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Determination of chiral catabolites from *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]glutathione, a proposed metabolite of L-histidine, by capillary electrophoresis

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Abstract

A new method for simultaneous determination of two diastereomers in each of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteine (**I**) and *N*-{*S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteinyl}glycine (**II**) was developed by electrophoresis using a neutral coated capillary with a separation buffer, pH 6.00, containing 80 m*M* hydroxypropyl-β-cyclodextrin at a field strength of 500 V cm^{-1} at 20°C. This method was applied to establishment of a catabolic pathway from *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]glutathione (**III**) to compound **I**. Incubation of either of compound **II** diastereomers as an enzyme substrate with rat kidney homogenate in a phosphate buffer, pH 7.4, resulted in a formation of compound **I** only having correspondent configurations on asymmetric carbon atoms of its molecule with those of the substrate, i.e. no occurrence of isomerization in the catabolism. Additionally, little difference in action as the substrate between two diastereomers of compound **II** was found. When an equimolar mixture of two diastereomers of compound **III** was allowed to react with the homogenate in the presence of glycylglycine, two diastereomers of compound **II** were formed in the same yield with each other and then these were catabolized gradually to both isomers of compound **I**. These results suggest that compound **II** is a metabolic intermediate for the formation of compound **I** from compound **III**, and that little variation in reactivities of two diastereomers of compound **III** as well as compound **II** with enzymes is given by the difference in stereoisomerism of asymmetric carbon atoms on their molecules.

Keywords: Diastereomer separation; Carboxy-1-(1H-imadazol-4-yl)ethyl derivatives; Glutathione conjugates; Imidazoles

1. Introduction

In the first step of L-histidine catabolism, urocanic acid is formed by the action of histidine ammonia-lyase (EC 4.3.1.3), which is found mostly in only liver and epidermis [1]. In liver, urocanic acid is metabolized by urocanate hydratase (EC 4.2.1.49) to 4,5-dihydro-4-oxo-5-imidazolepropanoic acid [2], and this compound is further catabolized to culminate in complete degradation to CO_2 and water. In the epidermis, however, histidine metabolism terminates with the formation of urocanic acid because urocanate hydratase is absent from the skin [3,4]. On the other hand, four novel imidazole compounds having sulphur-containing side chains were isolated from normal human urine and these were determined to be *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteine (**I**) [5,6], *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-2-mercaptoacetic acid [7], *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptolactic acid [8] and *N*-acetyl-*S*-[2-carboxy-1-(1*H*-imidazol-4-yl) ethyl]-L-cysteine [9]. The mercaptoacetate adduct and the mercaptolactate adduct were demonstrated to be products of enzymatic degradation of compound **I**

