Metabolic Fate of β-Aspartyl-14C-glycine in Normal Young Rats

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The metabolic fate of α- and β-L-aspartyl-[U-14C]glycine was investigated in normal young rats in vivo and in vitro. The radioactive dipeptides were synthesized from L-aspartic acid and [U-14C]glycine in our laboratory. When labeled β-aspartylglycine was given intraperitoneally, about 66% of the dose was excreted in the urine and 8% was recovered in the expired carbon dioxide over a 24-hr period. More than 90% of the urinary radioactivity was present in the β-aspartylglycine fraction of the urine. When labeled α-aspartylglycine was given, 3% and 22% of the dose were recovered, respectively, in the urine and expired carbon dioxide. In slice experiments with kidney, liver and small intestine from normal rats, α-aspartylglycine was rapidly and almost completely hydrolyzed, and a large amount of free 14C-glycine was released. In contrast, β-aspartylglycine was hardly hydrolyzed to the corresponding amino acids in liver and small intestine, and only slightly in kidney. These results suggest that in normal young rats most β-aspartylglycine, which may originate from endogenous tissue proteins, is hardly hydrolyzed and rapidly excreted into the urine.

Significant but small amounts of free and peptide-bound amino acids are normally excreted in urine.1-4 Since Kakimoto and Armstrong5 have demonstrated the excretion of β-asparthylhistidine in human urine, several workers6-8 have reported the isolation and identification of a number of β-aspartyl peptides from the urine. Buchanan et al.9 have demonstrated that the most abundant among these peptides is β-aspartylglycine, and Pisano et al.10 have reported that polymerized fibrin and Achilles tendon collagen contain the β-aspartyl linkage. It is also known that urinary hydroxyproline peptides are the endproducts of collagen degradation, and that prolylhydroxyproline accounts for about 60% of these hydroxyproline petides excreted in human urine.9-11 Thus, β-aspartyl peptides and hydroxyproline peptides occupy a large proportion of the urinary oligopeptides. However, the metabolism of these urinary peptides in the bodies of animals has not been sufficiently clarified. In our previous paper,12 we investigated the metabolic fate of γ-carboxyl-14C-prolylhydroxyproline in young rats to examine the origin of prolylhydroxyproline in the urine. Subsequently, in this paper we investigated the metabolic fate of synthetic α- and β-L-aspartyl-14C-glycine in vivo, and the hydrolysis of both labeled dipeptides in tissue slices in vitro from young normal rats.

Materials and Methods

Synthesis of α- and β-L-aspartyl-[U-14C]-glycine. The α- and β-L-aspartyl-14C-glycine were prepared from carboxbenzoxycarbonyl (Cbz)-L-aspartic acid and [U-14C]glycine (The Radiochemical Centre, Amersham) by the method of Buchanan et al.13,14 To Cbz-L-aspartic acid (37 mmol) was added acetic acid anhydride (210 mmol), and the solution was stirred for 5 hr and left overnight at room temperature. After evaporation in vacuo, the residue was dissolved in chloroform, then petroleum ether and diethyl ether were added slowly. The precipitate, Cbz-L-aspartic acid anhydride, was recrystallized by the same solvent system: yield 93%; mp. 109-115°C.

