urine was collected in 5% acetic acid at 12 hr intervals and the radioactivity was measured.

The animals were killed by decapitation 56 hr after the injection of the isotope. The liver was removed, and shed blood was combined with the carcass. The carcass and liver were homogenized and analyzed for the radioactivity

in their major components such as protein, lipid and soluble fractions. A portion of the sample was extracted with 20 parts of chloroform-methanol (C-M, 2:1, v/v) by the method of Folch *et al.*,⁸⁾ in which the extract was washed by mixing with 0.2 volume of 0.0003N calcium chloride. Total lipid in the resulting lower phase was measured for its radioactivity in a toluene scintillation solution. The residue of the C-M extraction was then treated with 20 parts of 10% cold trichloroacetic acid (TCA), and the TCA extract was combined with the upper phase solution by Folch's washing method to obtain the soluble fraction of the sample. The TCA insoluble residue was dissolved in 2N NaOH as the protein fraction, and the radioactivities of protein and soluble fractions were determined in the NT scintillation solution.⁹⁾ All the measurements of radioactivity were done with a liquid scintillation spectrometer (Packard, 3255). The efficiency of the counting system was estimated for each sample using an external standard.

Analysis of urine. The urine of three rats was pooled, lyophilized, and filled up to 50 ml with water. Total nitrogen was determined by the Kjeldahl method and hydroxyproline before and after acid hydrolysis by the method of Firschein and Shill.¹⁰⁾ Then the urine sample was divided into two aliquots. One was chromatographed directly on a Dowex 50 $\times$ 8 (20~50 mesh, H⁺, 2 $\times$ 20 cm) column, eluting with 100 ml of 0.1N acetic acid and subsequently with 200 ml of 3N ammonia. To the other aliquot of the sample was added concentrated ammonium hydroxide solution to bring it to approximately 0.2N, and it was heated for 16 hr at 50°C in a sealed tube. The solution was then evaporated to dryness, redissolved in water, and adjusted to pH 2 to 3 with N HCl. The sample was put on the column of Dowex 50 $\times$ 8 (H⁺, 2 $\times$ 20 cm) and eluted with 0.1N acetic acid (Fraction A)

and then 3N ammonia (Fraction B). Pro-Hyp diketopiperazine produced by alkaline treatment was contained in Fraction A, and amino acids and peptides in Fraction B. The radioactivities of Fractions A and B were determined. A sample of Fraction B was analyzed in the amino acid analyzer and the radioactivity was measured as described above.

RESULTS

Body weight, liver weight and urinary excretion of nitrogen and hydroxyproline are shown in Table I. The body weight gain during the isotope experiment was 3 g. The level of the daily urinary hydroxyproline excretion was about 1.4 mg. The free hydroxyproline was only 5% of the total excretion and thus most of the urinary hydroxyproline was present in peptide form. Hydroxyproline injected as Gly-

TABLE I. BODY WEIGHT, LIVER WEIGHT, AND URINARY EXCRETION OF NITROGEN AND HYDROXYPROLINE IN RATS

The values are mean $\pm$ standard errors for three rats. Urine was analyzed as a pooled sample of three rats.

Initial body weight (g)	170 $\pm$ 5.8
Final body weight (g)	173 $\pm$ 5.5
Liver weight (g)	8.2 $\pm$ 0.2
Urinary nitrogen (mg/day)	228
Urinary hydroxyproline (mg/day)	
Total	1.39
Free	0.07

FIG. 2. Expired Carbon Dioxide Production after Intraperitoneal Injection of Gly-¹⁴C-Pro-Hyp in Intact Rats.

Each value represents the mean of three rats. The vertical bars indicate standard errors.

FIG. 3. Urinary Excretion of ^{14}C after Intraperitoneal Injection of Gly- ^{14}C -Pro-Hyp in Intact Rats.

The value represents the mean of three rats. The vertical bars indicate standard errors.

Pro-Hyp was $3.8\ \mu\text{mol}$ (about $0.5\ \text{mg}$).

After intraperitoneal injection of the radioactive tripeptide, overall oxidative degradation occurred rapidly (Fig. 2). A sharp peak in the expired $^{14}\text{CO}_2$ production was observed at 1 to 2 hr and the excretion fell off rapidly thereafter. About 60% of the total $^{14}\text{CO}_2$ excretion was recovered during the first 3 hr.

