The Ion-exchange Chromatography of Imino Derivatives of Glycine

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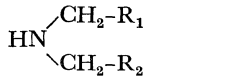
The resolution of the six imino derivatives of glycine (Gly) by ion-exchange chromatography is described. The imino compounds included iminodiacetonitrile (**1**), iminodiacetamide (**2**), iminodiacetic acid (**3**), α-(cyanomethylamino)acetamide (**4**), α-(cyanomethylamino)acetic acid (**5**), and α-(carbamoylmethylamino)acetic acid (**6**). A mixture of **1**—**6** was chromatographed along with Gly, glycinamide, aminoacetonitrile, and NH_3 with an automatic amino acid analyzer using Aminex A-4 resin column (0.25ϕ × 50 cm) and sodium citrate buffers. When the initial buffer of pH 3.25 was changed to pH 6.50 15 min after beginning the analysis, these ten components were completely resolved. The analysis was completed in about 4.5 h. The stability of **1**, **4**, and **5** in aqueous media at room temperature was also studied.

In connection with chemical evolution, Ponnamperuma and Woeller[1] demonstrated that aminoacetonitrile (AAN) could be formed by an electric discharge through an anhydrous mixture of methane and ammonia (a simulated Jovian atmosphere) which was considered to resemble the primitive atmosphere of the Earth. Consequently, the role of AAN in aqueous media, as a precursor to polypeptides and amino acids, became of primary importance.[2-4] Chadha *et al.*[3] studied the reaction of AAN in aqueous solutions of pH 9. When the reaction products were examined by ion-exchange chromatography, peptides such as diglycine were not detected. After the reaction products were hydrolyzed and derivatized for gas chromatography, they identified glycine (Gly), iminodiacetic acid (**3**), and a small amount of nitrilotriacetic acid by gas chromatography-mass spectrometry. Recently, we investigated the reaction of AAN in aqueous ammoniacal solutions. When the reaction mixtures were examined by ion-exchange chromatography, a number of ninhydrin reactive products were eluted along with Gly and glycinamide (Gly–NH_2). On the basis of the results obtained by Chadha *et al.*[3,4] these products were considered to be imino derivatives of Gly, as shown in Fig. 1. Therefore, the resolution of these imino compounds by ion-exchange chromatography was necessary for identification and determination of the reaction products of AAN.



1: $R_1 = R_2 = C{\equiv}N$
2: $R_1 = R_2 = CONH_2$
3: $R_1 = R_2 = COOH$
4: $R_1 = C{\equiv}N$, $R_2 = CONH_2$
5: $R_1 = C{\equiv}N$, $R_2 = COOH$
6: $R_1 = CONH_2$, $R_2 = COOH$

Fig. 1. Imino derivatives of Gly.

Since an automatic amino acid analyzer based on ion-exchange chromatography was first devised by Spackman *et al.*[5] in 1958, not only the resolution of amino acids and related compounds but also analysis time has been much improved by many investigators. However, substances analyzed by this method have been limited to those of biological origin such as hydrolyzates of proteins, physiological fluids, and tissue extracts.[6] This paper deals with the resolution of **1**—**6** by ion-exchange chromatography.

Results and Discussion

Ion-exchange Chromatography. The six imino compounds, that is, iminodiacetonitrile (**1**), iminodiacetamide (**2**), **3**, α-(cyanomethylamino)acetamide (**4**), α-(cyanomethylamino)acetic acid (**5**), and α-(carbamoylmethylamino)acetic acid hydrobromide (**6**·HBr) were prepared in the present study as the authentic samples for ion-exchange chromatography. The latter three (**4**, **5**, and **6**·HBr) are new compounds.

Because AAN was considered to give not only the imino compounds (**1**—**6**) but also hydrolysis products such as Gly, Gly–NH_2, and NH_3 in aqueous media, the resolution of a synthetic mixture of these ten compounds with an amino acid analyzer was examined.