To Cbz-L-aspartic acid anhydride (0.12 mmol) were added [U-14C] glycine (0.068 mmol, specific radioactivity: 2.97 mCl/mmol) and triethylamine (0.04 ml). The mixture was vigorously stirred for 30 min at room temperature, and evaporated to dryness. To Cbz-L-aspartyl-14C-glycine obtained was added a solution of 25% HBr in acetic acid (0.8 ml); this was left for 1 hr then evaporated to dryness, and the residue was washed with diethyl ether for four times. After diethyl ether was removed, the residue was dissolved in water and put on a column of Dowex 1 × 2 (pH 100-200 mesh, 2 × 3 cm), eluting with 15 ml of 0.1 N acetic acid and then with 100 ml of 1 N acetic acid. The fractions in which radioactivity was detected were pooled and evaporated. After this was dissolved in water, the solution was put on a column of Dowex 1 × 2 (100-200 mesh, formate form, 1 × 140 cm), eluting 650 ml of 0.1 N formic acid at 0.6 ml per min for the separation of the α- and β-14C-dipeptides (Fig. 1). The peptides were identified by co-chromatography with authentic peptides and by assay of amino acids before and after hydrolysis. The ninhydrin color of the β-dipeptide in a paper chromatogram was distinctly blue, but the α-dipeptide gave purple.14 The recovery of the radioactivity was 20.4% for the α-dipeptide and 19.7% for the β-dipeptide. The purity of each the preparation was tested in an amino acid analyzer (JEOL-6AHS) using a lithium citrate buffer system. As for α-L-aspartyl-14C-glycine, about 91% of the radioactivity was recovered in the α-dipeptide fraction and 9% in free glycine fraction, while for β-L-aspartyl-14C-glycine about 96% was recovered in the β-dipeptide fraction and 4% in free glycine fraction.

In vivo experiment. Young male rats of the Wistar strain were fed with a 20% casein diet for 7 days until the mean body weight reached about 160 g. The diet contained 200 g of casein, 100 g of corn oil, 40 g of

![Fig. 1. Separation of Synthetic α-Aspartyl-[U-14C]glycine and β-Aspartyl-[U-14C]glycine on Dowex 1 × 2 Column Chromatography.](image-url)
salt mixture, 20g of cellulose powder, 10g of vitamin mixture, and enough corn starch to complete 1 kg, as described previously. The animals were maintained in a temperature-controlled room (about 23°C) with a 12 hr light–12 hr dark cycle. For the labeled \( \alpha \)-dipeptide 4 rats were used, and 8 rats for the \( \beta \)-dipeptide. The \( \alpha \)-l-aspartyl-\( ^{14} \)C-glutamic acid (3.06 µCi/0.06 µmol in 0.4 ml saline) or \( \beta \)-l-aspartyl-\( ^{14} \)C-glutamic acid (1.48 µCi/4.38 µmol in 0.2 ml saline) was given by intraperitoneal injection. Immediately after injection, each animal was placed in a glass metabolism apparatus with water and the 20% casein diet. The expired carbon dioxide was collected in a mixture of monoethanolamine and ethylene glycol monomethyl ether (1:2, v/v) for 24 hr, and the radioactivity was measured by the method of Jeffay and Alvarez. Urine was also collected in 5% acetic acid, and the radioactivity was measured in an NT scintillation solution. 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 by an external standard ratio method.

Urine of the rats in each group was pooled, and the distribution of the radioactivity in urinary compounds was examined. To a sample of the urine were added non-labeled aspartic acid, glycine, and \( \alpha \) - and \( \beta \) - aspartylglycine as carriers. After acidification with 2N HCl, the sample was chromatographed directly on Dowex 50 x 8 (20–50 mesh, H+), 2 x 10 cm) by eluting with 100 ml of 0.1 N acetic acid then with 200 ml of 3 N ammonia. After removing the 3 N ammonia, the amino acids and peptides obtained were redissolved in water, and put on a column of Dowex 1 x 2 (100–200 mesh, formate form, 1 x 140 cm), eluting with 0.1 N formic acid as described above. The radioactivity of each fraction was measured and the recovery percentage of the radioactivity was calculated.