The urinary ^{14}C excretion after the injection of the radioactive tripeptide was maximal during the first 12 hr, about 93% of the total urinary ^{14}C excretion being recovered, and it declined greatly thereafter (Fig. 3).

The recoveries of the radioactivity in the expired carbon dioxide and urine during 56 hr were approximately 30 and 34% of the injected dose, respectively (Table II). The distribution of the radioactivity in the protein, lipid and soluble fractions of carcass and liver 56 hr after the injection of the isotope is also shown in Table II. The incorporations of ^{14}C from Gly- ^{14}C -Pro-Hyp into protein of carcass and liver were about 22 and 1.3% of the injected dose, respectively. The ^{14}C incorporated into tissue protein seemed to be derived from free ^{14}C -proline produced by hydrolysis of the injected tripeptide. The conversion of ^{14}C into the lipid in the carcass plus liver was 1.8% of the

TABLE II. DISTRIBUTION OF THE RADIOACTIVITY IN EXPIRED CARBON DIOXIDE, URINE, BODY PROTEIN, BODY LIPID, BODY SOLUBLE FRACTION, AND WHOLE PLASMA 56 HR AFTER INJECTION OF GLY- ^{14}C -PRO-HYP IN INTACT RATS

The values are expressed as % of the injected dose, and are mean $\pm$ standard errors of three rats.

Expired carbon dioxide	29.6 $\pm$ 0.5
Urine	34.2 $\pm$ 1.4
Protein	
Carcass	22.4 $\pm$ 1.1
Liver	1.28 $\pm$ 0.04
Lipid	
Carcass	1.73 $\pm$ 0.41
Liver	0.09 $\pm$ 0.01
Soluble fraction	
Carcass	0.11 $\pm$ 0.01
Liver	0.006 $\pm$ 0.0003
Plasma	0.53 $\pm$ 0.003

FIG. 4. Elution Pattern of Urinary ^{14}C in Fraction B in Amino Acid Analyzer.

Chromatographic analysis was done under the same conditions as in Fig. 1.

injected dose. The recoveries of ^{14}C in the soluble fractions of carcass and liver were 0.1 and 0.006% of the injected dose, respectively. About 0.5% of the injected dose was present in the whole plasma at 56 hr after the injection. The whole plasma volume was calculated by the method of Shiraki.¹¹ The formula used for calculating plasma volume was as follows:

$$\text{Plasma volume} = 3.00 \\ \times \text{body weight (100 g)} + 2.76$$

The total recovery percentage of the radioactivity was about 90% (the mean of three rats).

To characterize the urinary ^{14}C excreted after the injection of the isotope, we measured the distribution of ^{14}C in urinary metabolites as described in MATERIALS AND METHODS. The ^{14}C eluted by 0.1N acetic acid on the Dowex 50 $\times$ 8 column from the original urine sample was about 11% of urinary ^{14}C . The ^{14}C eluted by the same solvent from another urine sample treated with ammonia was about 27%. Kibrick *et al.*³⁾ have reported that the dipeptide Pro-Hyp is cyclized and quantitatively converted to its diketopiperazine by the treatment with 0.2N ammonia for 7 hr at 50°C. A similar result was obtained in our preliminarily experiment using authentic Pro-Hyp from Sigma. Since diketopiperazine cannot be adsorbed on the Dowex 50 $\times$ 8 column, the major part of the increment of ^{14}C eluted by acetic acid (Fraction A) may be due to cyclization of Pro-Hyp. Hence, about 16% of urinary ^{14}C may be attributed to ^{14}C -Pro-Hyp derived from the Gly- ^{14}C -Pro-Hyp injected. The 3N ammonia eluate (Fraction B) for the urine sample treated with 0.2N ammonia was evaporated to dryness and a suitable sample was analyzed in the amino acid analyzer for the radioactivity (Fig. 4). A large peak of Gly-Pro-Hyp and several minor peaks were found. About 40% of the urinary ^{14}C was recovered in the Gly-Pro-Hyp fraction, and 6% in the free proline fraction.

