NMR AND IR SPECTRA AND ELUTION BEHAVIORS DURING ION EXCHANGE CHROMATOGRAPHY OF GLUTAMIC ACID-CONTAINING DIPEPTIDES IN RELATION TO SEQUENCE DETERMINATION

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In this laboratory, many kinds of glutamyl peptides have been isolated from such higher plants as soybean seed[7] and seedling[6], green gram seed[8], ladino clover seed[9], azuki bean seed[14], buckwheat seed[15] and broad bean seed[10] and we have sought a method to determine simply and clearly with a small amount of isolated peptides whether the glutamic acid is the N-terminal or C-terminal component, and also whether the glutamyl residue is linked at α- or γ-position when glutamic acid is the N-terminal component.

In this paper, we summarize our studies on NMR[11] and IR spectra[12] and elution behaviors during ion exchange chromatography[11,13] of glutamic acid-containing dipeptides and add several data obtained thereafter.

MATERIALS AND METHODS

NMR Spectra were measured with Hitachi R-22 (90 MHz) spectrometer and chemical shifts were expressed relative to DSS as an internal standard. IR Spectra were obtained with a Hitachi EPI-S2 spectrophotometer in KBr disks. Elution behaviors of glutamyl peptides were recorded with a Hitachi KLA-2 amino acid analyer. Conditions for measuring NMR spectra and amino acid analysis are described in the note of the corresponding Tables and Figures.

Preparation of Glutamic acid-Containing Dipeptides

The method used by Buchanan *et al.* for synthesising α- and β-aspartyl peptides[4] was used in the synthesis of α- and γ-glutamyl dipeptides, but with N-carbobenzoxyglutamic anhydride in place of N-carbobenzoxyaspartic anhydride. α-Glutamyl-β-alanine and α- and γ-glutamyl dipeptides containing

asparagine, threonine, valine, proline, alanine, phenylalanine, glycine, α-aminoisobutyric acid and isopropylamine as C-terminal residue were prepared by this method, but γ-glutamyl-β-alanine could not be so obtained.

α-Glutamylleucine was prepared from the γ-benzylester of N-carbobenzoxyglutamic acid and leucine benzylester *p*-tosylate by mixed-anhydride method[3,23]. Isoglutamine was prepared from γ-benzylester of N-carbobenzoxyglutamic acid and ammonia.

Glycylglutamic acid and Valylglutamic acid were prepared from N-carbobenzoxyglycine and glutamic acid dibenzylester *p*-tosylate, and N-carbobenzoxyvaline and glutamic acid dibenzylester *p*-tosylate, respectively, by mixed anhydride method[3,23].

γ-Glutamylaspartic acid and γ-glutamyltyrosine were isolated from ladino clover seed[9], γ-Glutamylleucine, γ-glutamylmethionine and its sulfoxide and γ-glutamyl-γ-glutamylmethionine were obtained from green gram seed[8].

α-Glutamylaspartic acid, N^5-(4′-hydroxybenzyl) glutamine, γ-glutamylglutamic acid and γ-glutamyl-β-alanine were kindly donated by Dr. T. SHIBA[26], Dr. M. KOYAMA[15], Dr. E. TOMITA and Mrs. S. KAWAMURA (HASEGAWA)[5], respectively.

N-Glutaryl-α-aminoisobutyric acid and N-glutarylisopropylamine were prepared from glutaric anhydride and α-aminoisobutyric acid, and glutaric anhydride and isopropylamine, respectively.

Aspartylserine (α- and β-) and aspartylthreonine (α- and β-) were kindly supplied by Dr. H. SATO[24].

Amino acids used for preparation of the peptides were all in the L-form. Purity of all compounds prepared was confirmed by thin layer chromatography and elemental analysis.

RESULTS AND DISCUSSION

NMR Spectra

The position of the spectral line of a group adjacent to a potentially ionizable group dependents on the states of ionization of that group. As shown in Fig. 1, glutamic acid exists in α-zwitter ionic from in aqueous solution, so spectral line of α-H move to higherfield by addition of base and to lower field by addition of acid[20]. Similarly, γ-glutamyl dipeptides retain their α-zwitter ionic form in aqueous solution, so spectral line of glutamyl α-H should move upfield by addition of base and downfield by addition of acid. Spectral line of α-H of C-terminal component is expected to shift to higher field at basic condition. On the other hand, α-glutamyl

dipeptides exist in the diionic form in aqueous solution as shown in Fig. 1, then spectral line of glutamyl α-H should move upfield by addition of base and that of α-H of C-terminal residue should shift downfieldby addition of acid. For the dipeptides contaiding glutamic acid as C-terminal component and α-amino acid as N-terminal residue, it must be manifested that spectral line of glutamyl α-H shifts to lower field in acidic condition and that of α-H of N-terminal moiety move upfield by addition of base. Ionization states of glutamic acid-containing dipeptides in acidic, aqueous and basic solutions were summarized in Fig. 1 with the expected shifts of spectral lines of α-H of amino acid residues constituting the dipeptides.

The argument mentioned above has been well confirmed by the experimental results (Table I, II and III). For example, NMR sspectra of α- and

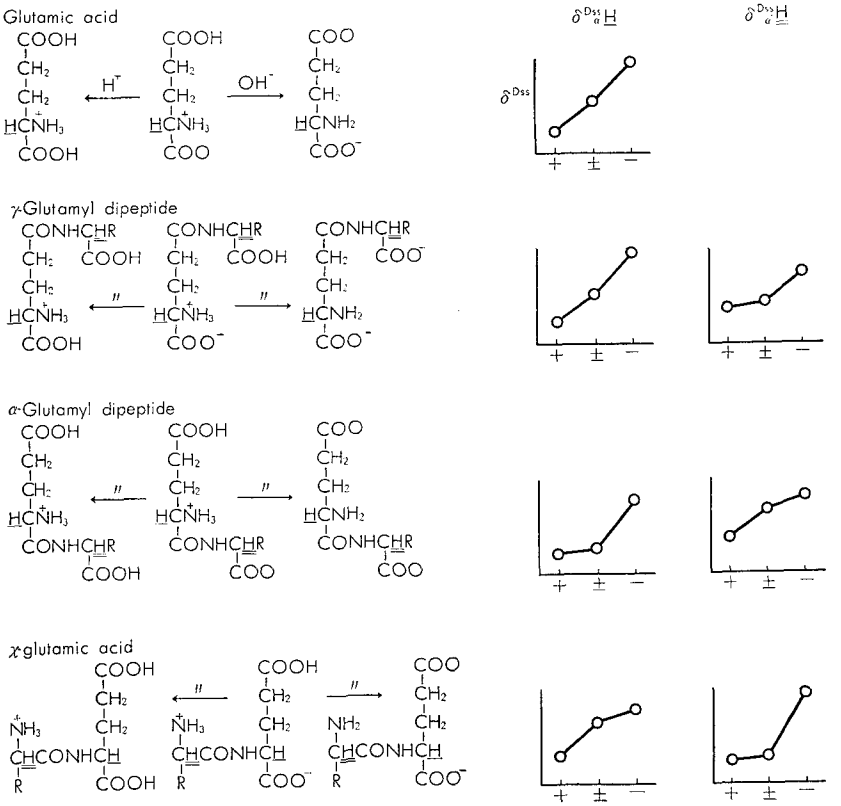

Fig. 1. Ionization State of Glutamic acid-Containing Dipeptides and Expected Shifts of Spectral Lines of α-CH of Amino Acid Residues Constituting the Dipeptides in Acidic, Aqueous and Basic Conditions.

