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# Dipeptide의 陽性子 磁氣共鳴硏究*

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# Proton Magnetic Resonance Studies of Dipeptides

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**요 약.** Glycine이 포함된 dipeptide인 glycyl-*L*-valine, *L*-valyl-glycine, glycyl-*DL*-alanine, glycyl-*DL*-serine 및 glycyl-*L*-aspartic acid의 중수용액속에서의 양성자 자기공명스펙트럼이 실온에서 pH의 함수로 연구되었다. 이들 스펙트럼의 분석으로부터 $C_\alpha H$, $C_\beta H$ 및 $C_\gamma H$ 양성자의 chemical shift는 pH에 따라 일단계의 적정곡선으로 변하고 있으며 spin-spin coupling constant는 거의 변하지 않고 있음을 알 수 있다. Dipeptide 용액속에 있는 구성 아미노산의 $C_\alpha H$ $C_\beta H$ 및 $C_\gamma H$ 양성자의 chemical shift가 각각 두개의 다른 값들로 나타나는것은 이들 용액속에 두개의 자기적으로 서로 다른 site들이 들어있음을 말해준다. 이 연구에서는 다섯개 dipeptide의 구조를 양성자 자기공명스펙트럼에 의해 확인하였으며 결과는 앞으로 oligopeptide에서의 구조 변화, conformation, 구성 아미노산의 순서 결정등을 양성자 자기공명스펙트럼의 분석에 의해 알아낼 수 있음을 암시하고 있다.

**ABSTRACT.** Proton magnetic resonance spectra of five glycine-containing dipeptides glycyl-*L*-valine, *L*-valyl-glycine, glycyl-*DL*-alanine, glycyl-*DL*-serine and glycyl-*L*-aspartic acid in $D_2O$ were investigated as a function of pH at room temperature. From the analysis of the spectra, it was found that the chemical shift of the $C_\alpha H$, $C_\beta H$ and $C_\gamma H$ protons varies with pH as a one-step titration curve, and that the spin-spin coupling constant remains almost unchanged. Two distinct values of the chemical shift for $C_\alpha H$, $C_\beta H$ or $C_\gamma H$ protons of constituent amino acids in dipeptide solutions indicate the existence of two magnetically non-equivalent sites in solution. From this study, the structures of the five dipeptides have been confirmed by proton magnetic resonance spectra and it has been suggested that the structural change, conformation and sequence determination can be explored for oligopeptides by an analysis of proton magnetic resonance spectra.

## 1. INTRODUCTION

Over the past decade high-resolution nuclear magnetic resonance (NMR) spectroscopy has become firmly established as one of the most powerful methods for structural analysis in both organic chemistry and biochemistry. In particular, high-resolution NMR spectra from an aqueous solution of amino acids and dipeptides can be completely analyzed to give the structure of these materials.

Nakamura and Jardetzky[1,2] have given some details of the NMR spectra of dipeptides in $D_2O$. However they did not study the effect of pH on NMR spectra in detail and most of earlier investigations[1~6] were restricted to a qualitative analysis of the proton NMR spectra. Recently, the first theoretical analysis of the proton magnetic resonance spectra of several amino acids has been successfully carried out by Rhee[7] using a quantum mechanical calculation of NMR transition frequencies and transition probabilities between spin states. For the analysis of proton magnetic resonance spectra of amino acids[7] and dipeptides[8,9] which contain more than ten protons, the method of "summation of distinct spectra" (SDS) has been applied to a first approximation, and has been validified[7,8].

In this paper, proton magnetic resonance spectra of five glycine-containing dipeptides were investigated as a function of pH in detail at room temperature. The proton resonance spectra were analyzed by the quantum mechanical "SDS" method[7~9] and the values of the chemical shift and spin-spin coupling constant were obtained precisely. This study was undertaken to investigate the structural change, conformation and the change in the chemical shift with pH in dipeptide solutions. From this study informations on the formation of peptide bond and sequence determination will be explored.