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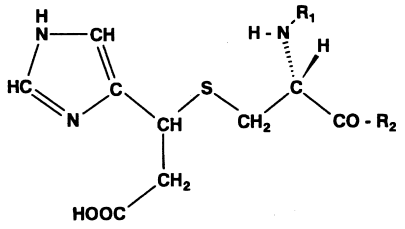
[7,8,10] through a formation of a metabolic intermediate *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-mercaptopyruvic acid [11]. The *N*-acetyl-L-cysteine adduct also was found to be an *N*-acetylated metabolite of compound **I** previously [9]. A precursor of compound **I** has been proposed to be *N*-{*S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteinyl} glycine (**II**) [10] which is formed possibly from *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]gluthathione (**III**) (*N* - {*S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-*N*-L-γ-glutamyl-L-cysteinyl}glycine) [7,10], an adduct of urocanic acid and glutathione. These findings suggest that histidine is metabolized in part by an alternative pathway initiated by the adduction of natural thiol compounds such as glutathione and L-cysteine to urocanic acid. Structural formulae of compounds **I** and **II** are shown in Fig. 1. These compounds each has two asymmetric carbon atoms; an α-carbon atom on L-cysteine moiety and a β-carbon atom on the skeletal imidazolylpropanoate part of the molecule. The α-carbon atom has an *R*-configuration because of L-cysteine as the origin. On the other hand, the β-carbon atom bears *R*- or *S*-configuration and this means the existence of two diastereomers (*R*,*R*- or *R*,*S*-) of compound **I** and **II** in each [9]. A chemical structure of compound **III** is also shown in Fig. 1. This has three asymmetric carbon atoms basing on α-carbon atoms of the L(*S*)-γ-glutamyl and the L(*R*)-cysteinyl residues, and a β-carbon atom on the imidazolylpropanoate part of the molecule. In the same sense employed for compound **I**, two diastereomers of compound **III** are existent. Recently [9], it was found that an *N*-acetylating enzyme to form the *N*-acetyl-L-cysteine adduct from compound **I** recognized stereoisomerism of asymmetric carbon atoms on the molecule of compound **I**. However, it had not been elucidated whether enzymes catalysing the formation of compound **I** from compound **III**, possibly via compound **II**, recognized the stereoisomerism of their asymmetric carbon atoms, as no method for chiral separation of their diastereomers except for compound **I** had been developed. Determination of each diastereomer of compound **I** was performed only by using an amino-acid analyser [9], although the method was time-consuming and required a volume of the sample for the analysis. A rapid method for simultaneous determination of every diastereomer of compound **I** and **II** with no derivatizing process has now been developed by capillary electrophoresis using hydroxypropyl-β-cyclodextrin as a chiral recognition agent and this has succeeded in yielding details of a catabolism from compound **III** to compound **I**. The present paper describes the capillary electrophoretic method and the findings that compound **II** is a metabolic intermediate and that neither isomerisation reaction nor enzymatic recognition of differences in stereoisomerism on the β-carbon atom on the imidazolylpropanoate part of their molecules occurs in the catabolism.



Compound I : R_1 = H; R_2 = -OH

Compound II : R_1 = H; R_2 = -NH-CH_2-COOH

Compound III: R_1 = -CO-$(CH_2)_2$-CH(NH_2)-COOH; R_2 = -NH-CH_2-COOH

Fig. 1. Structural formulae of *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl)-L-cysteine (**I**), *N*-{*S*-[2-carboxy-1-(1*H*-imidazol-4-yl) ethyl]-L-cysteinyl}glycine (**II**) and *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]glutathione (**III**).

2. Experimental

2.1. Materials

Ion-exchangers and chemicals used in the present work were as described previously [7,10]. Cyclodextrins involving β-cyclodextrin (β-CD), γ-cyclodextrin (γ-CD), hydroxypropyl-β-cyclodextrin (HP-β-CD) and dimethyl-β-cyclodextrin (DM-β-CD) were purchased from Beckman Instruments (Fullerton, CA, USA). Glycylglycine (Gly–Gly) and phthalic acid (1,2-dicarboxybenzene) were from Wako (Osaka, Japan). Two diastereomers of compound **I** were made by the addition of L-cysteine to urocanic acid [5], and isolated from each other by using the ion-exchange column chromatography [9].

In this work, one diastereomer having a peak at an elution time of 231 min [9] on the chromatogram of the amino acid analysis is defined to be compound **Ia** and this is identical with a physiological precursor of the urinary compound *N*-acetyl-L-cysteine adduct [9]. The absolute configuration on the asymmetric β-carbon atom on the skeletal imidazolylpropanoate part of compound **Ia** molecule has not been specified because of no detail of X-ray crystallographic data for the carbon atom [9]. The other having a peak at the elution time of 223 min [9] is termed to be compound **Ib** in this work. Two diastereomers of compound **II**, termed compound **IIa** and **IIb**, are defined as described in the results, Section 3. Compound **III** was prepared by the addition of glutathione to urocanic acid [7,10]. When the synthesized compound **III** was allowed to react with 6 *M* HCl, both compound **Ia** and **Ib** were formed in the same yield with each other [7] and this indicates that compound **III** used here is an equimolar mixture of its two diastereomers; an attempt to isolate compound **III** diastereomers from each other by ion-exchange column chromatography was unsuccessful because of their similar behaviours during the chromatography.