DISCUSSION

It has been generally accepted that urinary hydroxyproline peptides are end-products of collagen degradation. Collagen polypeptides contain a repeating sequence Gly-X-Y, where X or Y is any amino acid residue, and about 23% of hydroxyproline in collagen is present in a triplet Gly-Pro-Hyp.¹²⁾ Meilman *et al.*¹⁾ have demonstrated the excretion of Pro-Hyp (65% of total hydroxyproline excreted) and Gly-Pro-Hyp (2~4%) in human urine. Kibrick *et al.*³⁾ have reported a method for the quantitative measurement of Pro-Hyp by a isotope dilution method, about 40% of

total hydroxyproline excreted being present in this dipeptide. Szymanowicz *et al.*²⁾ have also reported that of hydroxyproline peptides more than 78 varieties from alpha chains of collagen were found in human urine, and 44% was Pro-Hyp. However, they could not isolate the tripeptide Gly-Pro-Hyp in human urine. Because the concentration of Gly-Pro-Hyp in serum is much higher than the minute amount of Pro-Hyp, the major hydroxyproline-containing peptide in urine is Pro-Hyp, Gly-Pro-Hyp is minor, and an enzyme obtained from swine kidney by Dehm and Nordwig¹³⁾ hydrolyzes Gly-Pro-Hyp to glycine and Pro-Hyp, Kibrick and Milhorat⁴⁾ have suggested that much of urinary Pro-Hyp comes from Gly-Pro-Hyp in blood serum. However, the quantitative direct relationship of urinary hydroxyproline peptide excretion to collagen catabolism has not been sufficiently examined. Only Weiss and Klein¹⁴⁾ have reported that, when tritiated Pro-Hyp is administered subcutaneously to rats, more than 80% of the dipeptide injected is excreted in urine, suggesting that this peptide linkage is not hydrolyzed to a significant extent *in vivo*. Noguchi *et al.*¹⁵⁾ have recently demonstrated that when ^{14}C -labeled urinary acid soluble peptide (ASP) is injected into rats, a significant amount of the radioactivity was recovered as urinary ASP within 24 hr, suggesting the presence of some peptides resistant to the *in vivo* proteolytic system.

In our investigation, we examined the metabolic fate of the radioactive tripeptide, Gly- ^{14}C -Pro-Hyp, in normal young rats *in vivo*. After the administration of the radioactive tripeptide, the recovery percentages of the ^{14}C in the expired carbon dioxide, body lipid and body protein were 30, 2 and 24% of the injected dose, respectively. The results suggest that not a small amount of Gly-Pro-Hyp linkage is hydrolyzed *in vivo* and that the free proline derived from its tripeptide is used for protein synthesis or further catabolized to expired CO_2 . Urinary excretion of the radioactivity was 35% of the injected

dose, in which more than 40% of the radioactivity in urine was present in the Gly-Pro-Hyp fraction, 16% in the Pro-Hyp fraction and 6% in the free proline fraction.

In our previous investigation,⁷⁾ when L-(U-¹⁴C)proline was administered to young rats fed a 30% protein diet, the recovery percentages of the radioactivity in the expired carbon dioxide, urine and body protein 12 hr after the injection were 30, 2 and 43% of the injected dose, respectively. Thus the urinary ¹⁴C excretion from free ¹⁴C-proline was much lower than that from Gly-¹⁴C-Pro-Hyp injected. The amount of the radioactive tripeptide injected was 2.1 μ mol per 100 g body weight in this experiment. This amount might be too large considering that the concentration of this peptide per 100 ml human serum was 0.15 μ mol, calculated from the data of Kibrick and Milhorat,⁴⁾ though the pool size might not be the same between rats and humans. The temporary high concentration of this tripeptide in the serum induced by such an injection might be too much to be hydrolyzed completely.

Because the ¹⁴C recovered in expired CO₂ plus body component is more than 55% of the injected dose even under such conditions, it seems to be quite all right to consider that this tripeptide can be hydrolyzed by tissue protease. Since Dehm and Nordwig^{13,16)} have demonstrated two proline-specific peptidases, X-prolyl-aminopeptidase and carboxypeptidase P, from swine kidney, the hydrolysis of Gly-Pro-Hyp in the body of rat may be attributed to such a proline-specific peptidase.

On the other hand, we does not exclude the

possibility that, in the course of collagen degradation *in vivo*, the Pro-Hyp linkage in collagen molecule is excreted into the urine not *via* Gly-Pro-Hyp, and the tripeptide injected is easily cleaved due to the presence of the glycine moiety.

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