Figure 2 illustrates the resolution of them with a pH 3.25—6.50 sodium citrate buffer system. All the ten components were completely resolved from each other and the analysis time was about 4.5 h. A column of 0.25ϕ × 50 cm packed with a Bio-Rad spherical resin, Aminex A-4 (8% crosslinkage; particle size 20± 4 μm) was found to be adequate for the present analysis. Among the basic components, **4** was always eluted at the break-through point of the second buffer. The buffer change time of 15 min was found to be optimum; the elutions with a buffer change time longer than 15 min or shorter than 15 min, resulted in merely a

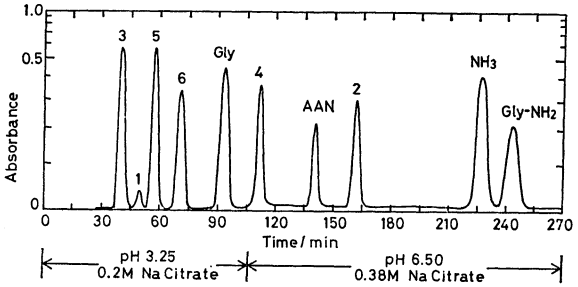


Fig. 2. Ion-exchange chromatogram of a mixture of imino derivatives of Gly (**1**—**6**) and related compounds. Conditions of chromatography are described in the Experimental Section. Charge amount of each component, 0.1 μmol. Elution was started using a pH 3.25 citrate buffer. A buffer change was made after 15 min to pH 6.50, and the break-through of the pH 6.50 buffer was observed at 108 min.

longer analysis time or the poor resolution of Gly and **4**, respectively.

As shown in Fig. 2, the components were eluted in a regular manner. Namely, compounds with the same *N*-substituent group (X) were eluted in the order of X–NH–CH_2–COOH (carboxylic acid), X–NH–CH_2–CN (nitrile), and X–NH–CH_2–$CONH_2$ (amide). For example, the compounds having carboxymethyl as an *N*-substituent group (X=CH_2–COOH) were eluted in the order of **3**, **5**, and **6**. The only exception is the case of the compounds (X=CH_2–CN), which were eluted in the order of **1** (nitrile), **5** (carboxylic acid), and **4** (amide). This reversed elution order of **1** and **5** could be explained in terms of the increased acidity of **1** due to a strong electron-attracting effect of the two cyano groups.

Figure 3 shows the elution behavior of the basic components with a pH 3.25—6.50 or a pH 3.25—5.28 citrate buffer system. Elution with a pH 3.25 buffer alone (D) resulted in a significantly longer analysis time, with the complete overlap of AAN and Gly–NH_2. The basic components, except for AAN and **2**, were eluted at almost constant positions regardless of the pH of a second buffer, so long as the buffer

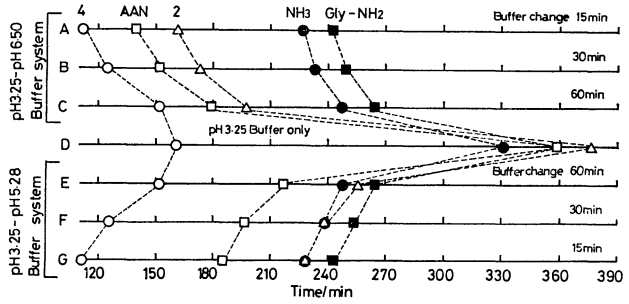


Fig. 3. Elution behavior of the basic components with various buffer systems.
Conditions of chromatography are described in the Experimental Section.

Table 1. Elution times and relative color constants of imino derivatives of Gly and related compounds[a)]

Compound	Elution time/min	Relative color constant[b)]
3	41	1.07
1	50	0.064
5	57	1.11
6	71	0.65
Gly	94	1.00
4	112	0.79
AAN	140	0.39
2	162	0.65
NH_3	227	1.23
Gly-NH_2	243	0.72

a) Conditions of chromatography are described in the Experimental Section. Elution was started using a pH 3.25 citrate buffer. A buffer change was made after 15 min to pH 6.50. b) The color constant refers to peak area per μmol of a compound. The peak area was calculated according to the conventional $H \times W$ method (Ref. 5). The color constant of Gly was 93.7 μmol^{-1}.