## 2. EXPERIMENTAL PROCEDURE

Dipeptides employed were glycyl-*L*-valine, glycyl-*DL*-alanine, glycyl-*L*-aspartic acid, glycyl-*DL*-serine, and *L*-valyl-glycine manufactured by Sigma Chemical Company. Dipeptide solutions were prepared in $D_2O$ obtained from Stohler Isotope Chemicals and had dipeptide concentrations of 0.2 *M* or less. For each di-

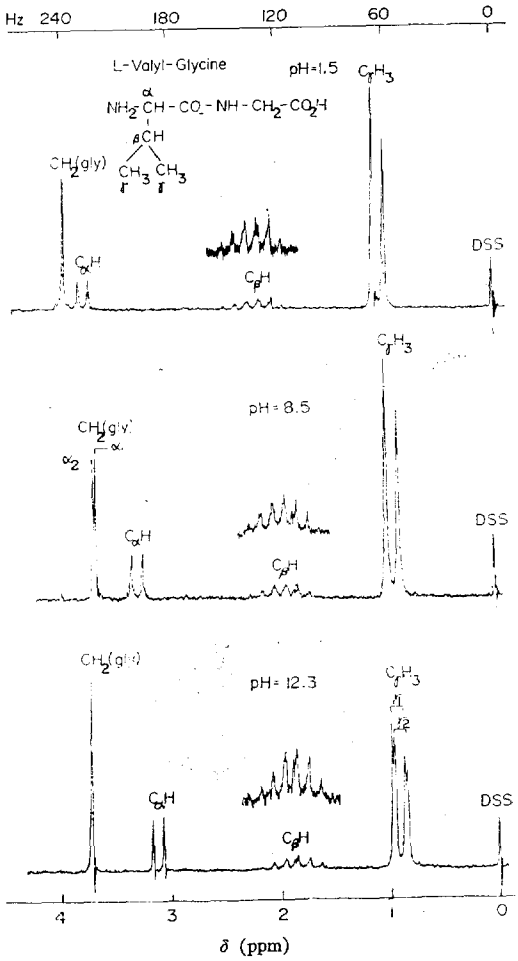


*Fig.* 1. Proton magnetic resonance spectra of solutions of *L*-valyl-glycine in $D_2O$ taken at a spectrometer frequency of 60 MHz at pH 1.5, 8.5 and 12.3.

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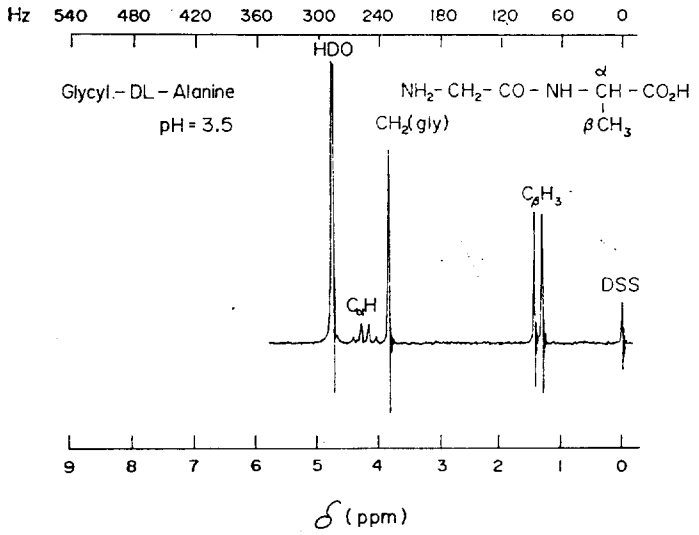


*Fig.* 2. Proton magnetic resonance spectra of solutions of glycyl-*DL*-alanine in $D_2O$ taken at a spectrometer frequency of 60 MHz at pH 3.5.