γ-glutamylalanine in acidic,aqueous and basic solutions are shown in Fig. 2 and 3. (See also Fig. 6 and 7 for α- and γ-glutamyl-α-aminoisobutyric acid, and Fig. 8 and 9 for α- and γ-glutamylglycine.) In the case of α-glutamyl peptides, the spectral line of glutamyl α-H shifted markedly upfield in the basic solution (about 0.7 ppm, Table I, column D and E), whereas that of α-H of C-terminal residue shifted down field in the acidic condition (about 0.2 ppm, Table I, column A and B). In the case of γ-glutamyl peptides, the spectral line of glutamyl α-H showed both of

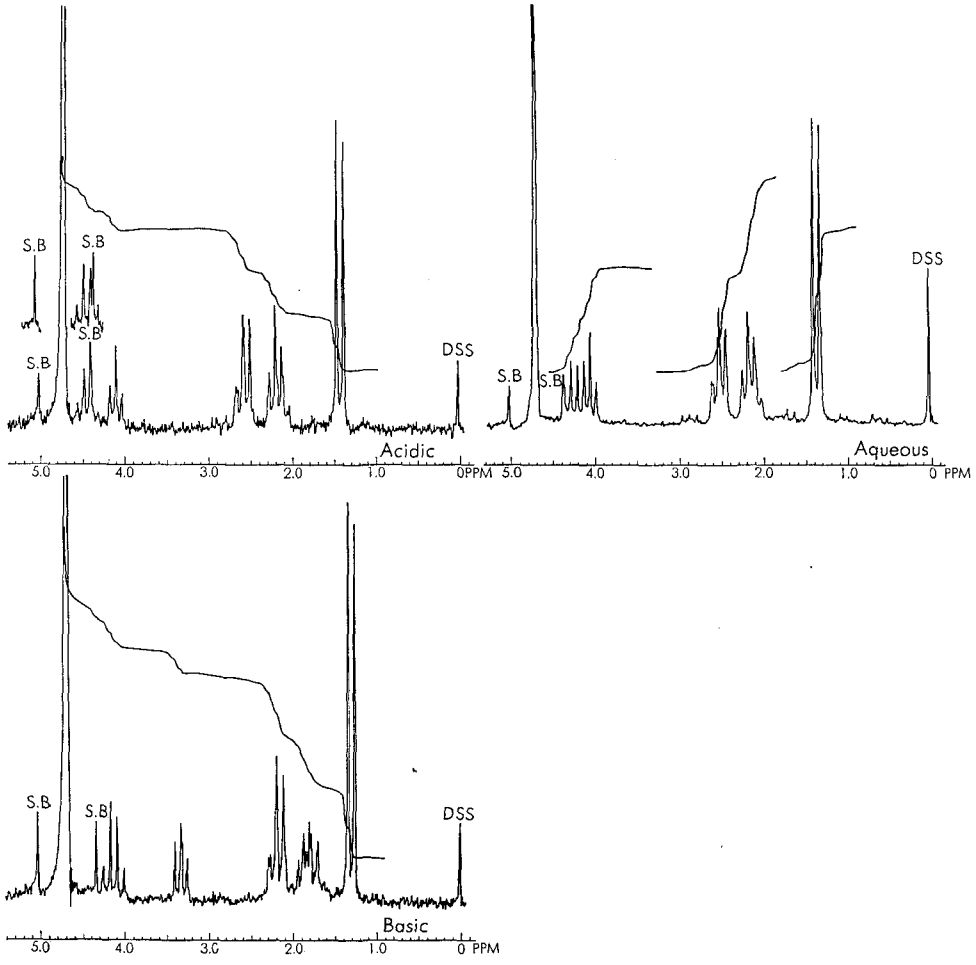

Fig. 2. NMR Spectra of α-L-Glutamyl-L-alanine in Acidic, Aqueous and Basic Solutions.
See the note of Table I for analytical conditions.

upfield shift in the basic condition (about 0.6 ppm, Table II, column D and E) and downfield shift in the acidic condition (about 0.3 ppm, Table II, column A and B) and α-proton of C-terminal residue shifted upfield in the basic solution (about 0.15 ppm, Table II, column D and E) contrary to the case with α-glutamyl peptides. In the dipeptides containing glutamic acid as the C-terminal moiety, the spectral line of glutamyl α-H shifted downfield in the acidic solution (Table III, column A and B) and α-H of the N-terminal moiety shifted markedly upfield in the basic solution

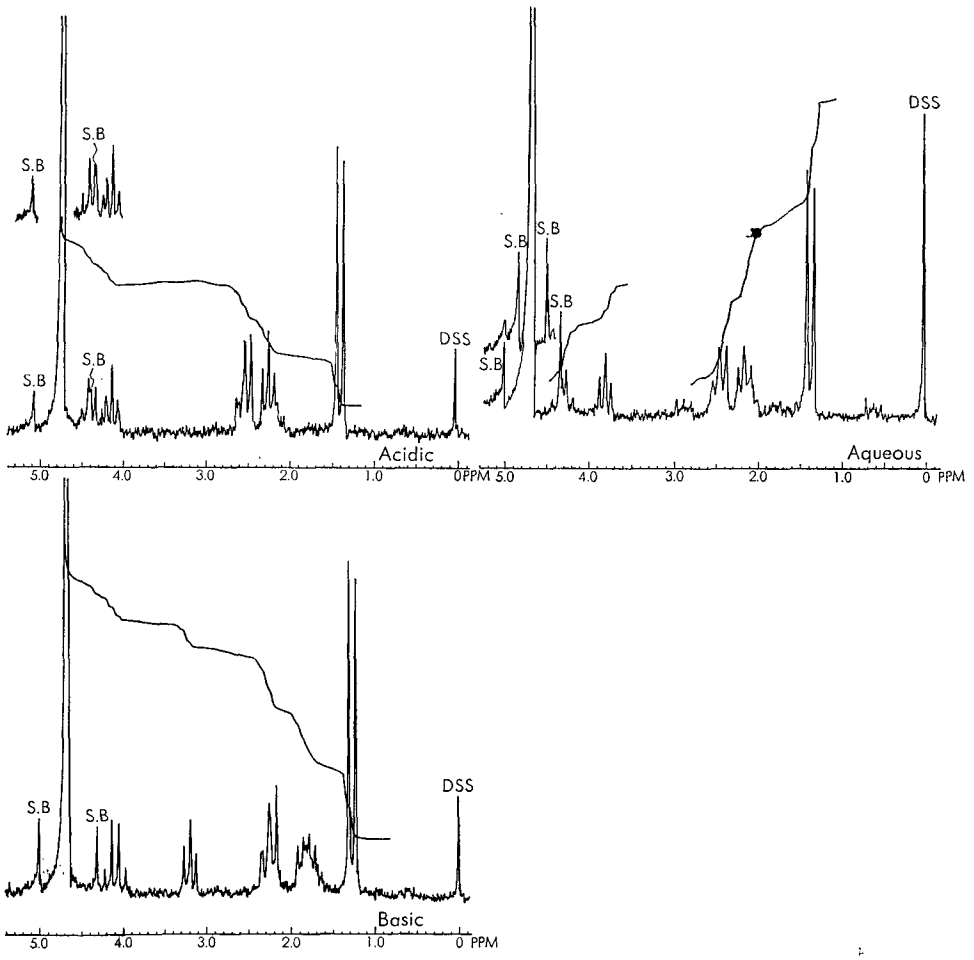

Fig. 3. NMR Spectra of γ-L-Glutamyl-L-alanine in Acidic, Aqueous and Basic Solutions.
See the note of Table II for analytical conditions.

(Table III, column D and E). These shifts coincided with data obtained for peptides containing α-monoamino monocarboxylic acid as their N-terminal moieties[25].

Furthermore, glutamyl α-H of α-glutamyl dipeptides resonates at lower field and that of γ-glutamyl dipeptides resonates at higher field than δ 3.9 in an aqueous solution (Table IV). This phenomenon should reflect the different chemical surroundings of glutamyl α-protons. Glutamyl α-H of γ-glutamyl dipeptides is surrounded by both cation ($-NH_3^+$) and anion ($-COO^-$), but that of α-glutamyl dipeptides has only adjacent cation ($-NH_3^+$) in aqueous solution (Fig. 1).

From these data it should be possible to determine the sequence of a dipeptide containing glutamic acid and also decide whether the glutamyl bond is α or γ when glutamic acid is the N-terminal component, by measuring the NMR spectra of the dipeptides in acidic, aqueous and basic

TABLE I. Chemical Shifts of Glutamyl α-CH and α-CH of the C-Terminal Amino Acid Residue of α-Glutamyl Dipeptides (δ_{DSS}, Amino acids which constitute the dipeptides are all in L-form.)