2.2. Spectroscopy and paper electrophoresis

Fast-atom-bombardment mass spectrometry (FAB–MS) with a direct-inlet system was carried out on a Shimadzu 9020-DF gas chromatography–mass spectrometer equipped with a Shimadzu SCAP 1123 data system and a 7240A plotter printer (Hewlett–Packard) according to a previous method [5]. Glycerol or thioglycerol (3-mercapto-1,2-propandiol) were used as a matrix. High-voltage paper electrophoresis was performed on Whatman 1 Chr paper (Whatman, Maidstone, UK) in a buffer (pH 3.1) consisting of pyridine–acetic acid–water (1:20:179, v/v) [12] at a potential gradient of 100 $V\,cm^{-1}$ for 50 min. The dried paper was sprayed with ninhydrin reagent, which was 2% (v/v) pyridine plus 1% (w/v) ninhydrin in acetone solution [7], or Pauly's reagent, a diazotized sulphanilic acid reagent for the detection of imidazole compounds [7,13,14]. Relative mobilities of compounds on the paper electrophoretogram, termed the mR_{ILA} value, were determined by comparison of the mobility for imidazol-4-yl-lactic acid as 1.00 [7].

2.3. Capillary electrophoresis

Capillary electrophoresis was carried out on a Beckman P/ACE 5510 system equipped with a Beckman P/ACE diode array detector and a System Gold Software for data collection. The instrument was used in the normal polarity, i.e. with the cathode (+) at the detector end. A Beckman eCAP capillary cartridge with 100×800 μm aperture contained a Beckman eCAP 37 cm (30 cm to detector) ×50 μm I.D. neutral coated capillary. The run temperature was set at 20°C. Separation buffers at the specified pH were prepared by mixing a Beckman eCAP chiral kit adjustable pH buffer (an initial pH, 2.50) with the chiral kit high pH buffer (an initial pH, 8.00); while continuously stirring, the pH was finally adjusted to the required value by carefully adding 1 *M* phosphoric acid or 1 *M* sodium hydroxide, 1 μl at a time. All buffer solutions were filtered through a 0.2-μm nylon syringe filter. High-purity electrophoresis grade water was always used to prepare electrophoresis materials and when rinsing the capillary. A sample was applied by pressure injection with nitrogen gas (>99.99%). To prevent any sample carry-over from previous separations, the capillary was rinsed by performing a 0.5-min high-pressure rinse with 0.1 *M* HCl followed by a 2-min rinse with water and finally a 5-min rinse with the separation buffer between every run. Compounds were detected by recording a change of absorbance at 210 nm. Resolution for diastereomers of compound **I** and **II** in each is expressed in the R_s value as followed: $R_s=2(t_{m1}-t_{m2})/(W_1+W_2)$, where t_{m1} and t_{m2} are migration times, W_1 and W_2 are the baseline width of the peaks, respectively.

2.4. Amino acid analysis

Amino acid analysis was carried out on a Hitachi KLA-5 amino-acid analyser as described previously [9] except for using an eluent of 500 ml, instead of 170 ml used previously, of 0.067 *M* sodium citrate buffer (pH 4.25) without ethanol.