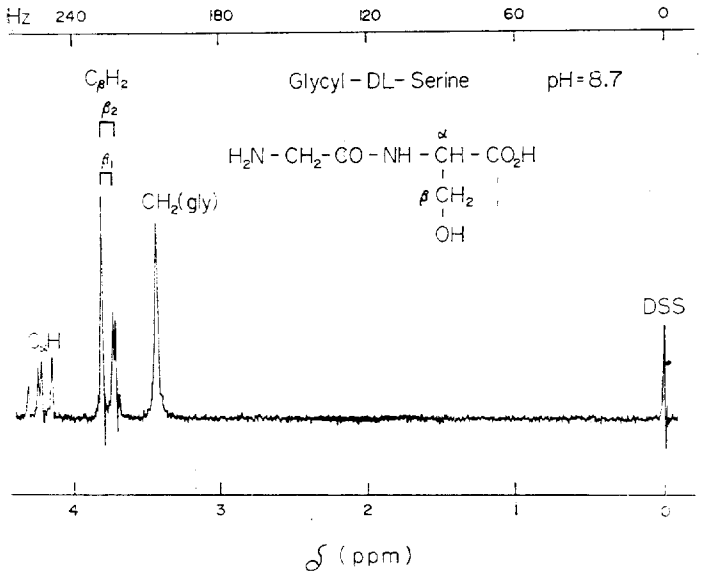


*Fig.* 3. Proton magnetic resonance spectra of solutions of glycyl-*DL*-serine in $D_2O$ taken at a spectrometer frequency of 60MHz at pH 8.7.

ptide, pH values of the solution of the same concentration were varied in the range of 0~13 by adding sufficient acid or alkali to produce a required pH value, and the proton magnetic peresonance spectra were investigated as a function of pH.

The pH of the solutions was measured with a Beckman pH meter at 25°C, the temperature at which the NMR spectra were observed. NMR spectra were obtained using a Japan Electron Optics Laboratory Model C-60 HL high-resolution NMR spectrometer operating at a fixed frequency of 60 MHz. Sweep width was calibrated against a standard sample furnished by the Japan Electron Optics Laboratory. Chemical shifts were referred to the methyl resonance of sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), the internal standard.

## 3. RESULTS AND DISCUSSION

Proton magnetic resonance spectra of solutions of *L*-valyl-glycine, glycyl-*DL*-alanine and glycyl-*DL*-serine are displayed as typical examples in *Figs.* 1~3. Assignment of resonances to corresponding chemical groups in a dipeptide was possible by counting the number of splittings of each resonance and by estimates of relative intensities of resonances from related chemical groups. For the determination of the chemical shifts and spin-spin coupling constants, the quantum mechanical "SDS" method[7~9] was used by a comparison of experimental spectra with

*Table* 1. Chemical shifts[a] and spin-spin coupling constants[b] for glycyl-*L*-valine in $D_2O$ measured at 25 °C.

| pH | Chemical shift | | | | Spin-spin coupling constant | |
|---|---|---|---|---|---|---|
| | $C_\alpha H$ (gly) | $C_\alpha H$ | $C_\beta H$ | $C_\gamma H$ | $J_{\alpha\beta}$ | $J_{\beta\gamma}$ |
| 0.05 | 3.96 | 4.36 | 2.27 | $\gamma_1$ 0.99 | 5.3 | $J_{\beta\gamma_1}$=6.8 |
| | | | | $\gamma_2$ 0.98 | | $J_{\beta\gamma_2}$=7.0 |
| 0.6 | 3.93 | 4.36 | 2.24 | $\gamma_1$ 0.99 | 5.5 | $J_{\beta\gamma}$=$J_{\beta\gamma_2}$=6.9 |
| | | | | $\gamma_2$ 0.97 | | |
| 1.9 | 3.93 | 4.34 | 2.24 | $\gamma_1$ 0.99 | 5.5 | $J_{\beta\gamma_1}$=$J_{\beta\gamma_2}$=7.1 |
| | | | | $\gamma_2$ 0.96 | | |
| 4.1 | 3.89 | 4.13 | 2.17 | $\gamma_1$ 0.95 | 5.5 | $J_{\beta\gamma_1}$=7.0 |
| | | | | $\gamma_2$ 0.91 | | $J_{\beta\gamma}$=6.9 |
| 5.7 | 3.89 | 4.10 | 2.15 | $\gamma_1$ 0.94 | 5.4 | $J_{\beta\gamma_1}$=6.9 |
| | | | | $\gamma_2$ 0.90 | | $J_{\beta\gamma}$=7.0 |
| 7.85 | 3.80 | 4.10 | 2.15 | $\gamma_1$ 0.95 | 5.4 | $J_{\beta\gamma_1}$=6.9 |
| | | | | $\gamma_2$ 0.91 | | $J_{\beta\gamma_2}$=7.0 |
| 9.85 | 3.41 | 4.09 | 2.14 | $\gamma_1$ 0.93 | 5.3 | $J_{\beta\gamma_1}$=6.8 |
| | | | | $\gamma_2$ 0.90 | | $J_{\beta\gamma}$=6.9 |
| 12.0 | 3.39 | 4.09 | 2.14 | $\gamma_1$ 0.93 | 5.5 | $J_{\beta\gamma_1}$=7.0 |
| | | | | $\gamma_2$ 0.90 | | $J_{\beta\gamma_2}$=6.9 |

