# Proton Magnetic Resonance Studies on Conformation and Association of Oligo-γ-benzyl-L-glutamates in Solution

TOYOKO IMAE and SHOICHI IKEDA, *Department of Chemistry, Faculty of Science, Nagoya University, Chikusa, Nagoya 464;* and YUJI KOBAYASHI and YOSHIMASA KYOGOKU, *Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan*

## Synopsis

The proton magnetic resonance spectra of *o*-nitrophenylthio-tetra- and hexa-γ-benzyl-L-glutamate ethylamides have been measured at different concentrations in CDCl$_3$ and CD$_{22}$Cl. The NH and α-CH resonances of the tetrapeptide show downfield shifts with increasing concentration, accompanying disappearance of their fine structure and line broadening. The apparent feature of chemical shifts against concentration is sigmoidal, and it can be interpreted by assuming the presence of a step or more of association–dissociation equilibria of tetrapeptide. With increasing concentration, small aggregates are formed by intermolecular hydrogen bonding, the size of which is not sufficiently large to exhibit critical micelle concentrations. In contrast to the tetrapeptide, the hexapeptide has constant chemical shifts of the NH and α-CH resonances, independent of concentration, which implies that only the unassociated molecules show observable sharp resonances. In the hexapeptide, the phenyl CH and benzyl CH$_2$ groups of the side chains exhibit new resonances above certain critical concentrations, indicating the restriction of rotational freedom of the side chains in the aggregated states.

## INTRODUCTION

Conformation of homooligopeptides in solution has been investigated by means of nmr spectra of NH and α-CH protons. Among them, the nmr spectra of methoxyethoxyethoxyacetyltri- and tetra-L-alanine ethyl esters,[1] *t*-butyloxycarbonyl-L-isoleucine,[1] L-norvaline,[2] or L-methionine[3] oligopeptide methyl esters, and carbobenzoxy-oligo-γ-ethyl-L-glutamate ethyl esters[4] have been measured in detail in deuterochloroform or its mixtures with dimethyl sulfoxide or trifluoroacetic acid. According to these investigations, the oligopeptides in chloroform are predominantly in the folded forms at lower concentrations, which are mainly stabilized by the intramolecular hydrogen bonds, and, with increasing concentration, these folded forms are transformed into the extended forms associated intermolecularly through peptide hydrogen bonds.

We have examined *o*-nitrophenylthio-di-, tetra-, and hexa-γ-benzyl-L-glutamate ethylamide, *o*-NO$_2$-C$_6$H$_4$-S-[Glu(OBzl)]$_x$-NHCH$_2$CH$_3$, with

$x = 2, 4$, and 6, in solution by means of ir spectra, CD, light scattering, and solution viscosity.[5-10] The dipeptide absorbs the amide I band at 1639 cm$^{-1}$ in the solid state,[5] and its conformation is assigned as the $\gamma$-structure.[11] The tetra- and hexapeptides have the amide I band around 1625 and 1690 cm$^{-1}$ in the solid state, and their conformation is the $\beta$-structure of antiparallel chains.[5,6]

According to our previous studies in solution,[9,10] the tetra- and hexapeptides are either in the $\sigma$- or $\beta$-conformation. The $\sigma$-conformation consists of residues of the $\sigma$-form that is in either of two states, i.e., hydrogen bond-free or intramolecularly hydrogen-bonded. A molecule in the $\sigma$-conformation is free from intermolecular association in solution. The $\beta$-conformation consists of residues of two forms, i.e., the $\sigma$- and $\beta$-form. A residue of the $\beta$-form is intermolecularly hydrogen-bonded. Thus, the $\beta$-conformation is intermolecularly associated in solution.

The tetrapeptide is in the $\sigma$-conformation at infinite dilution alone, while the hexapeptide can take up the $\sigma$-conformation below certain critical concentrations. With increasing concentration, either from infinite dilution for the tetrapeptide or beyond the critical micelle concentration for the hexapeptide, these residues are gradually transformed from the $\sigma$- to the $\beta$-form, and the molecules are intermolecularly hydrogen-bonded, forming the $\beta$-conformation.

In the present work we measure their proton nmr spectra in deuterochloroform (CDCl$_3$) and deuterated ethylene dichloride (CD$_2$ClCD$_2$Cl) and examine their conformations and association in these solvents.