2.5. Enzymatic degradation

Male Wistar rats (Clea Japan, Tokyo) weighing 300–350 g were maintained on a laboratory diet, MF (Oriental Yeast, Tokyo, Japan). Rat kidney homogenate was prepared by homogenizing with three volumes of 0.1 *M* potassium phosphate buffer (pH 7.4) containing 5 m*M* EDTA, and was used as an enzyme source without further purification. Enzymatic degradation of compound **IIa** or **IIb** as a substrate was performed by incubation of a 1-ml reaction mixture containing 5 m*M* of the substrate and 300 μl of the homogenate in the phosphate buffer, pH 7.4, for 30 min or 60 min at 37°C. The reaction was terminated by the addition of 2 ml of 15% (w/v) trichloroacetic acid, and the mixture was filtered through the 0.2-μm nylon syringe filter to obtain the filtrate. A 2.5-ml portion of the filtrate was then transferred onto a Dowex 50W-X8 column (H^+-form, 10×0.7 cm). After the column had been washed with 40 ml of water, reaction products were eluted with 25 ml of 2 *M* ammonia. The eluate was evaporated to dryness under the reduced pressure below 40°C. The residue obtained was dissolved in 1 ml of the buffer, pH 6.00, containing 0.5 m*M* of phthalic acid as an internal standard. The solution was then filtered through the nylon syringe filter, and the filtrate was used as a sample for capillary electrophoresis; the sample was diluted with the same buffer to the appropriate concentration if necessary. Reaction mixtures without the substrate, or with a homogenate pre-heated at 100°C for 3 min instead of the above homogenate were used as controls.

Enzymatic degradation of compound **III**, which is characterized to be an equimolar mixture of its two diastereomers as described above, as a substrate was carried out by incubating a 1-ml reaction mixture containing the substrate (5 m*M*), 60 m*M* of Gly–Gly and 50 μl or 300 μl of the rat kidney homogenate in the phosphate buffer, pH 7.4, at 37°C. The sample for capillary electrophoresis was prepared by the same method as that described above. Controls used were as described above. Recoveries of compounds **I**, **II** and **III** during the isolation steps were 94%, 93% and 95%, respectively. Experimental data indicated in this paper are uncorrected for recoveries.

3. Results

3.1. Isolation of two diastereomers of compound *II* from each other

Compound **II** was synthesized by a previous method [10]; on the chromatogram of amino-acid analysis, two broad peaks at 451 min and 463 min with similar shapes were found. When the synthesized compound was allowed to react with 6 *M* HCl at 100°C for 2 h, both compound **Ia** and **Ib** were formed in the same yield of 47% in each and this suggested an existence of two diastereomers of compound **II**. These diastereomers were isolated from each other by using a Dowex 50W-X8 column (45 cm×1.5 cm) equilibrated with sodium citrate buffer (pH 4.25). Compounds were chromatographed by using an eluent of the sodium citrate buffer of pH 4.25 with 6-ml portions being collected. One diastereomer having the peak at 463 min was found in eluates between 420 ml and 600 ml. This is defined to be compound **IIa** in this work. Analytical data calculated for $C_{11}H_{16}N_4O_5S$: C, 41.77; H, 5.10; N, 17.71%. Found: C, 41.72; H, 5.08; N, 17.67%. The HCl-treatment of compound **IIa** resulted in formations of only compound **Ia** (a yield, 95%) and glycine and this indicates that the configuration on the β-asymmetric carbon atom of the skeletal imidazolylpropanoate part of compound **IIa** molecule is identical with that of compound **Ia**. The other diastereomer having the peak at 451 min was found mainly in eluates between 180 ml and 300 ml. This is defined to be compound **IIb**. Analytical data found: C, 41.80; H, 5.13; N, 17.68%. The acid treatment of this compound resulted in formations of compound **Ib** and glycine without forming compound **Ia**. Thus, the configuration on the β-carbon atom of the skeletal part of compound **IIb** molecule is the same as that of compound **Ib**.

3.2. Chiral separation of imidazole compounds

Analytical conditions for chiral separation of compound **I** and **II** diastereomers were settled by the following steps: in the beginning, the most suitable type of cyclodextrin as the chiral recognition agent was selected. Results shown in Table 1 indicate that

Table 1
Chiral resolutions (R_s) for diastereomers of compound **I** and **II** with cyclodextrins (CDs)

CD types	Concentration of CD (m*M*)	R_s	
		Compound **Ia** and **Ib**	Compound **IIa** and **IIb**
β-CD	3	0.73	0.70
	15	0.91	0.75
γ-CD	10	0.4	0.5
	50	0.4	0
HP-β-CD	10	1.08	0.82
	100	1.40	1.28
DM-β-CD	10	0.5	0.5
	50	0.80	0.81

Conditions: [compound **Ia**]=[compound **Ib**]=0.5 m*M*; [compound **IIa**]=[compound **IIb**]=0.5 m*M*; 37 cm (30 cm effective) neutral coated capillary; a buffer pH of 8.00; an electric field strength at 500 V cm^{-1} (18.5 kV, average 25 μA); 20°C; 1-s pressure injection (approximately 5 nl s^{-1}).