[a]All values are in ppm downfield from DSS with an accuracy of ±0.05 ppm; [b]All values are in Hz with an accuracy of ±0.2 Hz.

*Table* 2. Chemical shifts[a] and spin-spin coupling constants[b] for *L*-valyl-glycine in $D_2O$ measured at 25 °C.

| pH | Chemical shift | | | | Spin-spin coupling constant | |
|---|---|---|---|---|---|---|
| | $C_\alpha H$(gly) | $C_\alpha H$ | $C_\beta H$ | $C_\gamma H$ | $J_{\alpha\beta}$ | $J_{\beta\gamma}$ |
| 0.5 | 4.00 | 3.83 | 2.18 | 1.04 | 6.0 | 6.8 |
| 1.5 | 3.98 | 3.79 | 2.16 | 1.04 | 6.0 | 6.7 |
| 3.6 | $\alpha_1$ 3.85 | 3.78 | 2.15 | 1.04 | 6.0 | 6.8 |
| | $\alpha_2$ 3.86 | | | | | |
| 5.5 | $\alpha_1$ 3.72 | 3.75 | 2.14 | 1.04 | 6.4 | 6.8 |
| | $\alpha_2$ 3.78 | | | | | |
| 7.4 | $\alpha_1$ 3.70 | 3.63 | 2.09 | 1.02 | 6.7 | 6.8 |
| | $\alpha_2$ 3.75 | | | | | |
| 8.5 | $\alpha_1$ 3.69 | 3.31 | 1.94 | 0.95 | 6.0 | 6.8 |
| | $\alpha_2$ 3.73 | | | | | |
| 10.7 | 3.69 | 3.11 | 1.86 | $\gamma_1$ 0.92 | 6.0 | $J_{\beta\gamma_1}$=6.8 |
| | | | | $\gamma_2$ 0.89 | | $J_{\beta\gamma_2}$=6.8 |
| 12.3 | 3.73 | 3.12 | 1.87 | $\gamma_1$ 0.92 | 6.0 | $J_{\beta\gamma_1}$=6.8 |
| | | | | $\gamma_2$ 0.90 | | $J_{\beta\gamma_2}$=6.8 |

[a]All values are in ppm downfield from DSS with an accuracy of ±0.05 ppm; [b]All values are in Hz with an accuracy of ±0.2Hz.

theoretical NMR transitions[7]. The results are given in *Tables* 1~6 for five dipeptides. The protons in the species —COOH, —$NH_2$ and HDO exchange rapidly and appear as a single

*Table* 3. Chemical shifts[a] and spin-spin coupling constants[b] for glycyl-*DL*-alanine in $D_2O$ measured at 25 °C.