## EXPERIMENTAL

Three samples of $o$-nitrophenylthio-oligo-$\gamma$-benzyl-L-glutamate ethylamides were the same as previously prepared and used.[5-10] They will be called the di-, tetra-, and hexapeptide, respectively, for $x = 2, 4$, and 6. Deuterochloroform (CDCl$_3$; enrichment isotope 99.95%) was obtained from Commissariate à l'Energie Atomique, Gif-sur-Yvette, France, and deuterated ethylene dichloride (CD$_2$ClCD$_2$Cl, minimum isotope purity 99 atom % D) was purchased from the Merck, Sharp, and Dohme Canada Ltd., Montreal, Canada. Both were filtered after being dried over the molecular sieve No. 4A and used immediately.

A solution for nmr measurement was prepared as follows: a weighed amount of oligopeptide sample was put directly in an nmr sample tube, a known volume of solvent was added, and the solution was kept overnight before each measurement. Concentration, $c$ (g cm$^{-3}$), of the solution was calculated from the weight of dissolved sample and the volume of solvent.

The proton nmr spectra at 360 MHz were measured on a Bruker WM 360-WB spectrometer operated in the Fourier-transform mode.

Chemical shifts, $\delta$ (ppm), of resonances were referred to internal tetramethylsilane. Measurements were performed at 25°C, unless otherwise stated. Temperature dependence of nmr spectra were measured by raising the temperature of a solution stepwise: a solution was kept for 30 min at each temperature before measurement, which assured the equilibrium. The nmr spectra recovered the original features when the solution was cooled down. Thus, the reversibility of the spectra was confirmed for the temperature change. Temperature of the solution was controlled to $\pm$ 0.1°C.

## RESULTS

### Assignment of Proton Resonances

The tentative assignment of proton resonances was achieved with reference to published data on oligo($\gamma$-ethyl-L-glutamate)s,[4] oligo(L-methionine)s,[3] and poly($\gamma$-benzyl-L-glutamate),[12] and also by the use of the spin-decoupling technique. The residues are abbreviated, omitting the symbol for benzyl ester, and numbered successively from the N- to the C-terminal by a superscript, as $Glu^i$ ($i = 1, 2, \ldots, x$). The o-nitrophenylthio group is abbreviated by Nps.

The typical proton nmr spectra of dilute solutions of oligopeptides in $CDCl_3$ are shown in Fig. 1. The sharp resonance observed at 7.26 ppm for $CDCl_3$ solutions or at 3.73 ppm for $CD_2ClCD_2Cl$ solutions can be assigned to the contaminant nondeuterated solvent, $CHCl_3$, or nondeuterated contaminants of $CD_2ClCD_2Cl$, respectively. The strong resonances arising from these contaminants sometimes disturbed the detection of proton resonances of the oligopeptides, especially in dilute solutions. In addition, both solvents still contained contaminant water, $H_2O$, which gave a sharp resonance around 1.6 ppm.

The strongest resonance around 7.3 ppm can be readily assigned to the phenyl CH group.[12] The benzyl $CH_2$ group has a resonance at 5.0–5.1 ppm. The resonances at 2.5 and 2.0 ppm can be attributed to the $\gamma$-$CH_2$ and $\beta$-$CH_2$ groups, respectively.[12]

Four proton resonances of the N-terminal Nps group are observed at the lowest fields, i.e., at 7.1–8.3 ppm. The C-terminal ethylamide shows a resonance at 3.25 ppm for its $CH_2$ group and a resonance at 6.27 ppm for its NH group. Its terminal $CH_3$ group has a reasonance at 1.13 ppm.