HP-β-CD is the most suitable agent for the chiral separation because highest resolutions for diastereomers of both compound **I** (R_s 1.40) and compound **II** (R_s 1.28) were obtained by the pH-8.00 buffer containing 100 m*M* HP-β-CD. Then, in order to select the best pH value of the separation buffer, different pH buffers containing 100 m*M* HP-β-CD were tested. Results obtained were as follows: by using a buffer of pH 7.00, R_s 1.42 and 1.38 were given for compound **I** and compound **II** diastereomers, respectively; in the use of a buffer of pH 6.00, resolutions markedly increased to 2.80 and 2.91 for compound **I** and compound **II**, respectively; however, no peak was detected by 45-min run when the pH 5.00 buffer was used. Thus, the most suitable pH of the separation buffer is 6.00 for both compound **I** and **II** diastereomers. The third step was a determination of the best concentration of HP-β-CD in the buffer. As shown in Fig. 2, the highest resolution of R_s 3.03 and 3.39 for respective compound **I** and **II** diastereomers were obtained by 80 m*M* HP-β-CD. Finally a strength of the electric field for run was selected. As shown in Table 2, high resolutions for both compound **I** and **II** were obtained at field strength between 450 V cm^{-1} and 550 V cm^{-1}, and a field strength of 500 V cm^{-1} was selected in this work. In the present work, the lowest available temperature of 20°C was used because higher temperatures resulted in shorter migration time but always lower resolutions for every compound.

To sum up, diastereomers of both compound **I** and **II** were completely separated by electrophoresis using a 37 cm (30 cm, effective) neutral coated capillary with the pH 6.00 buffer containing 80 m*M* HP-β-CD at the field strength of 500 V cm^{-1} (18.5 kV) at 20°C. Under these conditions, the migration time of 19.58, 20.31, 15.01 and 15.81 min were given for compound **Ia**, **Ib**, **IIa** and **IIb**, respectively. However, two diastereomers of compound **III** characterized above were not separated by this method and gave a peak at a migration time of 9.14 min. When a mixture of all imidazole compounds at less than 5 m*M* in each was applied by 1-s pressure injection, every peak on the electropherogram was

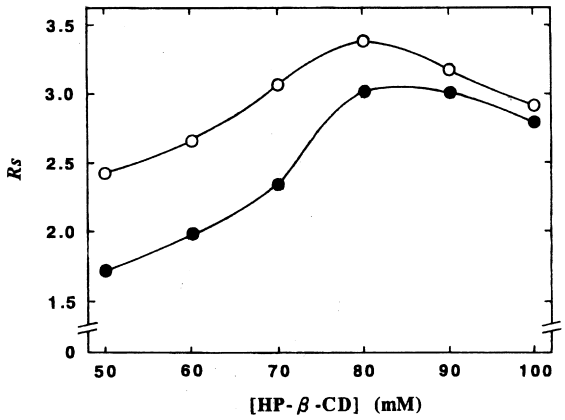


Fig. 2. Effects of concentrations of HP-β-CD on chiral resolutions (R_s) for compound **I** (●) and compound **II** (○) diastereomers. Analytical conditions: a buffer pH of 6.00; others are as described in the footnote of Table 1.