| pH | Chemical shfit | | | Spin-spin coupling constant $J_{\alpha\beta}$ |
|---|---|---|---|---|
| | $C_\alpha H$(gly) | $C_\alpha H$ | $C_\beta H$ | |
| 0 | 3.94 | 4.55 | 1.47 | 12.5 |
| 0.75 | 3.90 | 4.50 | 1.47 | 12.5 |
| 3.5 | 3.85 | 4.23 | 1.38 | 12.5 |
| 5.3 | 3.79 | 4.20 | 1.37 | 12.5 |
| 7.25 | 3.78 | 4.16 | 1.35 | 12.5 |
| 9.6 | 3.37 | 4.18 | 1.35 | 12.5 |
| 11.0 | 3.32 | 4.16 | 1.36 | 12.5 |
| 13.2 | 3.33 | 4.17 | 1.36 | 12.5 |

[a]All values are in ppm downfield from DSS with an accuracy of ±0.05 ppm; [b]All values in Hz with an accuracy of ±0.2 Hz.

*Table* 4. Chemical shifts[a] and spin-spin coupling constants[b] for glycyl-*DL*-serine in $D_2O$ measured at 25 °C.

| pH | Chemical shift | | | Spin-spin coupling Constant $J_{\alpha\beta}$ |
|---|---|---|---|---|
| | $C_\alpha H$ (gly) | $C_\alpha H$ | $C_\beta H$ | |
| 0.25 | 3.93 | 4.64 | 3.97 | 5.0 |
| 0.9 | 3.88 | 4.58 | 3.92 | 5.0 |
| 1.85 | 3.90 | 4.60 | 3.93 | 5.0 |
| 3.8 | 3.82 | 4.36 | 3.81 | 4.9 |
| 5.8 | 3.85 | 4.29 | $\beta_1$ 3.81 | $J_{\alpha\beta_1}$=5.0 |
| | | | $\beta_2$ 3.80 | $J_{\alpha\beta_2}$=5.0 |
| 7.3 | 3.78 | 4.25 | $\beta_1$ 3.78 | $J_{\alpha\beta_1}$=5.0 |
| | | | $\beta_2$ 3.77 | $J_{\alpha\beta_2}$=5.0 |
| 8.7 | 3.44 | 4.24 | $\beta_1$ 3.78 | $J_{\alpha\beta_1}$=4.9 |
| | | | $\beta_2$ 3.77 | $J_{\alpha\beta_2}$=4.9 |
| 9.8 | 3.34 | 4.26 | $\beta_1$ 3.79 | $J_{\alpha\beta_1}$=4.9 |
| | | | $\beta_2$ 3.78 | $J_{\alpha\beta_2}$=4.9 |
| 12.2 | 3.32 | 4.25 | 3.78 | 5.0 |

[a]All values are in ppm downfield from DSS with an accuracy of ±0.05 ppm; [b]All values are in Hz with an accuracy of ±0.2 Hz.

line whose position, a weighted average of the shifts in the three different environments, is close to that of HDO itself (4.73~4.78ppm) at a given pH.

In general, amino acids show two-stage titration curve with increasing pH, first one occurring in ionization by carboxyl group in acidic solution and another one by $-NH_3$ group in alkaline solution[10]. However, as in *Figs.* 4~7, plots of the chemical shift of protons as a function of pH for the dipeptide solutions show one-stage curve for $C_\alpha H$ protons of constituent amino acids due to the formation of peptide bond, instead of two-stage curve for each constituent amino acid. As shown in *Figs.* 4~7 the chemical shift for $C_\alpha H$-protons of constituent amino acid of dipeptides changes substantially with pH compared to a small change for the case of $C_\beta H$ or $C_\gamma H$-protons except for glycyl-*L*-aspartic acid. The spin-spin coupling constant

*Table* 5. Chemical shifts[a] and spin-spin coupling constants[b] for glycyl-*L*-aspartic acid in $D_2O$ measured at 25 °C.