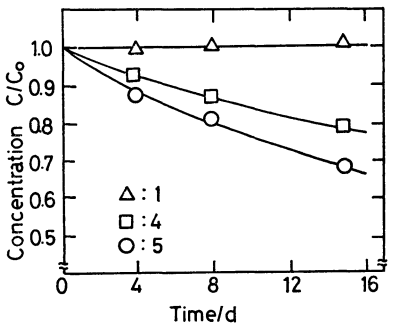


Fig. 4. Decomposition of α-amino nitriles (**1**, **4**, and **5**) in aqueous media (1 mM) at room temperature.

change time was unchanged. The elution times of AAN and **2** were very sensitive to the pH of a second buffer. Their elution was retarded remarkably with the pH 5.28 buffer, resulting in the poor resolution of NH_3, **2**, and Gly–NH_2 (E) or the complete overlap of NH_3 and **2** (F and G), in comparison with the elution with the pH 6.50 buffer. In this way, the basic components could be successfully resolved from each other by the use of the pH 3.25—6.50 buffer system.

Table 1 shows the elution times and the relative color constants of the ten components. It was evident that the color constant of **1** with ninhydrin was extremely poor compared with the other imino compounds, although the time of heating in the reaction bath of the amino acid analyzer was designed to be about 30 min.

Stability of the Imino Compounds in Aqueous Media. Among the imino compounds, α-amino nitriles such as **1**, **4**, and **5** were thought to be unstable in aqueous media due to the reverse Strecker reaction. Figure 4 shows the rate of decomposition of them in aqueous solutions (1 mM) (1 M=1 mol dm^{-3}) at room temperature. **4** and **5** were unstable and decomposed to 20.2 and 31.5%, respectively, in 15 d. Since **4** and **5** yielded Gly–NH_2 and Gly as the decomposition products, respectively, the unstability could be explained in terms of the reverse Strecker reaction as follows:

$$\mathrm{HN}\begin{matrix}\diagup \mathrm{CH_2\text{-}CN} \\ \diagdown \mathrm{CH_2\text{-}Y}\end{matrix} + \mathrm{H_2O} \longrightarrow \mathrm{H_2N\text{-}CH_2\text{-}Y} + \mathrm{CH_2O} + \mathrm{HCN}$$

(Y=$CONH_2$ or COOH)

Unlike **4** and **5**, **1** was stable under these conditions. Hence, in the case of such unstable compounds, it is desirable to calibrate an amino acid analyzer with freshly prepared standard solutions.

Experimental

All the melting points were determined with a Yanagimoto micro melting point apparatus and were not corrected. IR spectra were taken with a Hitachi Model 285 Infrared spectrophotometer. ^{1}H-NMR spectra were measured with a Hitachi R-24B spectrometer using TMS as an internal reference. Thin layer chromatography was carried out on silica gel 60 F_{254}(Merck) using the following solvent system: *n*-BuOH–AcOH–H_2O (4:1:1, v/v). *S*-(*p*-methoxybenzyloxycarbonyl)-4,6-dimethyl-2-pyrimidinethiol(Z(OMe)–SDP) and

25% HBr–AcOH were obtained from Protein Research Foundation.