			A In DCl[a]	B (A)-(C)	C In D_2O[b]	D (C)-(E)	E In NaOD[c]
α-Glutamylglycine	Glutamyl	α-CH	4.14	0.07	4.07	0.72	3.35
	Glycyl	α-CH	4.05	0.18	3.87[d]	0.12	3.75[d]
α-Glutamylalanine	Glutamyl	α-CH	4.08	0.07	4.01	0.67	3.34
	Alanyl	α-CH	4.48	0.25	4.23	0.10	4.13
α-Glutamylvaline	Glutamyl	α-CH	4.18	0.08	4.09	0.69	3.40
	Valyl	α-CH	4.30	0.21	4.09	0.05	4.04
α-Glutamylleucine	Glutamyl	α-CH	4.12	0.10	4.02	0.67	3.35
	Leucyl	α-CH	4.44	0.22	4.22	0.04	4.18
α-Glutamylasparagine	Glutamyl	α-CH	4.12	0.10	4.02	0.67	3.35
	Asparagyl	α-CH	[e]		4.52	0.04	4.48
α-Glutamyl-α-amino-isobutyric acid	Glutamyl	α-CH	4.02	0.08	3.94	0.69	3.25
α-Glutamylisopropyl-amide	Glutamyl	α-CH	3.95	0.05	3.90	0.65	3.25
Isoglutamine		α-CH	4.08	0.06	4.02	0.67	3.35

a) D_2O+DCl, pH<1.5, cationic form.
b) Zwitter ionic form.
c) D_2O+NaOD,pH>13, anionic form.
d) Average value of unequivalent two methylene protons giving four lines of AB pattern.
e) Overlapped with DOH signal.

TABLE II. Chemical Shifts of Glutamyl α-CH and α-CH of the C-Terminal Amino Acid Residue of γ-Glutamyl Dipeptides (δ_{DSS}, Amino acids which constitute the dipeptides are all in L-form.)

			A In DCl[a]	B (A)-(C)	C In D_2O[b]	D (C)-(E)	E In NaOD[c]
γ-Glutamylglycine	Glutamyl	α-CH	4.13	0.31	3.82	0.62	3.20
	Glycyl	α-CH	3.99	0.05	3.94	0.21	3.73
γ-Glutamylalanine	Glutamyl	α-CH	4.11	0.33	3.78	0.58	3.20
	Alanyl	α-CH	4.35	0.09	4.26	0.16	4.10
γ-Glutamylvaline	Glutamyl	α-CH	4.10	0.32	3.78	0.57	3.21
	Valyl	α-CH	4.24	0.06	4.18	0.14	4.04
γ-Glutamylleucine	Glutamyl	α-CH	4.10	0.32	3.78	0.59	3.19
	Leucyl	α-CH	4.36	0.04	4.32	0.17	4.15
γ-Glutamylasparagine	Glutamyl	α-CH	4.10	0.32	3.78	0.59	3.19
	Asparagyl	α-CH	d)		d)		4.50
γ-Glutamyl-α-amino-isobutyric acid	Glutamyl	α-CH	4.09	0.27	3.82	0.63	3.19
γ-Glutamylisopropyl-amide	Glutamyl	α-CH	4.10	0.36	3.74	0.54	3.20
Glutamine		α-CH	4.10	0.35	3.75	0.55	3.20

a) D_2O+DCl, pH<1.5, cationic form.
b) Zwitter ionic form.
c) D_2O+NaOD, pH>13, anionic form.
d) Overlapped with DOH signal.

TABLE III. Chemical Shifts of Glutamyl α-CH and α-CH of the N-Terminal Amino Acid Residue of the Dipeptides Containing Glutamic Acid as the C-Terminal Component (δ_{DSS}, valyl and glutamyl Residues are in L-form.)

			A In DCl[a]	B (A)-(C)	C In D_2O[b]	D (C)-(E)	E In NaOD[c]
Glycylglutamic acid	Glycyl	α-CH	3.86	0.02	3.84	0.53	3.31
	Glutamyl	α-CH	4.52	0.26	4.26	0.14	4.12
Valylglutamic acid	Valyl	α-CH	3.86	0.05	3.81	0.68	3.13
	Glutamyl	α-CH	4.48	0.22	4.26	0.13	4.13

a) D_2O+DCl, pH<1.5, cationic form.
b) Zwitterionic form.
c) D_2O+NaOD, pH>13, anionic form.

TABLE IV. Chemical Shifts of Glutamyl α-CH of α- and γ-Glutamyl Dipeptides ($\delta^{D_2O}_{DSS}$, Amino acids which constitute the dipeptides are all in L-form.)

	$\delta^{D_2O}_{DSS}\alpha$-CH	
	α-	γ-
Glutamylleucine	4.02	3.78
Glutamylvaline	4.09	3.78
Glutamylalanine	4.01	3.78
Glutamyl-α-aminoisobutyric acid	3.94	3.82
Glutamylphenylalanine	3.93	3.70
Glutamylthreonine	4.16	3.81
Glutamylproline	4.00	3.80
Glutamylasparagine	4.02	3.78
Glutamylasparic acid	4.13	3.75
Glutamyl-β-alanine	3.94	3.71
Glutamylglyeine	4.07	3.82
Gluamylisopropylamide	3.90	3.74
Isoglutamine	4.02	
Glutamine		3.75
Glutamyltyrosine	*	3.70
Glutamylmethionine	*	3.84
Glutamylmethioninesulfoxide	*	3.82
Glutamylglutamic acid	*	3.86

* Not measuaed.

TABLE V. Chemical Shifts of Aspartyl α-CH of α- and β-L-Aspartyl-L-serine (δ_{DSS}).

	A in DCl[a]	B (A)-(C)	C in D_2O[b]	D (C)-(E)	E in NaOD[c]
α-Aspartylserine	4.45	0.07	4.38	0.67	3.71
β-Aspartylserine	4.40	0.29	4.11	0.53	3.58

a) D_2O+DCl, pH<1.5, cationic form.
b) Zwitter ionic form.
c) D_2O+NaOD, pH>13, anionic form.

soolutions. The method is the most useful in the facts that samples are not destroyed and gives an unequivocal result with a few mg of quantity. Kristensen *et al.* have also obtained the similar results[16].

The same rule should hold also for dipeptides containing α-amino-α, ω-dicarboxylic acid other than glutamic acid. For example, NMR spectra of α- and β-aspartylserine in acidic, aqueous and basic solutions are shown in Fig. 4 and 5, and summarized in Table V.