**In slice experiment.** Four young male Wistar rats weighing about 200 g were fed on the 20% casein diet for 4 to 5 days before killing. After killing by decapitation, liver, kidney, and small intestine were quickly removed, rinsed with Krebs-Ringer phosphate solution (pH 7.4), and sliced by a VH-Slicer (Hottarika, Tokyo). The kidney slices were prepared from fresh cortices, and the intestine slices were obtained from lengths of jejunum starting 10 cm below the duodenojejunal junction. Tissue slices weighing 200 mg were placed in Warburg vessels containing 2 ml of the Krebs-Ringer phosphate solution (pH 7.4). Each slice was incubated for 20 min at 37°C with shaking (83 stroke/min). The reaction was stopped by addition of 0.5 ml of 20% trichloroacetic acid. The \( ^{14} \)CO\(_2\) generated during the incubation was absorbed in 0.5 ml of 1.5 N KOH placed in the center well of the vessel, and its radioactivity was measured in the NT scintillation solution. The pooled sample of the media and tissue slices of paired vessels for each rat was centrifuged, and the deproteinized supernatant solution was extracted three times with diethyl ether to remove trichloroacetic acid. The aqueous solution was then evaporated, and after it was redissolved in water the sample was chromatographed on a Dowex 50 x 8 (200–400 mesh, H\(^+\) form, 2 x 2 cm) column. The column was washed with 0.1 N acetic acid, and then amino acids and peptides were eluted with 3 N ammonia. After removal of ammonia, the amino acid and peptide fraction was analyzed in an amino acid analyzer (JEOL, 6AH) using a lithium citrate buffer system. The effluent of the column was divided into two currents, the first one being used to detect the amino acids by the ninhydrin reaction and the second allowing the radioactivity to be measured.

**Results**

**In vivo experiment.**

During the in vivo isotope experiment for 24 hr, the body weight gain of rats fed a 20% casein diet was about 5.4 g. The amounts of labeled \( \alpha \) - and \( \beta \)-l-aspartylglycine injected were 0.646 and 0.313 µmol per 100 g body weight, respectively, in this experiment. Since the tissue concentration of the dipeptides is unknown, it is obscure whether a drastic change of the pool size of the dipeptides is induced by the isotopes injected.

The patterns of the expired \( ^{14} \)CO\(_2\) during the 24-hr period after injecting intraperitoneally \( \alpha \) - or \( \beta \)-l-aspartyl-\( ^{14} \)C-glycine into young normal rats are shown in Fig. 2. In both the dipeptides the overall oxidative degradation occurred rapidly at an early stage of the experiment and thereafter the output fell off, more than 60% of the total \( ^{14} \)CO\(_2\) output being recovered for in the first 3 hr after the injection. The expired \( ^{14} \)CO\(_2\) output from the labeled \( \beta \)-dipeptide was much lower than that from the labeled \( \alpha \)-dipeptide. When the labeled \( \alpha \)-dipeptide was injected, about 22% of the injected dose was oxidatively degraded to expired carbon dioxide during the 24-hr period, but only 8% of the dose with the labeled \( \beta \)-dipeptide (Table 1). The urinary \( ^{14} \)C excretion from the labeled \( \beta \)-dipeptide (about 66% of the dose) was markedly greater than that from the \( \alpha \)-dipeptide. During the 24-hr period, about 74% of the radioactivity was eliminated in both the expired carbon dioxide and the urine and 26% of the dose was retained in the carcass of the animal when the labeled \( \beta \)-dipeptide was given, while 26% of the radioactivity was eliminated and 74% was retained in the carcass with the \( \alpha \)-dipeptide.

To characterize the radioactive compounds excreted in the urine after injecting \( \alpha \) - or \( \beta \)-aspartylglycine, we examined the distribution of the label in their metabolites as described in Materials and Methods. When the labeled \( \alpha \)-dipeptide was given intraperitoneally, a small quantity of the radioactivity was excreted into urine during 24 hr (3.2% of the dose, Table 1). Thus, the elution pattern of the urinary \( ^{14} \)C from the labeled \( \alpha \)-dipeptide was obscure on the chromatogram of the Dowex 1 x 2 column, though several minor peaks of radioactivity were recognized (Fig. 3A). The finding that a small amount of the label was recognized in the \( \beta \)-aspartylglycine fraction could probably be explained by the spontaneous isomerization of \( \alpha \)-
aspartylglycine to the β-isomer, as has been pointed out by Buchanan et al. On the other hand, the elution pattern for the labeled β-dipeptide was more distinct, and a large amount of the radioactivity was found in the fraction of β-aspartylglycine, about 97% of the total urinary radioactivity being recovered in this fraction (Fig. 3B).