Ion-exchange Chromatography. Ion-exchange chromatography was carried out with a Sibata amino acid analyzer AA-600 under the following conditions: column, 0.25ϕ × 50 cm packed with Aminex A-4(Bio-Rad, 8%-crosslinked, sulfonic acid type cation exchange resin, particle size 20± 4 μm); flow rate of an eluent, 6 ml/h; flow rate of a ninhydrin solution,[5] 3 ml/h; jacket temperature, 30 °C; chart speed, 120 mm/h. Absorbance at 570 nm was measured by continuous photometry. Sodium citrate buffers of pH 3.25 (0.2 M Na), pH 5.28(0.35 M Na), and pH 6.50(0.38 M Na) were used as eluents. The former two buffers were obtained from Wako Pure Chemical Industries, Ltd. The pH 6.50 buffer was prepared according to the method of Moore *et al.*[7] using caprylic acid instead of phenol. Elution was started with the pH 3.25 buffer and the initial buffer was changed to a second buffer(pH 5.28 or 6.50) at an appropriate later time. A calibrated equimolar(0.1 μmol) mixture of the imino compounds(**1**—**5**, and **6**·HBr), Gly, Gly–NH_2·HBr, AAN·1/2 H_2SO_4, and NH_4·1/2 SO_4 was applied on the column of the amino acid analyzer.

Aminoacetonitrile Neutral Sulfate(AAN·1/2 H_2SO_4). Concd H_2SO_4(25.5 g, 0.25 mol) was gradually added to ethanol (EtOH) (125 ml). The temperature of the solution rose, but it was adjusted to 65—70 °C. Methyleneaminoacetonitrile (MAAN)[8] (34 g, 0.5 mol) was added gradually to it. In the beginning, MAAN dissolved, but after a certain point it ceased to dissolve. The mixture was warmed for 1 h(60 °C) on a water bath, and then kept in a refrigerator overnight. The precipitates were dissolved in H_2O(100 ml) and the undissolved material(MAAN) was removed by filtration. The product was precipitated by the addition of EtOH(600 ml) to the filtrate and then recrystallized from H_2O–EtOH; yield, 20 g(38%); mp, 160 °C(decomp)(lit,[9] mp, 165 °C); R_f, 0.27. Found: C, 23.03; H, 4.72; N, 26.76%. Calcd for $C_2H_5N_2O_2S_{0.5}$: C, 22.85; H, 4.79; N, 26.68%.

*Iminodiacetonitrile (**1**).* A solution of freshly distilled HCN(70 ml, 1.81 mol) and concd H_2SO_4(1 ml) in methanol (MeOH) (175 ml), was added to a suspension of MAAN (119 g, 1.75 mol) in MeOH (70 ml). The resulting suspension was warmed for 3 h(60 °C), and then refluxed at 75 °C for a further 30 min on a water bath, in a draft chamber. After the reaction mixture had been cooled to room temperature, the precipitates(MAAN) were removed by filtration. When the filtrate was cooled to 0 °C, **1** precipitated; this was processed with charcoal and then recrystallized from EtOH; yield, 85.7 g(52%); mp, 76—77 °C(lit,[10] 75 °C); R_f, 0.62.

*Iminodiacetamide (**2**).* A solution of dimethyl iminodiacetate hydrochloride(1.97 g, 10 mmol) in MeOH(15 ml) was treated with triethylamine (Et_3N) (1.4 ml, 10 mmol) at 0 °C. When diethyl ether(Et_2O) (60 ml) was added to the resulting solution, Et_3N·HCl precipitated and was removed by filtration. The filtrate was evaporated to give an oil (dimethyl iminodiacetate). Ammonolysis of the crude oil according to the method of Jongkees[11] gave **2**, which was recrystallized from MeOH: yield, 0.85 g(65%); mp, 141—143 °C(lit,[11] 143 °C); R_f, 0.17.

*Iminodiacetic Acid (**3**).* A solution of Gly(18.8 g, 0.25 mol) and chloroacetic acid(23.6 g, 0.25 mol) in 3 M LiOH (250 ml) was allowed to stand at room temperature for 6 d. When the solution was acidified with 6 M HCl(60 ml) under cooling, crystals precipitated; these were collected by filtration. The crystals(yield, 12.5 g; mp, 152—154 °C) were identified to be nitrilotriacetic acid by elementary analysis(Found: C, 37.53; H, 4.72; N, 7.47%. Calcd for $C_6H_9NO_6$: C, 37.70; H, 4.75; N, 7.33%. Lit,[12] mp, 142 °C). The filtrate was concentrated to give a syrup, which was dissolved in a minimum amount of H_2O. The half volume of the solution was applied on a column(Amberlite CG-120, acid form, 2ϕ × 90 cm), and then eluted with H_2O. The fractions containing **3** were collected and evaporated to give **3**. The remaining solution was also treated similarly. Recrystallization was performed from H_2O–EtOH; yield, 12.8 g(39%); mp, 225—228 °C(lit,[13] 225 °C); R_f, 0.09.