Table 2
Effects of the strength of the electric field on chiral resolutions (R_s) for diastereomers of compound **I** and **II**

Strength of the electric field (V cm^{-1})	R_s	
	Compound **Ia** and **Ib**	Compound **IIa** and **IIb**
300	2.37	2.91
400	2.93	3.24
450	3.02	3.38
500	3.03	3.39
500	3.02	3.36
600	2.91	3.24
700	2.61	2.52

Conditions: a buffer, pH 6.00, containing 80 m*M* HP-β-CD; others are as described in the footnote of Table 1.

completely separated from each other and this indicated a possibility of a simultaneous determination of these compounds.

Quantitative analyses of these imidazole compounds were carried out by comparison of the peak area for 0.5 m*M* phthalic acid as the internal standard which gave a peak at a migration time of 4.30 min in the present method. Values obtained in this manner were linear at 0.03–5.0 m*M* of these compounds. This method was applicable for determination of products formed by enzymatic reaction of compound **II** or **III** diastereomers with rat kidney homogenate as described below.

*3.3. Enzymatic formation of compound **I** from compound **II***

Incubation of compound **IIa** (5 μmol) as the substrate with 300 μl of rat kidney homogenate for 30 min or 60 min resulted in a formation of compound **Ia** in yields of 24.8±2.8% or 39.4±4.6% (in mean±S.D., $n=4$), respectively, without a formation of compound **Ib**. In the same reaction of compound **IIb** instead of compound **IIa** with the homogenate, compound **Ib** was yielded in 25.3±3.2% for the 30-min incubation, or 38.9±5.2% (in mean±S.D., $n=4$) for the 60-min incubation, accompanied by no compound **Ia**. These results indicate that no isomerization on the β-asymmetric carbon atom of the skeletal part of either compound **IIa** molecule or compound **IIb** molecule occurs in their catabolism. Additionally, it is suggested that there is little difference in reactivities as the enzyme substrate between two diastereomers of compound **II** because nearly identical yields of compound **Ia** and **Ib** with each other are given by the above reactions.

*3.4. Formation of compound **I** via compound **II** by enzymatic degradation of compound **III***

In order to elucidate whether enzymatic recognition of the difference in stereoisomerism between two diastereomers of compound **III** occurs in the catabolism, a mixture of compound **III** (5 μmol) and Gly–Gly (60 μmol) was incubated with 50 μl of rat kidney homogenate. As shown in Table 3, amounts of compound **IIa** and **IIb** formed for each reaction time were much similar to each other and this suggests that the reactivity of each diastereomer of compound **III** as the enzyme substrate is much the same. Namely, an enzyme catalysing the formation of compound **II** from compound **III** hardly recognizes the difference in stereoisomerism between two diastereomers of compound **III**, although the enzyme has not been specified completely.

On the electropherogram of the sample obtained by the 60-min reaction (Fig. 3A), a small amount of compound **Ia** and **Ib** were detected, and these were determined to be 0.08±0.04 μmol and 0.09±0.05 μmol (in mean±S.D.), respectively; in the 40-min reaction, these compounds were detected to be less than 0.03 μmol i.e. trace.

In order to determine compound **II** to be a metabolic intermediate for the formation of compound **I** from compound **III**, the larger amount of rat kidney homogenate was added to the reaction mixture. When 300 μl, instead of 50 μl, of the homogenate was used as the enzyme source in the similar

Table 3
Enzymatic formations of compound **IIa** and **IIb** from compound **III**[a]

Reaction time (min)	Amount of compound **III** unreacted (μmol)	Products formed (μmol)	
		Compound **IIa**	Compound **IIb**
Controls[b]	4.77±0.18	n.d.[c]	n.d.[c]
10	2.48±0.21	1.25±0.09	1.24±0.13
20	1.22±0.14	1.82±0.14	1.83±0.12
40	0.41±0.11	2.25±0.12	2.26±0.10
60	0.36±0.12	2.30±0.11	2.28±0.12

[a] Incubation of a 1-ml reaction mixture containing 5 mM compound **III**, 60 mM glycylglycine and 50 μl of rat kidney homogenate in a phosphate buffer, pH 7.4, at 37°C. Values represent the mean±S.D., $n=4$.
[b] The incubation for 60 min with the kidney homogenate pre-heated at 100°C for 3 min.
[c] n.d.=No detection of the compound on the electropherogram.