An interesting differences in NMR spectra between α- and γ-glutamyl-

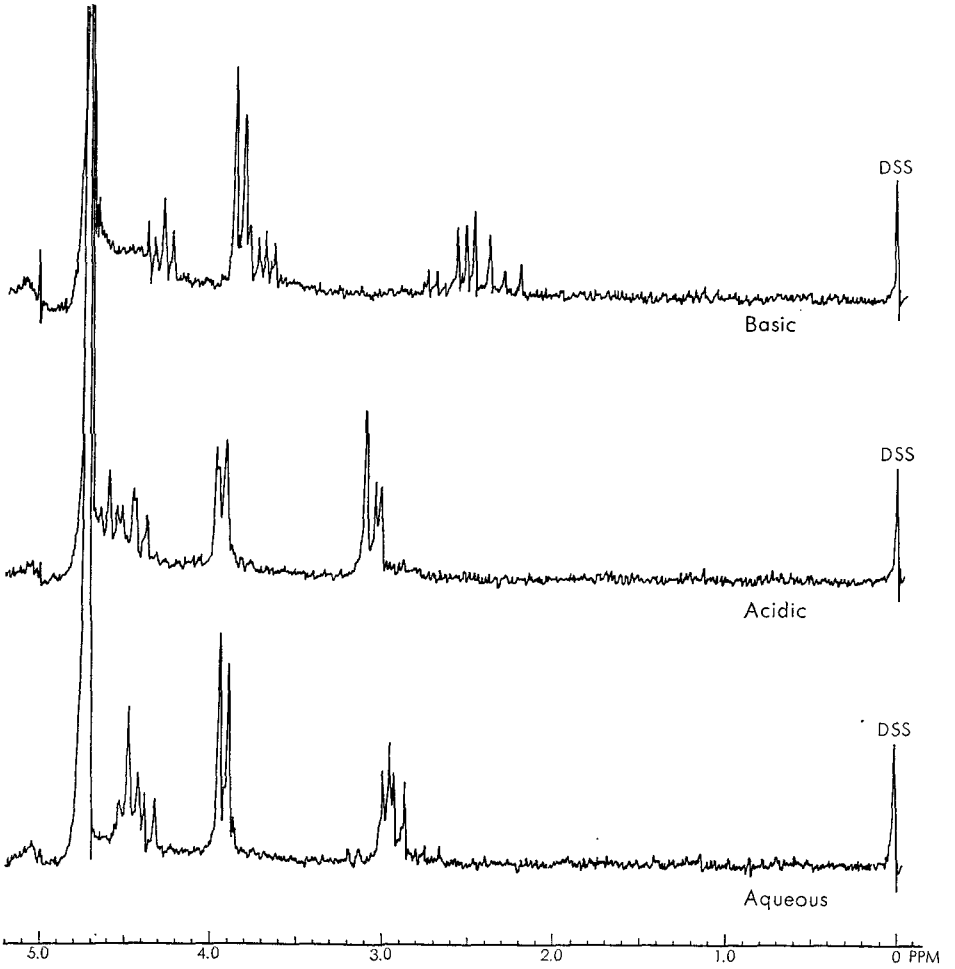

Fig. 4. NMR Spectra of α-L-Aspartyl-L-serine in Acidic, Aqueous and Basic Solutions.
See the note of Table V for analytical conditions.

α-aminobutyric acid were observed. As shown in Table VI and Fig. 6, two methyl groups of α-glutamyl-α-aminoisobutyric acid, which were equivalent in an acidic solution, became unequivalent in the aqueous and basic solutions. But two methyl groups of α-glutamylisopropylamine corresponding to the compound which lost the C-terminal carboxyl group of α-glutamyl-α-aminoisobutyric acid were always equivalent in cationic, zwitter ionic and anionic forms. Two methyl groups of N-glutaryl-α-aminoisobutyric acid corresponding to the compound which lost the N-

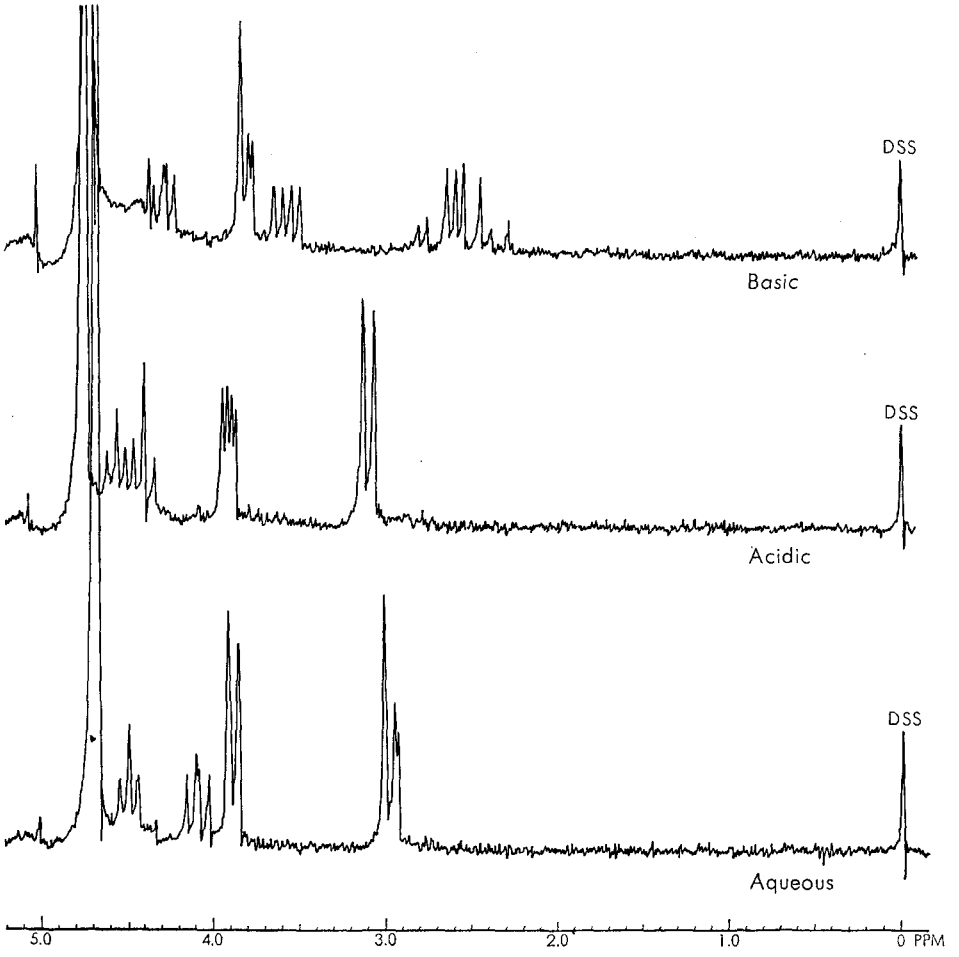

Fig. 5. NMR Spectra of β-L-Aspartyl-L-serine in Acidic, Aqueous and Basic Solutions.
See the note of Table V for analytical condition.

terminal amino group of α-glutamyl-α-aminoisobutyric acid were also equivalent in any ionic from. The unequivalence of the two methyl groups was also not recognized in the case of N-glutarylisopropylamine having no N-terminal amino group and C-terminal carboxyl group. Two methyl groups of γ-glutamyl-α-aminoisobutyric acid (Fig. 7) and γ-glutamylisopropylamide were invariably equivalent. Consequently the presence of α-amino group and ionized C-terminal carboxyl group seems to be required for the appearance of unequivalence of two methyl groups of α-glutamyl-α-amino-

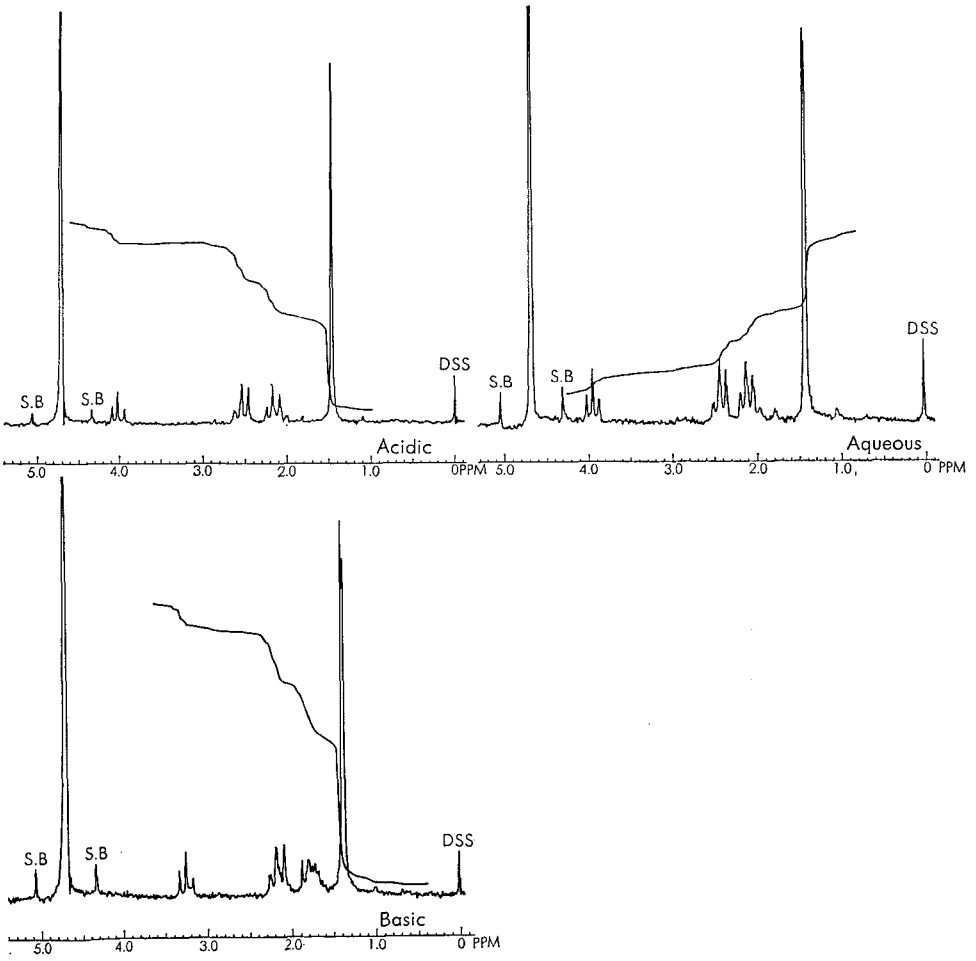

Fig. 6. NMR Spectra of α-L-Glutamyl-α-aminoisobutyric acid in Acidic, Aqueous and Basic Solutions.
See the note of Table VI for analytical conditions.