*α-(Cyanomethylamino)acetamide (**4**).* A mixture of Gly–NH_2·HBr(5.27 g, 34 mmol) and formalin(2.7 ml, 34 mmol) was cooled below 0 °C on an ice salt bath, and then a solution of NaCN(1.64 g, 34 mmol) in a minimum amount of H_2O was added dropwise with stirring. After the addition was completed, the stirring was continued for 3 h. The reaction mixture was extracted with ethyl acetate(AcOEt) (40 ml × 7), and the extract was dried (Na_2SO_4), filtered, and then evaporated to give **4**, which was recrystallized from AcOEt–Et_2O; yield, 0.90 g(27%); mp, 124—126 °C; R_f, 0.41: IR (KBr), 3380(NH), 2225(C≡N), and 1650 cm^{-1} (amide I); NMR(DMSO-d_6), δ=7.24, 7.05(2H, br, $CONH_2$), 3.63(2H, s, CH_2), 3.14(2H, s, CH_2), and 2.81(1H, br, NH). Found: C, 42.25; H, 6.07: N, 37.18%. Calcd for $C_4H_7N_3O$: C, 42.47; H, 6.24; N, 37.14%.

*Ethyl α-(Cyanomethylamino)acetate (**7**).*[14] A mixture of glycine ethyl ester hydrochloride(41.9 g, 0.3 mol) and formalin(25 ml, 0.3 mol) was cooled below 0 °C, and a solution of NaCN(14.7 g, 0.3 mol) in H_2O(30 ml) was added dropwise with stirring. After the addition was completed, the stirring was continued for 4 h. The reaction mixture was extracted with Et_2O(30 ml × 2), and the ethereal extract was dried (Na_2SO_4), filtered, and then evaporated to give a pale yellowish oil; yield, 32 g(75%); R_f, 0.49; NMR($CDCl_3$), δ= 4.20(2H, q, J=7 Hz, $C\underline{H}_2CH_3$), 3.59(2H, s, CH_2), 3.49 (2H, s, CH_2), 2.31(1H, s, NH), and 1.27(3H, t, J=7 Hz, $CH_2C\underline{H}_3$); ion-exchange chromatography (a pH 3.25—5.28 buffer system with the buffer change of 30 min), elution time 137 min.

*N-(p-Methyloxybenzyloxycarbonyl)-α-(cyanomethylamino) acetic Acid Dicyclohexylammonium Salt (**8**·DCHA).* A mixture of **7** (7.10 g, 50 mmol), Z(OMe)–SDP(9.13 g, 30 mmol), and Et_3N(7 ml, 50 mmol) in $CHCl_3$ (50 ml) was stirred for 24 h at room temperature. The reaction mixture was washed with H_2O(25 ml × 5) and the $CHCl_3$ layer was dried(Na_2SO_4), filtered, and then evaporated to give a brown oil. The oily ethyl *N*-(*p*-methoxybenzyloxycarbonyl)-α-(cyanomethylamino)acetate was taken up in acetone(20 ml) and saponified as follows. 1 M NaOH(30 ml) was added to it and the resulting solution was stirred at room temperature for 2 h. After being acidified to pH 3 with 1 M HCl, the solution was extracted with AcOEt(50 ml × 5). The extract was washed with H_2O(50 ml × 3), dried(Na_2SO_4), filtered, and then evaporated to give a brown oil. The oil was dissolved in AcOEt(20 ml) and treated with DCHA(5 ml). When the solution was kept in a deep freezer overnight, **8**·DCHA precipitated and was recrystallized from $CHCl_3$–Et_2O; yield, 2.3 g(17%); mp, 152—154 °C; R_f, 0.61; IR (KBr), 2750—2400(NH_2^+), 2230(C≡N), and 1630 cm^{-1} (COO^-); NMR($CDCl_3$), δ=8.53(2H, br, NH_2^+), 7.45—6.70(4H, m, aromatic), 5.06(2H, s, CH_2O), 4.38(2H, s, CH_2), 3.90(2H, s, CH_2), 3.78(3H, s, OCH_3), and 2.90, 1.87—1.20(22H, m, cyclohexyl). Found: C, 65.12; H, 8.30; N, 8.86%. Calcd for $C_{25}H_{37}N_3O_5$: C, 65.34; H, 8.11; N, 9.14%.