The NH and $\alpha$-CH resonances of $Glu^1$ of the dipeptide appear at 3.52 and 3.38 ppm, respectively, locating rather strongly upfield. This is caused by the effect of the electronegative S atom of the neighboring Nps group. The NH and $\alpha$-CH groups of $Glu^2$ of the dipeptide have resonances at 7.05 and 4.44 ppm, respectively. These groups of residues other than $Glu^1$ of the tetra- and hexapeptides also have resonances at 7–8 ppm and 4.0–4.5 ppm, respectively, and their locations are
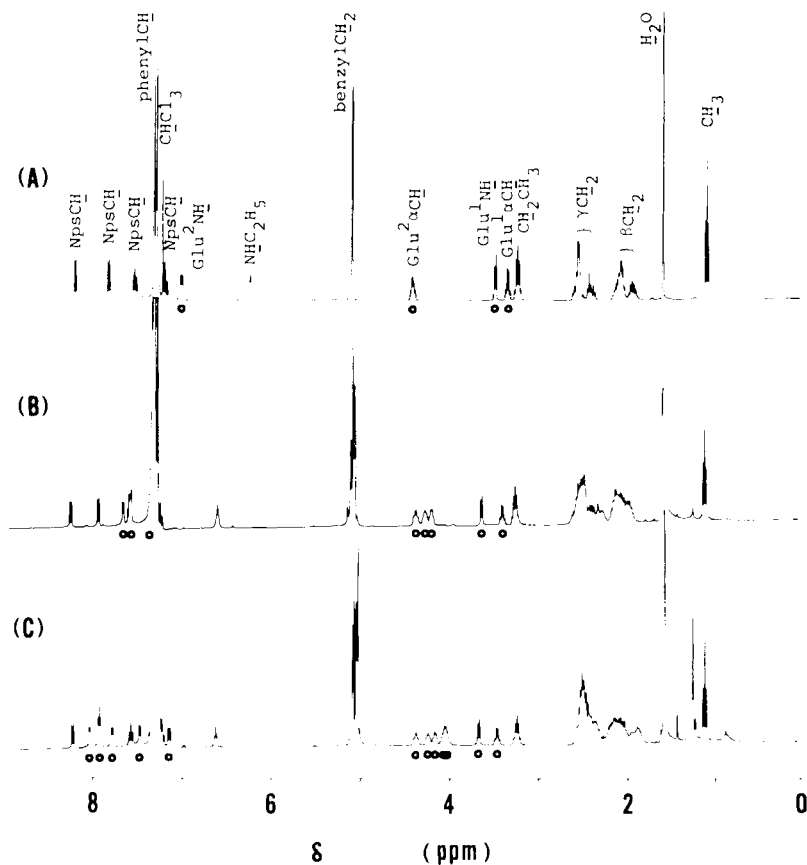
Fig. 1.  Typical nmr spectra of Nps-[Glu(OBzl)]$_x$-NHC$_2$H$_5$ in CDCl$_3$. (A) $x$ = 2; concentration, 0.38 $\times$ 10$^{-2}$ g cm$^{-3}$. (B) $x$ = 4; concentration, 0.19 $\times$ 10$^{-2}$g cm$^{-3}$. (C) $x$ = 6; concentration, 0.20 $\times$ 10$^{-2}$ g cm$^{-3}$. Circles (○) indicate the NH and $\alpha$-CH resonances.

consistent with those for the other oligopeptides published so far.[1-4,13-15] Except for the $\alpha$-CH groups of the hexapeptide, the number of resonances assignable to the NH and $\alpha$-CH groups is equal to the number of residues, as indicated by circles under the resonances in Fig. 1.

Table I lists the values of chemical shifts of NH and $\alpha$-CH resonances of the dipeptide in CDCl$_3$, most of which remain unaltered at concentrations up to 1.95 $\times$ 10$^{-2}$g cm$^{-3}$, or 3.07 $\times$ 10$^{-2}M$. This means that the dipeptide does not undergo any concentration-dependent conformational change. However, the NH resonances of Glu$^2$ and ethylamide shift downfield by 0.1 ppm on increasing concentration.

Table I also lists the values of these chemical shifts of the tetrapeptide at infinite dilution and of the hexapeptide below the critical concentrations, both in CDCl$_3$ and CD$_2$ClCD$_2$Cl. This assignment was tentatively performed as follows.

TABLE I
Chemical Shifts of Proton Resonances of Unassociated Oligopeptides in Solution

| | Dipeptide, CDCl$_3$ | Tetrapeptide | | Hexapeptide | |
|---|---|---|---|---|---|
| | | CDCl$_3$ | CD$_2$ClCD$_2$Cl | CDCl$_3$ | CD$_2$ClCD$_2$Cl |
| NH resonances | | | | | |
| Glu$^3$ | | 7.65 | 7.50 | 8.02 | 7.95 |
| Glu$^4$ | | 7.55 | 7.38 | 7.92 | 7.72 |
| Glu$^5$ | | | | 7.78 | 7.66 |
| Glu$^6$ | | | | 7.47 | 7.39 |
| Glu$^2$ | 7.03 | — | 7.14 | 7.14 | 7.06 |
| Glu$^1$ | 3.51 | 3.64 | — | 3.68 | 3.66 |
| Ethylamide | 6.25 | 6.58 | 6.53 | 6.62 | 6.60 |
| α-CH resonances | | | | | |
| Glu$^2$ | 4.43 | 4.36 | 4.22 | 4.37 | 4.20 |
| Glu$^6$ | | | | 4.24 ⎫ | ⎫ |
| Glu$^5$ | | | | 4.16 ⎬ | 4.10 |
| Glu$^3$ | | 4.25 ⎫ | 4.15 ⎫ | 4.05 | 4.02 |
| Glu$^4$ | | 4.18 ⎬ | ⎬ | | 3.98 |
| Glu$^1$ | 3.37 | 3.40 | 3.42 | 3.47 | 3.48 |