| pH | Chemical shift | | | Spin-spin coupling constant $J_{\alpha\beta}$ |
|---|---|---|---|---|
| | $C_\alpha H$(gly) | $C_\alpha H$ | $C_\beta H$ | |
| 0.2 | 3.93 | 4.86 | 3.03 | 6.0 |
| 0.55 | 3.97 | 4.93 | 3.06 | 6,0 |
| 1.45 | 3.90 | 4.86 | 3.01 | 6.0 |
| 3.8 | 3.89 | 4.66 | $\beta_1$ 2.86 | $J_{\alpha\beta_1}$=6.0 |
| | | | $\beta_2$ 2.85 | $J_{\alpha\beta_2}$=7.5 |
| 5.5 | 3.84 | 4.48 | $\beta_1$ 2.68 | $J_{\alpha\beta_1}$=4.5 |
| | | | $\beta_2$ 2.58 | $J_{\alpha\beta_2}$=9.0 |
| 7.2 | 3.84 | 4.48 | $\beta_1$ 2.68 | $J_{\alpha\beta_1}$=5.0 |
| | | | $\beta_2$ 2.57 | $J_{\alpha\beta_2}$=7.5 |
| 8.6 | 3.65 | 4.50 | $\beta_1$ 2.68 | $J_{\alpha\beta_1}$=4.5 |
| | | | $\beta_2$ 2.60 | $J_{\alpha\beta_2}$=8.3 |
| 9.7 | 3.41 | 4.48 | $\beta_1$ 2.66 | $J_{\alpha\beta_1}$=4.5 |
| | | | $\beta_2$ 2.62 | $J_{\alpha\beta_2}$=8.3 |
| 11.7 | 3.36 | 4.47 | $\beta_1$ 2.66 | $J_{\alpha\beta_1}$=4.5 |
| | | | $\beta_2$ 2.62 | $J_{\alpha\beta_2}$=9.0 |

[a]All values are in ppm downfield from DSS with an accuracy of ±0.05 ppm; [b]All values are in Hz with an accuracy of ±0.2 Hz.

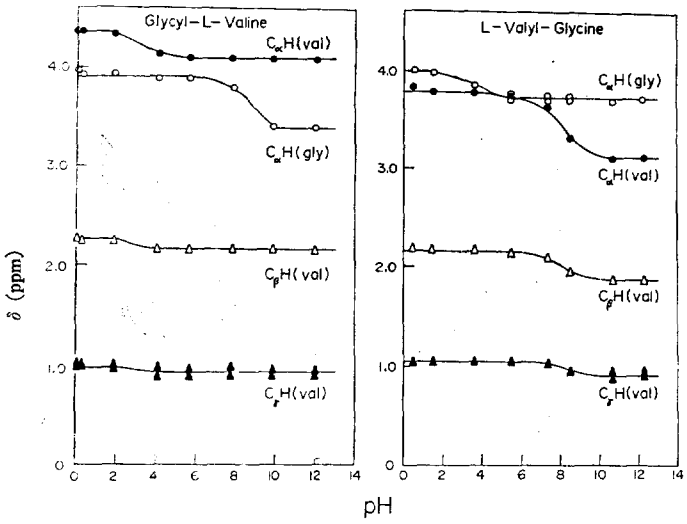


*Fig.* 4. Chemical shifts of protens plotted as a function of pH in glycyl-*L*-valine and *L*-valyl-glycine solutions.

remains almost unchanged with pH except for the case of glycyl-*L*-aspartic acid (see *Tables* 1~5). For glycyl-*L*-aspartic acid, the chemical shift of $C_\beta H$ protons of aspartic acid-part changes substantially with pH (*Fig.* 7) and the coupling constant changes with pH (*Table* 5). which will be discussed later.

According to Roberts and Jardetzky[11], the N-terminal and C-terminal titration shifts are transmitted to the side chain protons in amino acids and to the $C_\alpha H$ protons of the neighboring residue in a dipeptide chain, but not beyond. However, for the dipeptides studied, it is seen that the N-terminal and C-terminal shifts are transmitted not only to the $C_\alpha H$, but also to the $C_\beta H$ and $C_\gamma H$ protons. The magnitude of N-terminal and C-terminal shifts is smaller for the $C_\beta H$ and $C_\gamma H$ protons than for the $C_\alpha H$ protons, which explains that the peptide bond formation