TABLE VI. Chemical Shifts of Methyl Groups of α- and γ-L-Glutamyl-α-aminoisobutyric acid and Some Related Compounds (Hz from DSS as an internal standard, 90 MHz.)

	In DCl Solution[a]	In D_2O Solution[b]	In NaOD Solution[c]
α-L-Glutamyl-α-amino-isobutyric acid (1)	131 (s, 6H)	126 (s, 3H), 125 (s, 3H)	125 (s, 3H), 123 (s, 3H)
γ-L-Glutamyl-α-amino-isobutyric acid (2)	128 (s, 6H)	130 (s, 6H)	121 (s, 6H)
α-L-Glutamylisopro-pylamide (3)	102 (d, J=7Hz, 6H)	102 (d, J=7Hz, 6H)	100 (d, J=7Hz, 6H)
γ-L-Glutamylisopro-pylamide (4)	98 (d, J=7Hz, 6H)	98 (d, J=7Hz, 6H)	98 (d, J=7Hz, 6H)
N-Glutaryl-α-amino-isobutyric aicd (5)	128 (s, 6H)	128 (s, 6H)[d]	123 (s, 6H)
N-Glutarylisopro-pylamine (6)	88 (d, J=7Hz, 6H)	98 (d, J=7Hz, 6H)[d]	99 (d, J=7Hz, 6H)

$$HOOCCHCH_2CHCONHC(CH_3)_2$$
$$\quad\quad R_1 \quad\quad R_2 \quad\quad\quad R_3$$

(1) R_1=H, R_2=NH_2, R_3=COOH.
(2) R_1=NH_2, R_2=H, R_3=COOH.
(3) R_1=H, R_2=NH_2, R_3=H.
(4) R_1=NH_2, R_2=H, R_3=H.
(5) R_1=R_2=H, R_3=COOH.
(6) R_1=R_2=R_3=H.

a) D_2O+DCl, pH<1.5, cationic form.
b) Zwitter ionic form.
c) Carboxy group does not dissociate as in acidic solution.
d) D_2O+NaOD, pH>13, anionic form.

TABLE VII. Chemical Shifts of Glycyl Methylene Protones of α- and γ-L-Glutamylglycine (Hz from DSS as an internal standard, 90 MHz)

	In DCl Solution[a]	In DOSolution[b]	In NaOD Solution[c]
α-L-Glutamylglycine	365 (s, 2H)	352 (d, J=18Hz, 1H) 342 (d, J=18Hz, 1H)	341 (d, J=17Hz, 1H) 332 (d, J=17Hz, 1H)
γ-L-Glutamylglycine	359 (s, 2H)	354 (s, 2H)	334 (s, 2H)

a) D_2O+DCl, pH<1.5, cationic form.
b) Zwitter ionic form.
c) D_2O+NaOD, pH>13, anionic form.

isobutyric acid.

The same phenomenon was manifested for glycyl methylene group of glutamylglycine (Table VII, Fig. 8 and 9). The glycyl methylene protons of α-glutamylglycine which were equivalent in the acidic solution became unequivalent in the aqueous and basic solutions and gave four lines of AB pattern due to geminal coupling. The same conditions as in the case of α-glutamyl-α-aminoisobutyric acid may be required for the appearance of the unequivalence of the glycyl methylene protons of α-glutamylglycine,

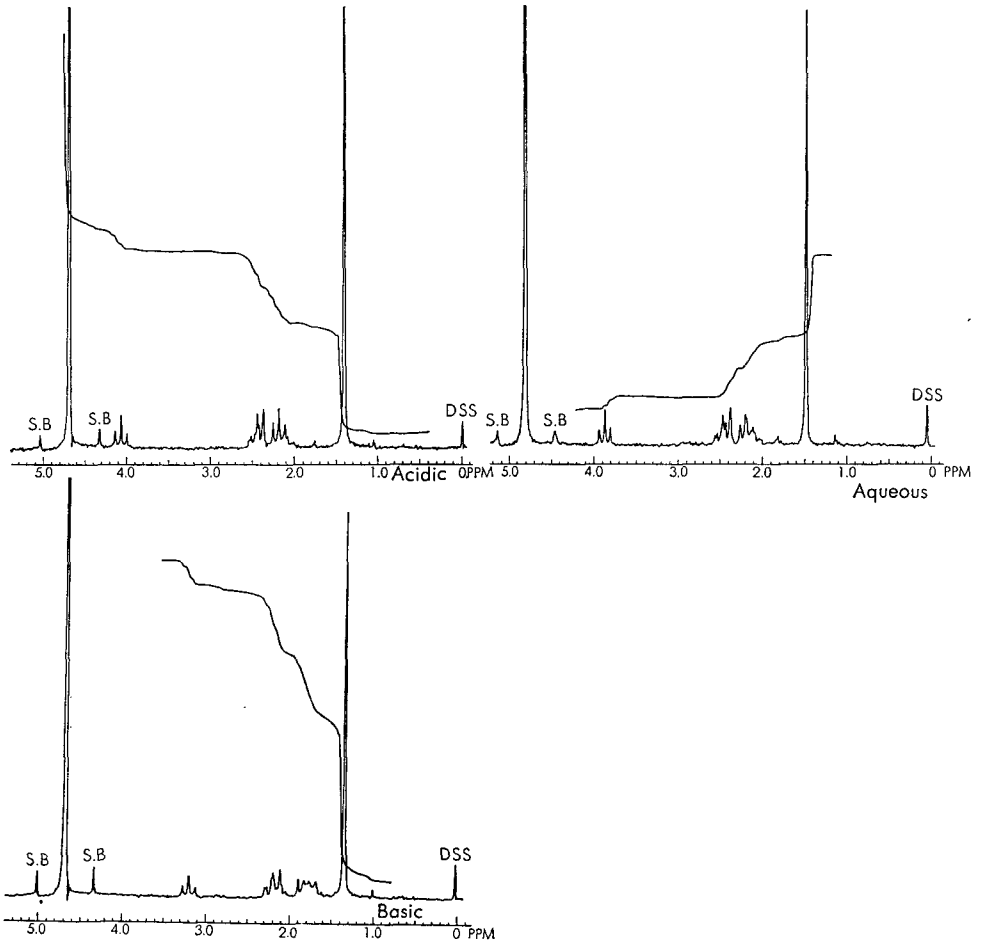

Fig. 7. NMR Spectra of γ-L-Glutamyl-α-aminoisobutyric acid in Acidic, Aqueous and Basic Solutions.
See the note of Table VI for analytical conditions.

namely, α-amino and ionized C-terminal carboxyl groups are essential. The unequivalence of glycyl methylene protons has been observed in a many kind of dipeptides containing glycine as C-terminal constituent[2,18,19,21,22].