The locations of NH resonances of ethylamide and Glu$^1$ and of α-CH resonance of Glu$^1$ would not differ from those of the dipeptide but remain at the highest fields, respectively. The NH group of Glu$^2$ would also be subject to the effect of the S atom either directly or indirectly, and it is hydrogen-bond-free in the intramolecularly hydrogen-bonded structure proposed below. Thus, the NH resonance of Glu$^2$ of the Nps oligopeptides could be at the high fields, similar to that of Glu$^1$ of the other N-substituted oligopeptides.[3,4] Then the NH resonance of Glu$^2$ remains around 7.1 ppm for the three Nps oligopeptides. The mutual connectivity of the NH and α-CH resonances permits assignment of the α-CH resonance of Glu$^2$ at the lowest field around 4.4 ppm.

For the NH groups of the other Glu residues, the NH resonances are assigned in the order of increasing field from Glu$^3$ to Glu$^x$, in accord with Ribeiro et al.[3,4] Owing to the strong effect of electronegative S atom of Nps group, even this assignment might still be perturbed. The spin-decoupling technique determines the mutual connectivity of the NH and α-CH resonances, and the assignment of the α-CH resonances as shown in Table I can be derived.

## NMR Spectra of the Tetrapeptide

In contrast to the dipeptide, the nmr spectra of the tetrapeptide vary markedly with a change in concentration. Its spectra in CDCl$_3$ at different concentrations are shown in Fig. 2. As the concentration of the tetrapeptide is increased, the NH resonances of Glu$^2$ to Glu$^4$ and of
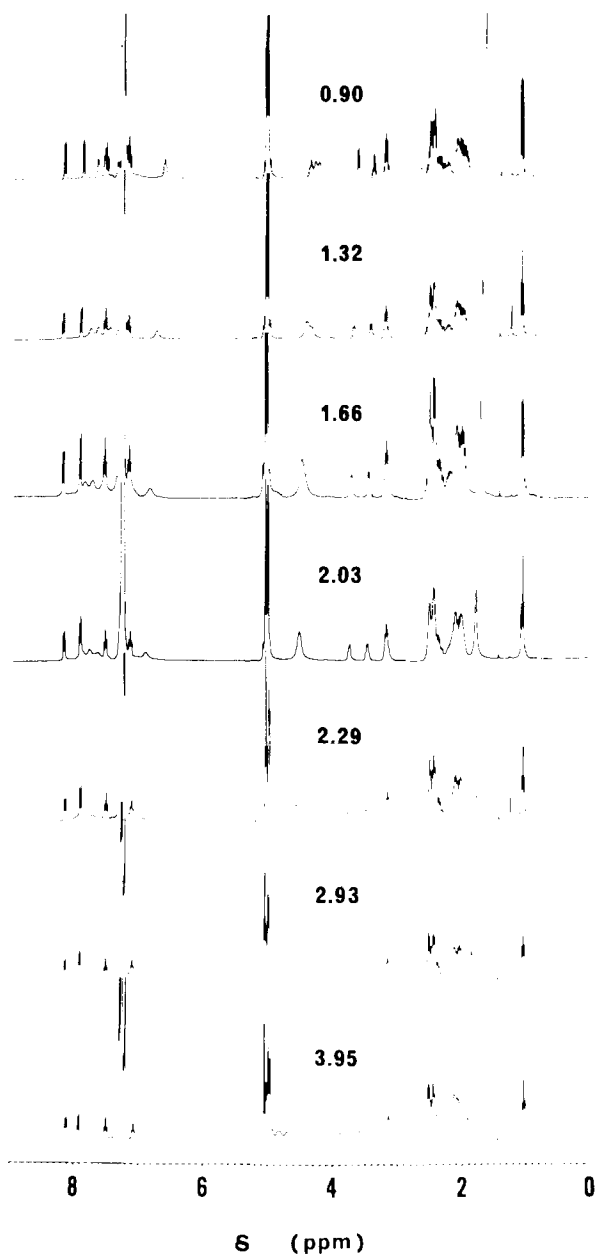
Fig. 2. The nmr spectra of the tetrapeptide in $CDCl_3$ at different concentrations. Concentrations are expressed as $10^{-2}g$ $cm^{-3}$.