manner as that described above, gradual formations of compound **Ia** and **Ib** from compound **IIa** and **IIb**, respectively, were clearly observed. Fig. 3B shows an electropherogram of the sample obtained for the 120-min incubation. In this instance, a yield of compound **Ia** was nearly the same as that of compound **Ib**; amounts of compound **IIa** and **IIb** remained in the reaction mixture were also identical with each other. The same result was obtained at every reaction period of 10, 30, 60 and 90 min. Changes in total amounts of compound **I** diastereomers, compound **II** diastereomers and compound **III** in reaction mixtures for times are shown in Fig. 4. Results suggest that compound **II** is formed in the first step of compound **III** catabolism apparently at

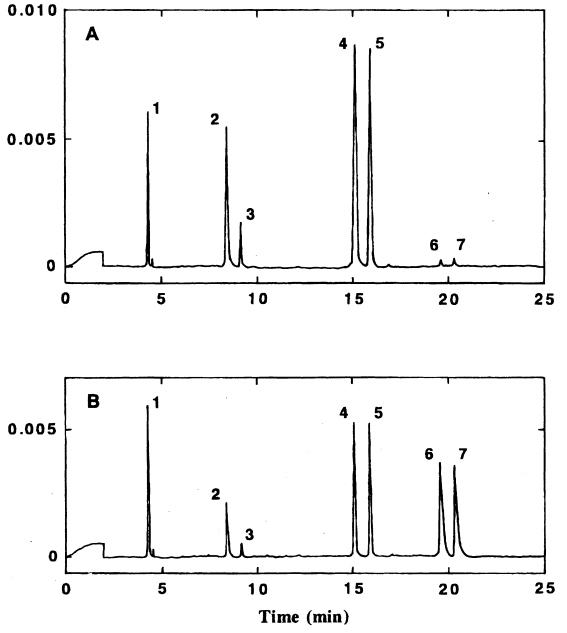


Fig. 3. Capillary electropherograms of products formed by enzymatic degradation of compound **III**. Incubation of a 1-ml reaction mixture containing 5 m*M* compound **III**, 60 m*M* glycylglycine and 50 μl of rat kidney homogenate for 60 min (A), or 300 μl of the homogenate for 120 min (B). Analytical conditions: a neutral coated capillary (37 cm×50 μm I.D.); a buffer, pH 6.00, containing 80 m*M* HP-β-CD; 500 V cm^{-1} at 20°C. Peaks are as follows: 1, 0.5 m*M* phthalic acid as the internal standard; 2, γ-Glu–Gly–Gly; 3, compound **III**; 4, compound **IIa**; 5, compound **IIb**; 6, compound **Ia**; 7, compound **Ib**.

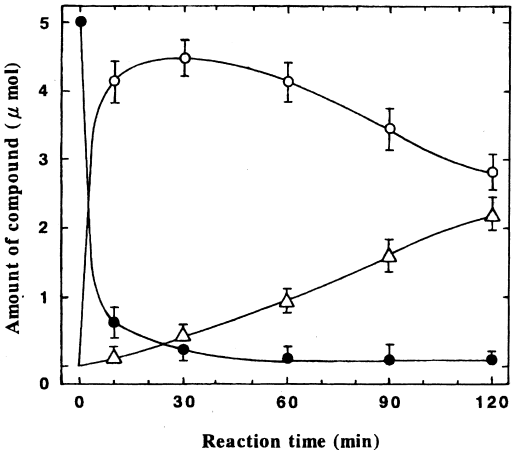


Fig. 4. Change curves of total amounts of compounds **Ia** and **Ib** (△) formed, compounds **IIa** and **IIb** (○) formed and compound **III** (●) unreacted by incubation of a 1-ml reaction mixture containing 5 m*M* compound **III**, 60 m*M* Gly–Gly and 300 μl of rat kidney homogenate in a phosphate buffer, pH 7.4, at 37°C; four experiments.

an initial velocity of 4 μmol min^{-1} ml^{-1} of the homogenate and then further metabolized to compound **I** at the lower velocity under these reaction conditions.