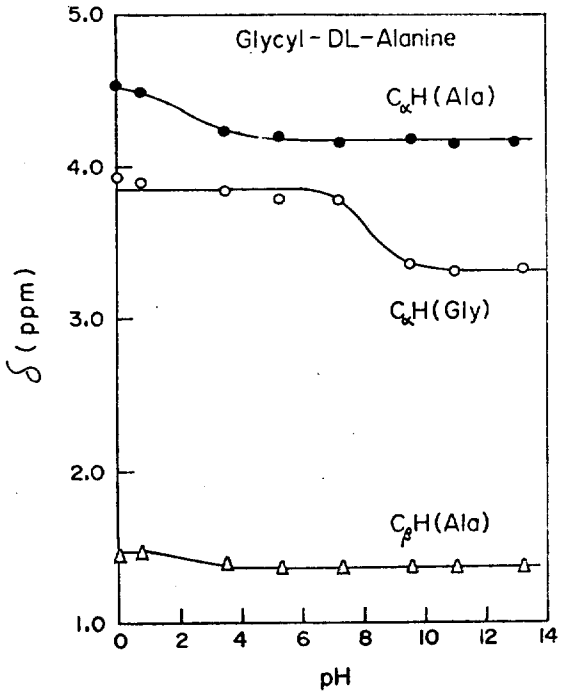


*Fig.* 5. Chemical shifts of protons plotted as a function of pH in glycyl-*DL*-alanine solution.

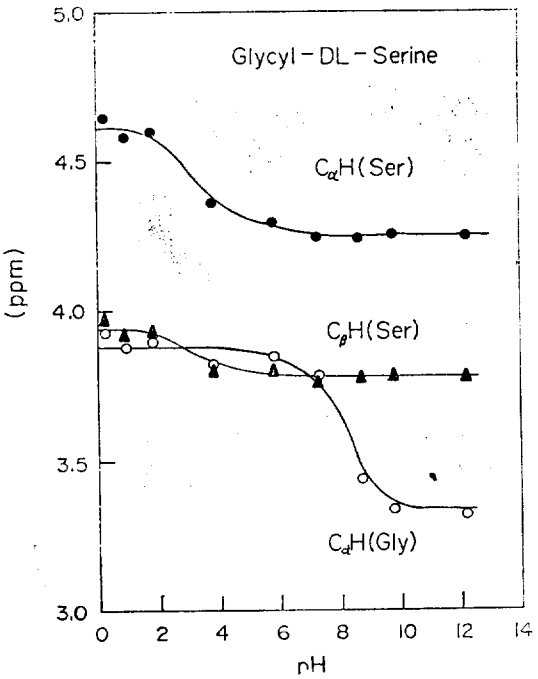


*Fig.* 6. Chemical shifts of protons plotted as a function of pH in glycyl-*DL*-serine solution.

occurs next to the $C_\alpha$ atom of the constituent amino acids.

For glycyl-*L*-valine, the two sets of $C_\gamma H$ protons are magnetically non-equivalent in the pH range of 0∼12 (see *Table* 1). This may arise from either two different rotamers about the $C_\alpha$–$C_\beta$ bond or from two distinct $C_\gamma H$ proton sites. Considering only staggered rotamers about the $C_\alpha$–$C_\beta$ bond, it is unlikely that two different rotamers can exist.

For *L*-valyl-glycine, $C_\gamma H$ protons also have two different values of the chemical shift in the pH range of 10∼12 and $C_\alpha H$ protons of glycyl-part have two magnetically non equivalent sites in the pH range of 3.6∼8.5 (see *Table* 2), indicating that the rotation about the N-$C_\alpha$ bond of glycyl-part is quite hindered. Restricted rotation leads to the existence of preferred rotamers. Nakamura and Jardetzky[1] have shown that the restricted rotation in aminoacyl-glycine defines a limited range of allowed conformations where the two glycine $C_\alpha H$ protons are non-equivalent. In the pH range of 3.6∼8.5 not far from pH of *L*-valyl-glycine there may be an appreciable amount of zwitterionic form. In the zwitterionic form, rotamers in which the positive ($-\overset{+}{N}H_3$) and negative charges ($-COO^-$) are *cis* to each other will be favored, thus further restricting the freedom of rotation and increasing the non-equivalence of the two $C_\alpha H$ protons. The energy barriers for the rotation of an N-terminal glycine (glycyl-amino acid)-about the $C_\alpha$–CO bond are lower than those for the rotation of a C-terminal glycine (aminoacyl-glycine) about the N-$C_\alpha$ bond[1]. This accounts for the preponderance of chemical shift non-equivalence in the latter. It is easy to distinguish the sequence of consituent amino