ethylamide gradually shift downfield. Their intensities decrease considerably above $1 \times 10^{-2}$g cm$^{-3}$, and their locations are obscure above $2.2 \times 10^{-2}$g cm$^{-3}$. The proton resonances of the NH group of Glu$^1$ and the $\alpha$-CH groups of all the residues also shift downfield and broaden, although their broadening is less than that of the NH resonances of Glu$^2$ to Glu$^4$ and of ethylamide. Nevertheless, the $\alpha$-CH resonances initially overlap but later resolve with the increase in concentration. The other resonances of the terminal groups and side chains are less sensitive to the increase in concentration. The chemical shifts of resonances of the tetrapeptide in CDCl$_3$ are plotted against concentration in Fig. 3.

The nmr spectra of the tetrapeptide in CD$_2$ClCD$_2$Cl are very similar to those in CDCl$_3$, and their chemical shifts gradually change with an increase in concentration, although the concentration region where a sharper change occurs is lower in CD$_2$ClCD$_2$Cl than in CDCl$_3$. In CD$_2$ClCD$_2$Cl the proton resonances of the NH and $\alpha$-CH groups shift markedly downfield at concentrations above $0.075 \times 10^{-2}$g cm$^{-3}$ and already begin to broaden at $0.40 \times 10^{-2}$g cm$^{-3}$. Specifically, four NH resonances of Glu$^2$ to Glu$^4$ and of ethylamide broaden markedly above $0.78 \times 10^{-2}$g cm$^{-3}$. The corresponding $\alpha$-CH resonances overlap with one another at higher concentrations. Chemical shifts of the resonances in CD$_2$ClCD$_2$Cl are plotted against concentration in Fig. 3.

In both CDCl$_3$ and CD$_2$ClCD$_2$Cl the resonances of the NH and $\alpha$-CH protons shift sigmoidally with changing concentration. Among them, the shifts of the NH and $\alpha$-CH resonances of Glu$^2$ to Glu$^4$ and of the NH resonance of ethylamide are more intense, while those of the NH and $\alpha$-CH resonances of Glu$^1$ are much less.