The observed unequivalence should reflect the restriction of rotation in molecules by interaction between α-amino group and ionized C-terminal carboxyl group. It seems unlikely that the unequivalence of the two methyl groups of α-glutamyl-α-aminoisobutyric acid and that of the glycyl methylene protons of α-glutamylglycine are due to the influence of the

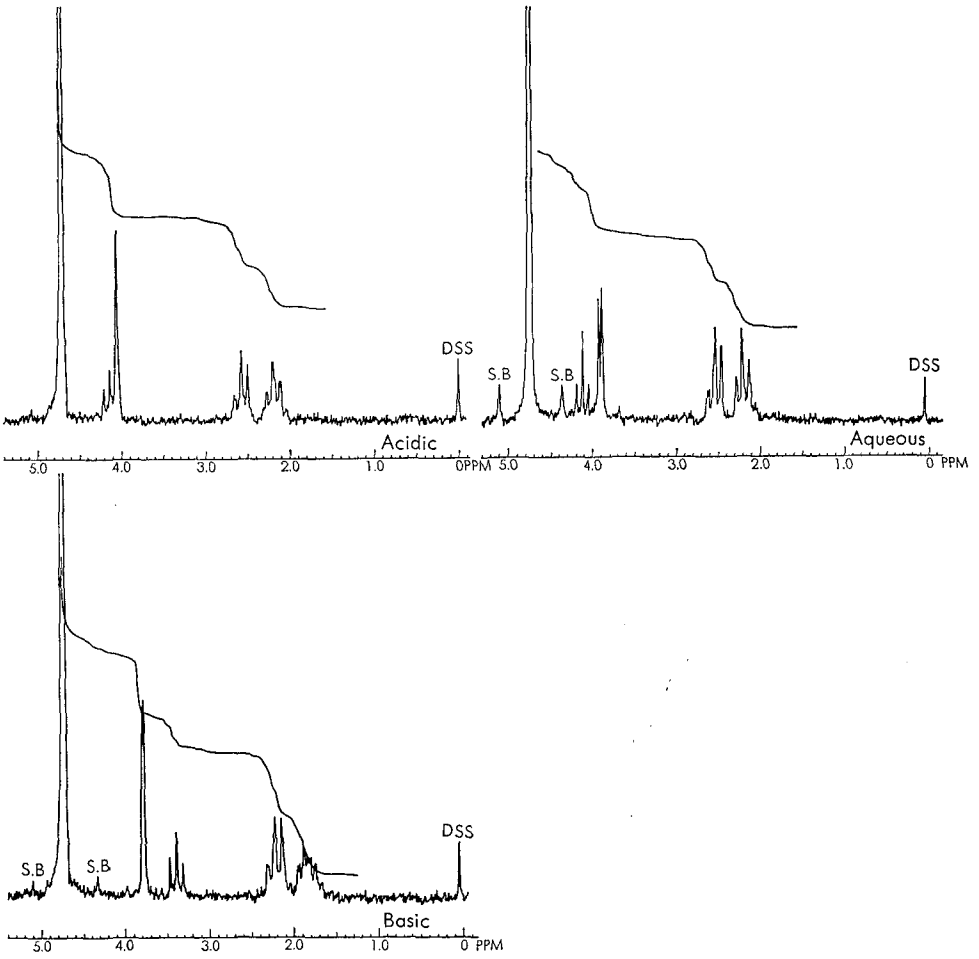

Fig. 8. NMR Spectra of α-L-Glutamylglycine in Acidic, Aqueous and Basic Solutions.
See the note Table VII for analytical conditions.

cis-trans isomerism of the peptide bond[27], because such an unequivalence is not manifested in α- and γ-glutamylisopropylamide, γ-glutamyl-α-aminoisobutyric acid, N-glutaryl-α-aminoisobutyric acid, N-glutarylisopropylamine and γ-glutamylglycine. It is also not interpreted by the *cis-trans* isomerism of the peptide bond that the glycyl methylene protons of α-glutamylglycine give four lines of AB pattern and that the peak areas of unequivalent two methyl groups of α-glutamyl-α-aminoisobutyric acid are equal. If the unequivalence of the two methyl groups of α-glutamyl-α-aminoisobutyric

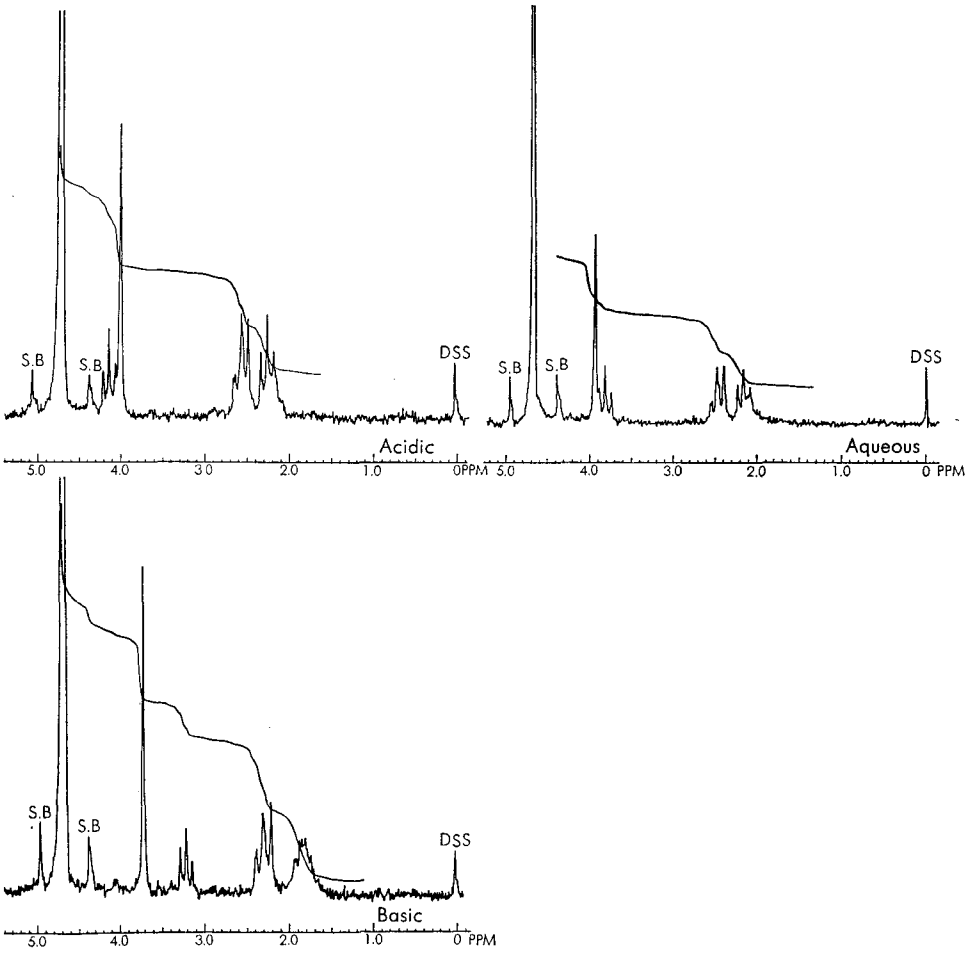

Fig. 9. NMR Spectra of γ-L-Glutamylglycine in Acidic, Aqueous and Basic Solutions.
See the note of Table VII for analytical conditions.

acid were due to the *cis-trans* isomerism of the peptide bond, the amount of *cis*-form should be equal to that of *trans*-form, because the peak areas of these unequivalent two methyl groups are equal. But LaPlanche *et al.* have described[17] that eighty-eight percent of N-isopropylformamide exists in *trans*-form, and N-isopropylacetamide and N-isopropylisobutylamide are present only in *trans*-form. According to LaPlanche *et al.* it is not conceivable that a half of α-glutamyl-α-aminoisobutyric acid having larger substituents on both of amide-carbon and nitrogen atoms than in the case of N-isopropylacetamide exists in *cis*-form.

IR Spectra

Carbonyl stretching vibration of carboxyl group of α-amino acid hydrochloride appears at higher wave number by influence of ionized amino group on α-position than that of usual carboxyl group, but the effect of the amino group is no longer observed in the case of β-amino acid. This fact suggests that carbonyl stretching vibration of peptide bond (amide I) of α-glutamyl dipeptides which has an amino group on α-position appears at higher wave number than that of γ-glutamyl dipeptides having no amino group on α-position. This validity was judged by experimental results. As indicated in Table VIII, carbonyl stretching vibration of α-glutamyl peptide bond was observed at higher wave number (1685~1665 cm^{-1}) than that of γ-glutamyl peptide bond (1660~1635 cm^{-1}). Amide II band was indistinguishable from two other strong absorption of $-NH_3^+$ group (deformation) and $-COO^-$ group (stretching) occurring near amide II region.