In vitro experiment by tissue slices
The hydrolysis of radioactive α- and β-aspartylglycine in the slices of kidney, liver, and small intestine of rats fed the 20% casein diet is shown in Table II. The measurement of free 14C-glycine liberated from the dipeptides in the incubation medium plus the tissues was done by an amino acid analyzer (Fig. 4). Although the 14CO2 formation from the α-dipeptides during a 60-min period of the incubation was markedly depressed in each of the three tissues, the value for kidney slices was higher than that for liver or intestine. When the labeled α-dipeptide was incubated in the slices of liver and intestine, most of the radioactivity was recovered in the free glycine fraction but only a small amount of the label was in the substrate dipeptide fraction. In the kidney slices, 67% of the radioactivity was found in the free glycine fraction, and 24% was in the substrate

Table II. Cleavage of α-Aspartyl-[U-14C]glycine and β-Aspartyl-[U-14C]glycine in Tissue Slices of Kidney, Liver, and Intestine in Normal Young Rats
The values are mean ± standard errors for four rats. The slices (about 200 mg) were incubated in 2 ml of Krebs-Ringer phosphate solution (pH 7.4) at 37°C for 60 min.

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<thead>
<tr>
<th>Substrates</th>
<th>Recovery of Radioactivity in:</th>
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<tbody>
<tr>
<td></td>
<td>CO2 (%)</td>
</tr>
<tr>
<td>α-Asp-[14C]Gly</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>Liver</td>
<td>0.6 ± 0.02</td>
</tr>
<tr>
<td>Intestine</td>
<td>0.2 ± 0.01</td>
</tr>
<tr>
<td>β-Asp-[14C]Gly</td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Liver</td>
<td>0.1 ± 0.03</td>
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<tr>
<td>Intestine</td>
<td>0.1 ± 0.01</td>
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Discussion
Many kinds of oligopeptides are excreted in human urine, which are probably associated with the resistance of these peptides to hydrolysis by peptidases and with poor renal reabsorption during passage of the original urine down the proximal convoluted tubules. To examine the origin of these urinary oligopeptides, we have investigated the metabolic fate of synthetic α- and β-l-aspartyl-[U-14C] glycine in vivo and the hydrolysis of these labeled dipeptides in the tissue slices of kidney, liver, and small intestine in vitro. When the labeled β-dipeptide was given intraperitoneally, about 66% of the dose was excreted into the urine, almost all of the label excreted being recovered as the β-aspartylglycine. But about 8% of the radioactivity was oxidatively degraded to the expired carbon dioxide, and a considerable amount of the label, about 26% of the dose, was retained in the carcass, most of which might be used for tissue protein synthesis. Thus, these results suggest that a small but significant amount of β-aspartylglycine is hydrolyzed and that the free glycine liberated may be used for body protein synthesis or further catabolized to expired
carbon dioxide. On the other hand, when the radioactive \( \alpha \)-dipeptide was given, only 3% of the radioactivity was excreted in the urine, while about 74% of the label was retained in the carcass and 22% in the expired carbon dioxide. We reported previously that, when [U-\(^{14}\)C]glycine given intraperitoneally to rats fed on an adequate egg protein diet, the recovery percentages of the radioactivity in the expired carbon dioxide, urine and body protein were 24, 3, and 55% of the dose, respectively, 12 hr after the injection.\(^{1-3}\) Thus, the urinary \(^{14}\)C excretion from the labeled \( \alpha \)-dipeptide is similar to that from labeled free glycine, but the label excreted in the urine from the \( \beta \)-dipeptide is markedly higher than that from \( \alpha \)-dipeptide, suggesting that the \( \beta \)-aspartyl linkage is resistant to hydrolysis in the tissues of rats. These results obtained in normal young rats in this study are consistent with those obtained in human subjects by Buchanan et al.,\(^{13}\) who have reported that, when \( \beta \)-aspartyl-[1-\(^{14}\)C]glycine was given intravenously to human subjects, more than 70% of the dose was rapidly excreted in the urine in the same chemical form but only 2.3% of the dose in the expired carbon dioxide and that \( \beta \)-aspartylglycine was not an artifact brought about during the isolation procedures.