Infrared spectral studies[9] and light-scattering studies[10] have demonstrated that the tetrapeptide undergoes concentration-dependent conformational changes and association–dissociation equilibria in both chloroform and ethylene dichloride. The sigmoidal changes of chemical shifts with concentration suggest that concentration-dependent equilibria between two kinds of hydrogen-bonded states of residues, i.e., between the $\sigma$- and $\beta$-form, should occur simultaneously among more than two residues of the same kind. Otherwise, the change of chemical shift with concentration should appear with a concave downward curvature. Thus, intermolecular hydrogen bonds would be formed among more than two molecules, giving multimers of aggregates larger than dimer. The midpoints of the conformational changes or the association–dissociation equilibria are about $2.1 \times 10^{-2}$g cm$^{-3}$ in CDCl$_3$ and $0.8 \times 10^{-2}$g cm$^{-3}$ in CD$_2$ClCD$_2$Cl, respectively, if the sigmoidal curves for the $\alpha$-CH protons are referred to. The appearance of a single resonance for each proton represents the rapid rates of exchange between the hydrogen-bond-free state and the intramolecularly hydrogen-bonded state of the $\sigma$-form, and also between the $\sigma$- and $\beta$-form.
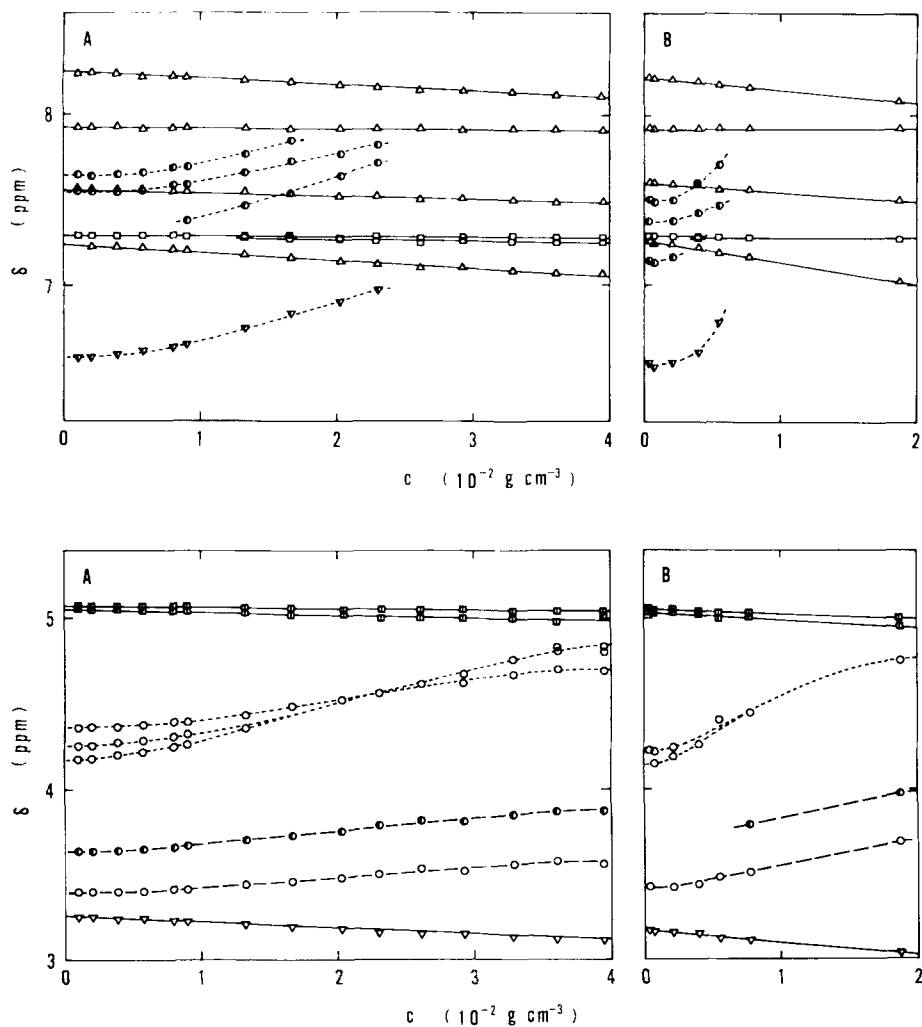
Fig. 3. Chemical shifts of various resonances of Nps-[Glu(OBzl)]$_4$-NHC$_2$H$_5$ as functions of concentration. Solvent: A, CDCl$_3$; B, CD$_2$ClCD$_2$Cl. (A) $\delta$ = 6.2 – 8.6 ppm: ($\triangle$), Nps C$H$; ($\bigcirc$), N$H$; ($\square$), phenyl C$H$; ($\triangledown$), N$H$C$_2$H$_5$. (B) $\delta$ = 3.0 – 5.4 ppm: ($\square$) benzyl C$H_2$; ($\bigcirc$), $\alpha$-C$H$; ($\bigcirc$), Glu$^1$N$H$; ($\triangledown$), NHC$H_2$CH$_3$.

Since all the other resonances shift upfield linearly with increasing concentration, they reflect neither conformational change nor association–dissociation equilibrium. Among them, the phenyl CH resonances shift much less than the others.

## NMR Spectra of the Hexapeptide

The nmr spectra of the hexapeptide in CDCl$_3$ and CD$_2$ClCD$_2$Cl also change with increasing concentration, but the character of these changes is quite different from that of the tetrapeptide. The spectra

of the hexapeptide in $CDCl_3$ remain unaltered at concentrations below $0.20 \times 10^{-2}$g cm$^{-3}$ or $1.3 \times 10^{-3}M$. As shown in Fig. 4, the NH and $\alpha$-CH resonances of Glu[2] to Glu[6] and the NH resonance of ethylamide lose their fine structures but still keep their intensities nearly unaltered as the concentration is further increased. Their intensities remain almost constant when compared with the constant intensity of contaminant $H_2O$ resonance, in spite of a 20-fold difference in concentration. Note also that the fine structures of the NH and $\alpha$-CH protons of Glu[1] disappear above moderate concentrations. The fine structures are completely absent from the NH and $\alpha$-CH resonances of the terminal groups and the side chains of all the residues in most concentrated solutions.

Furthermore, new resonances appear at 7.11 and 4.75 ppm with an increase in concentration more than $0.20 \times 10^{-2}$g cm$^{-3}$. The intensities of these resonances increase with increasing concentration, while the intensities of the phenyl CH and benzyl $CH_2$ resonances at 7.3 and 5.0 ppm decrease at higher concentrations. Thus, new resonances can be assigned to the protons of phenyl CH and benzyl $CH_2$ groups subject to the restricted rotations. At higher concentrations, these resonances, together with those of the $\beta$- and $\gamma$-$CH_2$ groups, become stronger, but the other resonances in the main chain and the terminal groups do not increase their intensities.