NH Stretching vibration of α-glutamyl bond was observed at lower wave number than that of corresponding γ-glutamyl bond. For example, IR spectra of glutamyl-α-aminoisobutyric acid and some related compounds were illustrated in Fig. 10. Amide I of α-glutamyl-α-aminoisobutyric acid (1685 cm^-) appeared at higher wave number than that of corresponding γ-isomer (1655 cm^-). The same rule holded for α- and γ-glutamylisopropylamide (1685 cm^{-1} for α- and 1640 cm^{-1} for γ-). Amide I of N-glutaryl-α-aminoisobutyric acid which has not N-terminal amino group and N-glutarylisopropylamine having no N-terminal amino and C-terminal carboxyl groups were observed in lower wave number region (1620 cm^{-1} and 1640 cm^{-1}, respectively). On the other hand, NH stretching vibration of peptide bond of both α-glutamyl-α-aminoisobutyric acid and α-glutamylisopropylamide appeared at 3280 cm^{-1} and the value was lower than those of their corresponding γ-isomers (3370 cm^{-1} for γ-glutamyl-α-aminoisobutyric acid and 3330 cm^{-1} for γ-glutamylisopropylamide). Furthermore, NH stretching vibration of γ-glutamyl-α-aminoisobutyric acid was at the same region

TABLE VIII. Carbonyl and NH Stretching Vibrations of Peptide Bond of α- and γ-Glutamyl Dipeptides and Some Related Compounds (cm^{-1}, KBr disks. Amino acids which constitute the dipeptides are all in L-form.)

	NH	CO
α-Glutamylleucine	3250	1680
γ-Glutamylleucine	3350	1650
α-Glutamylvaline	3260	1675
γ-Glutamylvaline	3350	1645
Valyglutamic acid	3250	1680
α-Glutamylalanine	3250	1675
γ-Glutamylalanine	3350	1655
α-Glutamylglycine	3320	1685
γ-Glutamylglycine	3350	1650
α-Glutamylaspartic acid	*	1670
γ-Gluatmylaspartic acid	*	1635
α-Glutamylphenylalanine	3230	1670
γ-Glutamylphenylalanine	3350	1655
α-Glutamyl-β-phenyl-β-alanine	*	1665
γ-Glutamyl-β-phenyl-β-alanine	3330	1640
α-Glutamyl-β-alanine	3330	1680
γ-Glutamyl-β-alanine	3380	1650
α-Glutamyl-α-aminoisobutyric acid	3280	1685
γ-Glutamyl-α-aminoisobutyric acid	3370	1655
N-Glutaryl-α-aminoisobutyric acid	3370	1620
N-Glutarylisopropylamine	3330	1640
α-Glutamylisopropylamide	3280	1685
γ-Glutamylisopropylamide	3330	1640
γ-Glutamyltyrosine	*	1660
γ-Glutamylmethionine	3360	1660
γ-Glutamylmethioninesulfoxide	*	1640
γ-Glutamylglutamic acid	3380	1640
N^5-(4′-Hydroxybenzyl)glutamine	3290	1645

* Overlapped with other absorption.

as the N-glutaryl-α-aminoisobutyric acid (3370 cm^{-1}) which corresponded to the deaminated derivative of the former. The same relation holded for γ-glutamylisopropylamide and its deaminated derivative, N-glutarylisopropylamine (both 3330 cm^{-1}).

Amide I band of N-glutaryl-α-aminoisobutyric acid appeared at particularly low wave number (1620 cm^{-1}), so that IR spectra of some N-acetyl-

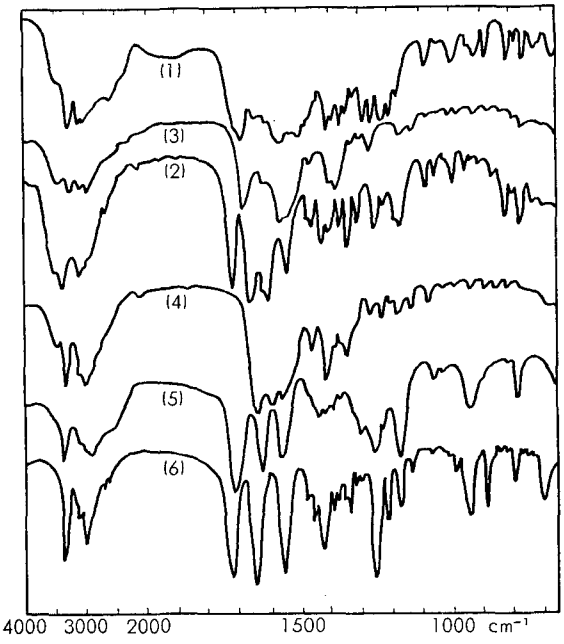

Fig. 10. IR Spectra of α-L-Glutamyl-α-aminoisobutyric acid (1), γ-L-Glutamyl-α-aminoisobutyric acid (2), α-L-Glutamylisopropylamide (3), γ-L-Glutamylisopropylamide (4), N-Glutaryl-α-aminosiobutyric acid (5) and N-Glutarylisopropylamine (6) (KBr disks).

TABLE IX. Carbonyl and NH Stretching Vibrations of Amino Bond of N-Acetyl Amino Acids (cm^{-1}, KBr disks. Amino acids are all in L-form)

	NH	CO
N-Acetylleucine	3350	1620
N-Acetyltyrosine	3370	1595
N-Acetylglycine	3370	1585
N-Acetyl-α-aminoisobutyric acid	3380	1620

amino acids were measured for comparison (Table IX). Carbonyl stretching vibrations of N-acetyl group of N-acetylamino acids were observed near 1600 cm^{-1}.

Amide I band of α-aspartyl dipeptides was observed at higher wave number than that of corresponding β-isomer as in the case of α- and γ-glutamyl dipeptides (Table X).

TABLE X. Carbonyl Stretching Vibration of Peptide Bond of α- and β-Aspartyl Dipeptides (cm^{-1}, KBr disks. Aspartyl, seryl and threonyl residues are in L-form.)

	CO
α-Aspartylserine	1665
β-Aspartylserine	1645
α-Aspartylthreonine	1660
β-Aspartylthreonine	1640

NH Stretching vibration overlapped with other absorption.

Elution Behavior during Ion Exchange Chromatography

The γ-glutamyl-α-amino acids have lower pK_2-values than α-glutamyl-α-amino acids. During ion exchange chromatography with strongly basic ion exchange resin in the acetate form, α-glutamyl dipeptide is always eluted before its γ-isomer with acetic acid, because the elution order is determined by the pK_2-value[16]. The same rule holds for α- and β-aspartyl dipeptides[4]. Thus the mixture of α- and γ-glutamyl dipeptides or α- and β-aspartyl dipeptides can be separated to each compound with the use of strongly basic ion exchange resin in the acetate form and acetic acid as eluent.

The opposite elution pattrn is expected when the chromatography is carried out with a strongly acidic ion exchange resin, namely, γ-glutamyl dipeptides should be eluted before its α-isomer, because γ-glutamyl peptides have α-amino acid unit and α-isomes have not. As a column of an amino acid analyzer is packed with strongly acidic ion exchange resin (Amberlite CG–120), we analyzed a many kind of α- and γ-glutamyl peptides with an amino acid analyzer. The elution time of α- and γ-glutamyl peptides are shown schematically in Fig. 11; together with that of the C-terminal amino acid of each peptide. γ-Glutamyl dipeptide was eluted faster and its α-isomer was slower than individual amino acid constituting the C-terminal

residue of the peptide, except for α-glutamyl-β-alanine which was eluted faster than β-alanine. The elution pattern of glutamic acid-containing peptides used so far are summarized in Fig. 12, together with that of a standard amino acid mixture.