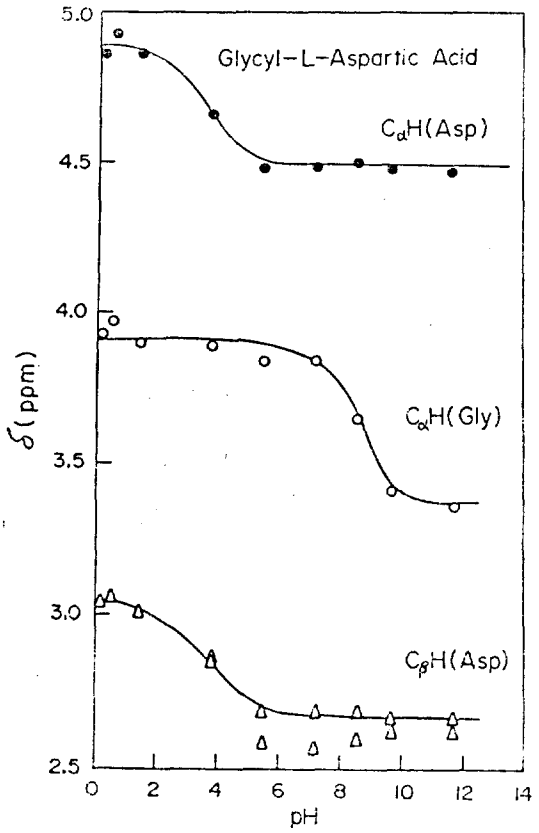


*Fig.* 7. Chemical shifts of protons plotted as a function of pH in glycyl-*L*-aspartic acid solution.

acid-linkage of glycyl-*L*-valine and *L*-valyl-glycine from the N-terminal and C-terminal titration shifts as shown in *Fig.* 4.

For glycyl-*DL*-serine, two $C_\beta H$ protons are magnetically nonequivlent (See *Table* 4), which may arise from the hindered rotation about $C_\alpha$–$C_\beta$ bond due to the existence of internal hydrogen bonding formed between OH group and anionic form of carboxyl group in serine part.

For glycyl-*L*-aspartic acid there are two distinct results from the other four dipeptides; first the greatest pH dependence of the chemical shifts of the $C_\beta H$ protons and second the changes in the spin-spin coupling constants with pH. This peptide has three ionizable groups, while the others have two. The effect of the third ionizable group (—COOH) on the $C_\beta$ atom may be transmitted to the $C_\beta H$ titration shifts, showing the great pH dependence of the chemical shifts. Changes in the spin-spin coupling constants with pH reflect changes in rotamer populations[11]; it is qualitatively clear that relative rotamer populations for $C_\beta H$ protons vary with pH in glycyl-*L*-aspartic acid solutions in the pH range of 4~12.

The following conclusions have been drawn:

1. Proton magnetic resonance spectra of five glycine-containing dipeptides can be analyzed completely by an analytical method, and their structures have been confirmed by proton magnetic resonance spectra.

2. The analysis of proton NMR spectra gives the informations on the existence of different rotamers in dipeptide solutions and the changes in rotamer population with pH in glycyl-*L*-aspartic acid.

The method of analysis used for five dipeptides employed could be applicable to an analysis of the proton magnetic resonance spectra of oligopeptides and to sequence determination of unknown dipeptides or oligopeptides in the future.

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