The chemical shifts of all the resonances are shown as functions of concentration in Fig. 5. In contrast to the tetrapeptide, there is no concentration dependence of chemical shifts. However, there is discontinuity of the spectra around $(0.2-0.4) \times 10^{-2}$g cm$^{-3}$, as noted above.

A similar situation is also observed for the solutions of the hexapeptide in $CD_2ClCD_2Cl$. That is, the NH and $\alpha$-CH resonances of Glu[2] to Glu[6] and the NH resonance of ethylamide have constant chemical shifts and intensities, independent of concentration. In addition, however, the loss of the fine structure and the drastic change such as the appearance of the new resonance occur at concentrations above $(0.05-0.07) \times 10^{-2}$g cm$^{-3}$. Figure 5 gives chemical shifts as functions of concentration.

Our measurements of ir spectra[6,9] and light scattering[7,8,10] have demonstrated that the hexapeptide in solution undergoes conformational change and micelle formation concomitantly above certain critical concentrations. Thus, the appearance of new resonances associated with the side-chain groups can be connected with the micelle formation accompanying conformational change. However, the constant chemical shifts and intensities of resonances associated with the main-chain groups can be interpreted well if only the hexapeptide in the $\sigma$-conformation gives observable resonances associated with their main chains.

Figure 6 shows the dependence of intensities of the new resonances on the concentration, based on the intensity of a proton resonance of
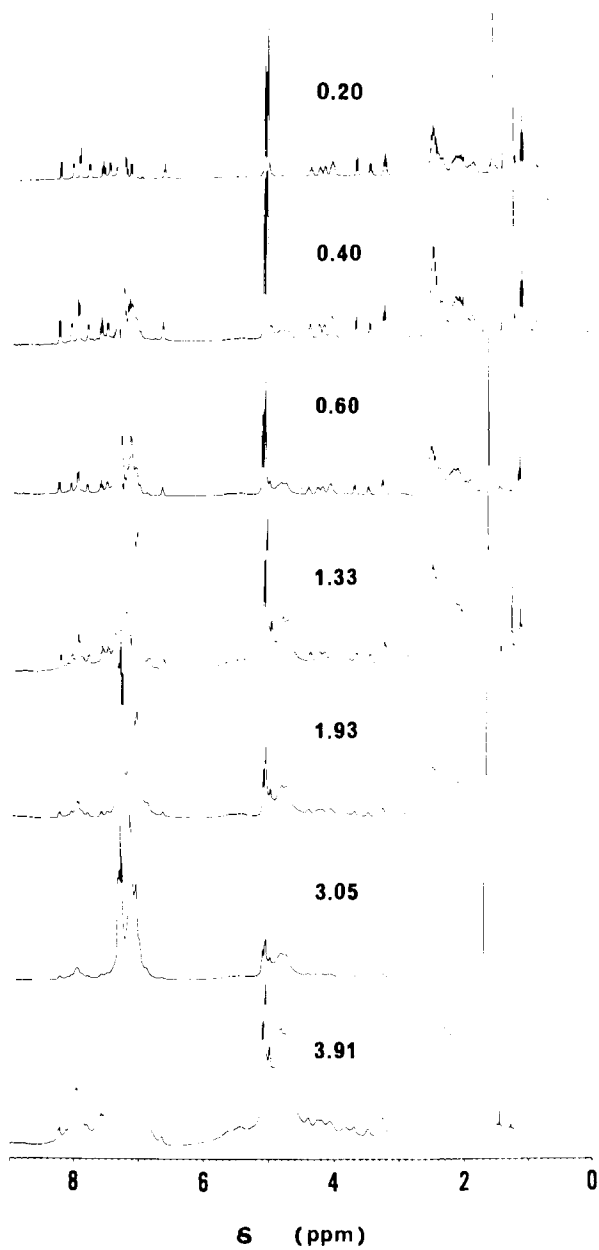
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0.20

0.40

0.60

1.33

1.93

3.05

3.91

8          6          4          2          0

δ    (ppm)