During an analysis of γ-glutamylaspartic acid with an amino acid analyzer, we noticed that the peptide was eluted as two peaks under certain conditions. As shown in Fig. 13, γ-glutamylaspartic acid was eluted as a single peak at 60 min when the volume of sample solution applied to the column was 0.3 ml or less, and also as asingle peak at 74 min when the volume applied was 0.7 ml or more, but the peptide appeared as two peaks (at 60 and 74 min) when the volume of sample applied was in the range of 0.4 to 0.6 ml. The change in elution behavior of γ-glutamylaspartic acid by alterations in the volume of sample solution applied to the column may be due to a change in its ionization state, for the sample is dissolved in 0.2N Na-citrate buffer (pH 2.2), loaded on an analyzer and developed with 0.2N Na-citrate buffer (pH 3.25) under a standard condition[28]. This idea was supported by the fact that γ-glutamylaspartic acid gave always one peak at 57 min regardless of the sampling volume when

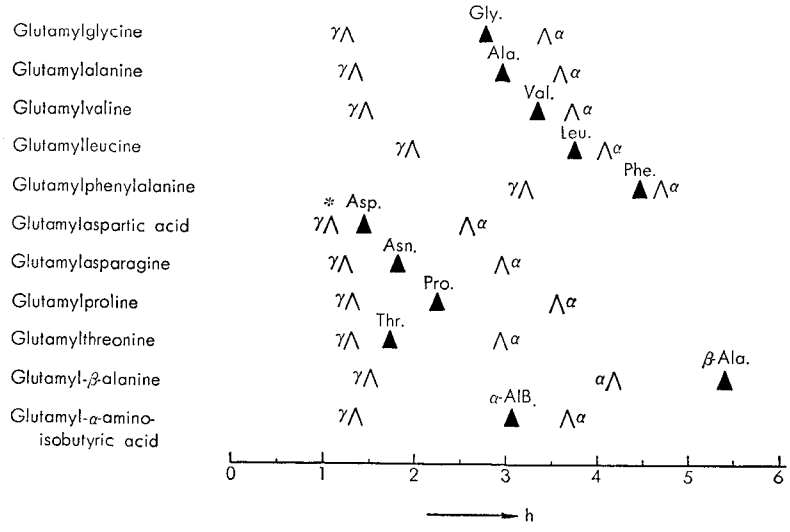

Fig. 11. Elution Time of α- and γ-Glutamyl Dipeptides.
Amino acids which constitute the dipeptides are all in L-form.
Analytical conditions. Column; Amberlite CG-120 (400 mesh) (0.9×50 cm). Column temperature; 50°C. Eluent; 0.2 N Na-citrate buffer (pH 3.25 followed by 0.2 N Na-citrate buffer (pH 4.25) at 90 min. Flow rate; 30 ml/h.
* Sampling volume is 0.3 ml. See Fig. 13 for more detail.

the peptide was dissolved in 0.2N Na-citrate buffer (pH 3.25) and developed with the same buffer. Other α- and γ-glutamyl peptides examined so far were eluted as one peak when 0.5 ml of sample solution dissolved in pH 2.2 buffer was applied and developed with pH 3.25 buffer. Attention should be drawn to the sampling volume when quantitative or qualitative analysis of this peptide is carried out with the use of an amino acid analyzer.

Zacharius and Talley have reported the elution behavior of many kinds of ninhydrin-positive compounds, including several γ-glutamyl peptides, during ion exchange chromatography[29]. Arai *et al.* have described the

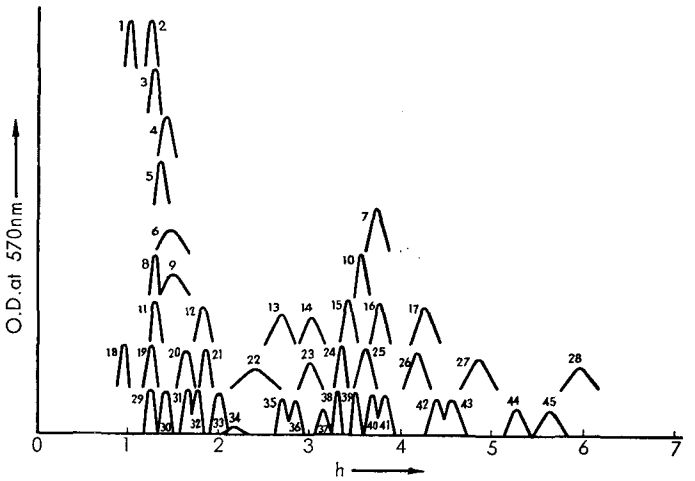

Fig. 12. Elution Patterns of α- and γ-Glutamyl Dipeptides and Free Amino Acids.

Amino acids which constitute the peptides are all in L-form. See the note of Fig. 11 for analytical conditions.

1, γ-glutamylaspartic acid (sampling volume, 0.3 ml). 2, γ-glutamylaspartic acid (sampling volume, 0.7 ml). 3, γ-glutamyl-γ-glutamylmethionine. 4, γ-glutamyl-α-amino-isobutyric acid. 5, γ-glutamylalanine. 6, γ-glutamylproline. 7, α-glutamyl-α-amino-isobutyaic acid. 8, γ-glutamylglycine. 9, γ-glutamylvaline. 10, α-glutamylalanine. 11. γ-glutamylthreonine. 12, γ-glutamylmethionine. 13, α-glutamylaspartic ocid. 14, α-glutamylasparagine. 15, α-glutamylglycine. 16, α-glutamylvaline. 17, α-glutamyl-β-alanine. 18, γ-glutamylmothionine sulfoxide. 19, γ-glutamylasparagine. 20, γ-glutamyl-β-alanine. 21, γ-glutamylisopropylamide. 22, γ-glutamylleucine. 23, α-glutamylthreonine. 24, γ-glutamyltyrosine and γ-glutamylphenylalanine. 25, α-glutamylproline. 26, α-glutamylleucine. 27, α-glutamylphenylalanine. 28, α-glutamylisopropylamide, 29, methionine sulfoxide. 30, aspartic acid. 31, threonine. 32, serine, asparagine and glutamine. 33, glutamic acid. 34, proline. 35, glycine. 36, alanine. 37, α-aminoisob utyric acid. 38, valine. 39, methionine. 40, isoleucine. 41, leucine. 42, tyrosine. 43, phenylalanine. 44, β-alanine. 45, isoglutamine.

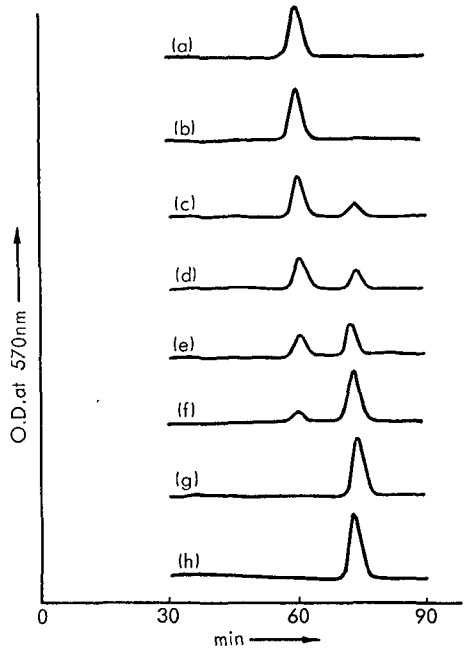

Fig. 13. Elution Behavior of γ-L-Glutamyl-L-aspartic acid.

Ten μmoles of γ-glutamylaspartic acid were dissolved in 10 ml of 0.2N Na-citrate buffer (pH, 2.2); 0.2 ml portions of the solution were diluted with 0.2, 0.4, 0.6, 0.7, 0.8, 1.0, 1.2 and 1.4 ml of the same buffer solution and half of each diluted sample, which contained 0.1 μmole of γ-glutamylaspartic acid, was loaded on the colume, then 1.0 ml of 0.01N hydrochloric acid was added, and elution was carried out as described in the note of Fig. 11.

elution patterns of twelve α-glutamyl peptides in relation to their tastes[1].

Summary

1. γ-Glutamyl dipeptide could be distinguished easily and clearly from its α-isomer by measuring the NMR spectra of the peptide in acidic, queous and basic solutions.

2. Characteristic difference in NMR spectra pattern between some α- and γ-glutamyl dipeptides in aqueous and basic conditions was detailed.

3. In IR spectra, difference in carbonyl and NH stretching vibrations between α- and γ-glutamyl dipeptides were illustrated.

4. Distinction between α- and γ-glutamyl dipeptides by means of an amino acid an alyzer was also described and discussed from view point of their analyses.

5. An elution behavior of γ-glutamylaspartic acid changed with alteration in the volume of sample solution applied to the column of an amino acid analyzer.

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