*α-(Cyanomethylamino)acetic Acid (**5**).* To a suspension

of **8**·DCHA(1.0 g, 2.2 mmol) in AcOEt(50 ml) was added 0.2 M H_2SO_4 dropwise with shaking until the solution became free of suspended material. The AcOEt layer was washed with H_2O(20 ml×3), dried (Na_2SO_4), filtered, and then evaporated to give a pale brown oil. When the oil was dissolved in trifluoroacetic acid(3 ml) at 0 °C, the solution turned red. After 5 min, anisole(1 ml) was added to the solution; the mixture became colorless on standing at room temperature for 30 min. Et_2O(20 ml) was added and the precipitates were dissolved in H_2O(20 ml). After the undissolved materials had been removed by filtration, the filtrate was concentrated to a small volume. On the addition of EtOH and a large amount of Et_2O, free **5** precipitated; yield, 80 mg(32%); mp, 142—144 °C; R_f, 0.21: IR(KBr), 2750—2500(NH_2^+), 2250(C≡N, weak), and 1580 cm^{-1} (COO^-); NMR(DMSO-d_6), δ=6.48(2H, br, NH_2^+), 3.65 (2H, s, CH_2), and 3.31(2H, s, CH_2). Found: C, 41.95; H, 5.34; N, 24.91%. Calcd for $C_4H_6N_2O_2$: C, 42.11; H, 5.30; N, 24.55%.

*α-(Carbamoylmethylamino)acetic Acid Hydrobromide (**6**·HBr).* **8** prepared by decomposition of its DCHA salt(1.0 g, 2.2 mmol), was treated with anhydrous 25% HBr–AcOH(1.5 ml) at room temperature to give a clear solution with the evolution of CO_2. After 30 min, crystals were precipitated by the addition of Et_2O(30 ml); these were dissolved in H_2O (5 ml). After filtration, the solution was concentrated to a small volume and EtOH(10 ml) was added. When the solution was kept in a deep freezer, **6**·HBr precipitated and was recrystallized from H_2O–EtOH; yield, 180 mg (38%); mp, 211—213 °C; R_f, 0.42; IR(KBr), 2750—2600 (NH_2^+), 1740(COOH), and 1680 cm^{-1}(amide I); NMR (DMSO-d_6), δ=8.90(2H, br, NH_2^+), 7.90, 7.54(2H, br, $CONH_2$), 3.91(2H, s, CH_2), and 3.77(2H, s, CH_2). Found: C, 22.82; H, 4.39; N, 13.20%. Calcd for $C_4H_9N_2O_3Br$: C, 22.55; H, 4.26; N, 13.15%.

Decomposition of the Imino Compounds. An aqueous solution of each imino compound(1 mM) was prepared and allowed to stand at room temperature. An aliquot(0.1 ml) of the solution was analyzed with the amino acid analyzer at appropriate intervals. Decomposition products were identified by comparing their elution times with those of the authentic samples. **4** and **5** yielded Gly–NH_2(19.3%) and Gly(26.5%), respectively, on decomposition with H_2O in 15 d. The other imino compounds were found to be stable under these conditions.

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