3.5. Action of Gly–Gly as the γ-glutamyl acceptor

On electropherograms shown in Fig. 3, another product having a peak at a migration time of 8.41 min was also detected. This compound, however, was formed neither by removing Gly–Gly from the reaction mixture nor by the reaction of compound **II** with the homogenate. The product was isolated by using a Dowex 1-X8 column (acetate form, 0.7×10 cm) with an eluent of 35 ml of 0.2 *M* acetic acid after the column had been washed with 80 ml of 0.05 *M* acetic acid. The product was determined to be L-γ-glutamylglycylglycine (γ-Glu–Gly–Gly) by the following results: on the FAB-mass spectrum, peaks were assigned to be *m*/*z* 262 (MH^+, the molecular ion plus proton), 187 (MH^+ − glycine), 133 (MH^+ − pyroglutamic acid), 131 (MH^+ − glycylglycine), 115 (133 − H_2O) and 75 (MH^+ − γ-glutamylglycyl group); reaction with 6 *M* HCl gave a product consisting of glutamic acid–glycine (1:2, by mol); on the paper electrophoretogram, mR_{ILA} 0.15 to the cathode, i.e. an acidic compound; a positive reaction with ninhydrin reagent, but negative one with Pauly's reagent. The formation of γ-Glu–Gly–Gly suggests that Gly–Gly acts as a γ-glutamyl acceptor from the donor compound **III**.

4. Discussion

From these results, every diastereomer of compound **I** and **II** was accurately determined by the present method and this allowed to give details of the metabolism from compound **III** to compound **I** via compound **II** as described above. Judging from the formation of γ-Glu–Gly–Gly, the reaction from compound **III** to compound **II** is catalysed possibly by the action of γ-glutamyl transpeptidase (EC 2.3.2.2). In other experiments (in preparation), both diastereomers of compound **II** were formed in a similar yield to each other by incubating a reaction mixture of compound **III** and Gly–Gly with bovine kidney γ-glutamyl transpeptidase (Wako), or with γ-glutamyl transpeptidase purified partially from rat kidney by the method of Tate and Meister [15]; moreover, the formation of compound **II** was suppressed by L-serine/boric acid, an inhibitor of γ-glutamyl transpeptidase [16,17]. On the other hand, we previously found that *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-*N*-L-γ-glutamyl-L-cysteine was not formed by reaction of compound **III** with rat tissue homogenates [10] and this suggested that compound **III** was hardly cleaved by actions of some carboxypeptidases. It is therefore possible that the glutathione moiety of compound **III** molecule is metabolized to the L-cysteinylglycine moiety of compound **II** by the action of γ-glutamyl transpeptidase and then further catabolized to the L-cysteine moiety of compound **I** with other enzymes such as L-cysteinylglycine hydratase (EC 3.4.13.6) and/or α-aminoacyl-peptide hydratase (microsomal) (EC 3.4.11.2), like the metabolism of glutathione.

Present results suggest that the configuration of the β-asymmetric carbon atom on the imidazolylpropanoate part is maintained during the catabolism of compound **III**, and that both diastereomers of compound **III** as well as those of compound **II** act as the substrate with the same reactivity to each other. Compound **Ia** was previously determined to be a physiological precursor of the urinary compound *N*-acetyl-*S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-L-cysteine [9]. Thus, it is possible that one diastereomer of compound **III** having the same configuration on the β-carbon atom as that of compound **Ia** is first formed in the body and then metabolized to compound **IIa**, compound **Ia** and the *N*-acetyl-L-cysteine adduct in this order. Physiological actions of these imidazole compounds have not been elucidated yet, although formations of these imidazole compounds participate in part in the accumulation and the elimination of the epidermal urocanic acid under conditions of sunlight irradiation as described previously [7].

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