Weiss and Klein\(^{18}\) found that, tritiated prolylhydroxyproline was given subcutaneously to rats, more than 80% of the radioactivity from the dipeptide was excreted in the urine, suggesting that this peptide linkage was hardly hydrolyzed \textit{in vivo}. In our previous paper,\(^{12}\) however, we demonstrated that only about 30% of the label was excreted in the urine when glycyl-\(^{14}\)C-prolylhydroxyproline was injected to rats to prevent the conversion of prolylhydroxyproline to diketopiperazine. Therefore, it is suspectable that the tritiated dipeptide used by them has not been the cyclic one, and rather it seems to us that they administered their diketopiperazine which is not degraded in the tissues and is rapidly excreted in the urine. As compared to the labeled hydroxyproline tripeptide, a large amount of the \( \beta \)-aspartylglycine is more rapidly excreted in the urine. Moreover, Pisano et al.\(^{17}\) demonstrated that feeding gelatin to humans caused a marked increase in the urinary excretion of \( \beta \)-aspartylglycine and that this peptide could be isolated from enzymic digests of collagen. These results suggest that \( \beta \)-aspartylglycine in the urine may be a good index of collagen catabolism as well as hydroxyproline peptides. Noguchi et al.\(^{19}\) reported that when the \(^{14}\)C-labeled urinary acid soluble peptide (ASP) was given to rats, about 50% of the dose was recovered as urinary ASP within 24 hr, and that urinary excretion of ASP form amino acids could reflect the rate of whole body protein degradation.\(^{20}\) Thus, the study on the metabolic behavior of radioactive urinary oligopeptides in the body of animal or human has been done by several workers,\(^{12,13,18,19}\) and the results suggest that these peptides are of endogenous origin and are less sensitive to hydrolysis by tissue peptidase(s).

In the tissue slices experiment, the \(^{14}\)CO\(_2\) formation from both the aspartyl dipeptides was very low for a 60-min incubation, but the liberation of labeled free glycine from the \( \alpha \)-dipeptide in the medium and the tissue soluble fractions was very high, showing that the linkage of \( \alpha \)-aspartylglycine is promptly hydrolyzed by peptidase(s) of these tissues. On the other hand, the hydrolyzation activity against \( \beta \)-aspartylglycine was very low in the three tissues. Among these tissues, the radioactivity found in the glycine fraction was somewhat higher in the kidney slices than in the liver and small intestine. The subcellular distribution of dipeptidase activity in the small intestinal mucosa has indicated that the bulk of the hydrolyzation activity resides in the cytosol fraction.\(^{1,2,21}\) Some may assume from this evidence that the lower activity in the hydrolysis of \( \beta \)-aspartylglycine as compared with \( \alpha \)-aspartylglycine is attributed to the failure of transport of the \( \beta \)-dipeptide across the plasma membrane, so that the dipeptidase which is highly active in the cytosol can not attack the substrate dipeptide. This assumption, however, would be excluded, since we obtained experimental evidence of very slow hydrolyzation activity against the \( \beta \)-dipeptide even in the cytosol fraction of several tissues, which will be published elsewhere.

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\textbf{References}


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