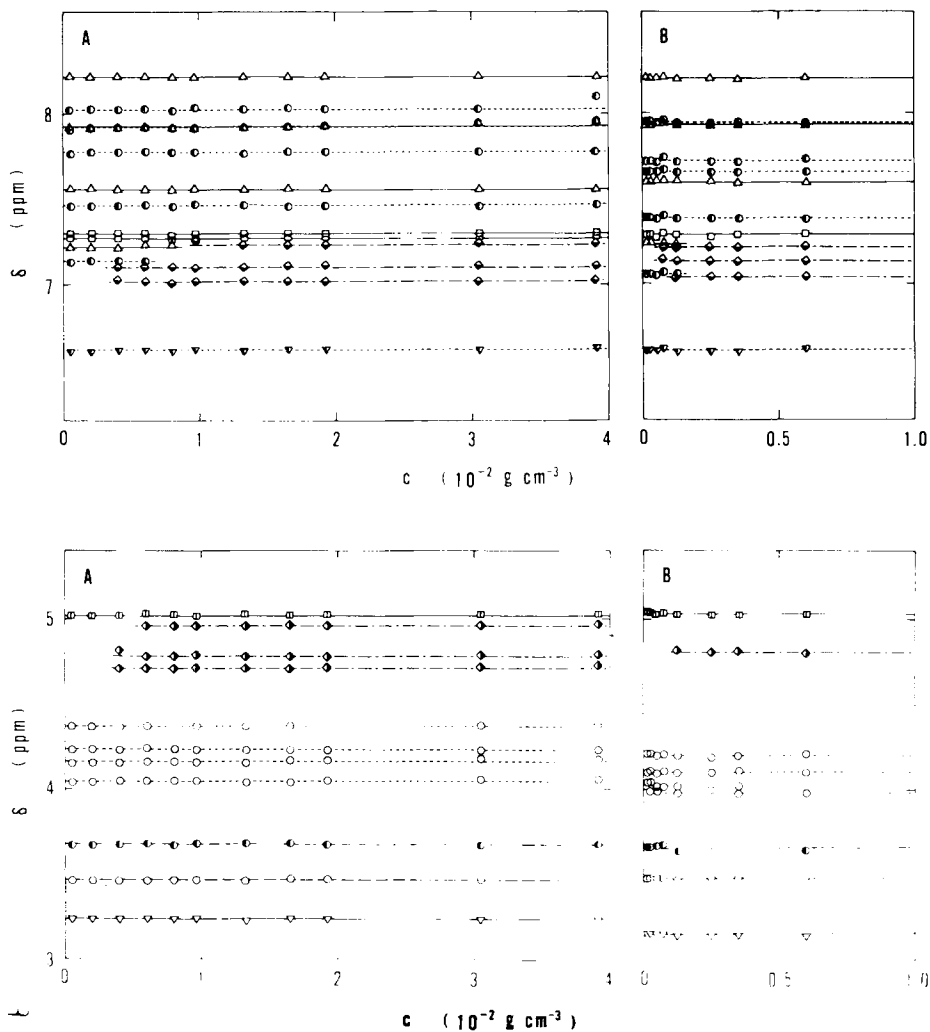Fig. 4. The nmr spectra of the hexapeptide in CDCl₃ at different concentrations (10⁻²g cm⁻³).

Fig. 5. Chemical shifts of various resonances of Nps-[Glu(OBzl)]$_6$-NHC$_2$H$_5$ as functions of concentration. Solvent: A, CDCl$_3$; B, CD$_2$ClCD$_2$Cl. (A) $\delta$ = 6.2–8.6 ppm: ($\triangle$), Nps C$H$; ($\bullet$), N$H$; ($\square$) and ($\blacklozenge$), phenyl C$H$; ($\blacktriangledown$), NH$C_2$H$_5$. (b) $\delta$ = 3.0–5.4 ppm: ($\square$) and ($\blacklozenge$), benzyl C$H_2$; ($\bigcirc$), $\alpha$-C$H$; ($\bullet$), Glu$^1$N$H$; ($\bigtriangledown$), NHC$H_2$CH$_3$.

the Nps group at 8.22 ppm. These intensities increase above the critical concentrations at 0.32 × 10$^{-2}$g cm$^{-3}$ in CDCl$_3$ and at 0.05 × 10$^{-2}$g cm$^{-3}$ in CD$_2$ClCD$_2$Cl, and these critical concentrations are comparable with the critical micelle concentrations derived from the other methods,[6-10] although the nmr spectra measured in deuterated solvents give somewhat lower values. Thus, the nmr spectra responds to the micelle formation of the hexapeptide through the change in rotational freedom of its side chains and the broadening and disappearance of main-chain resonances.
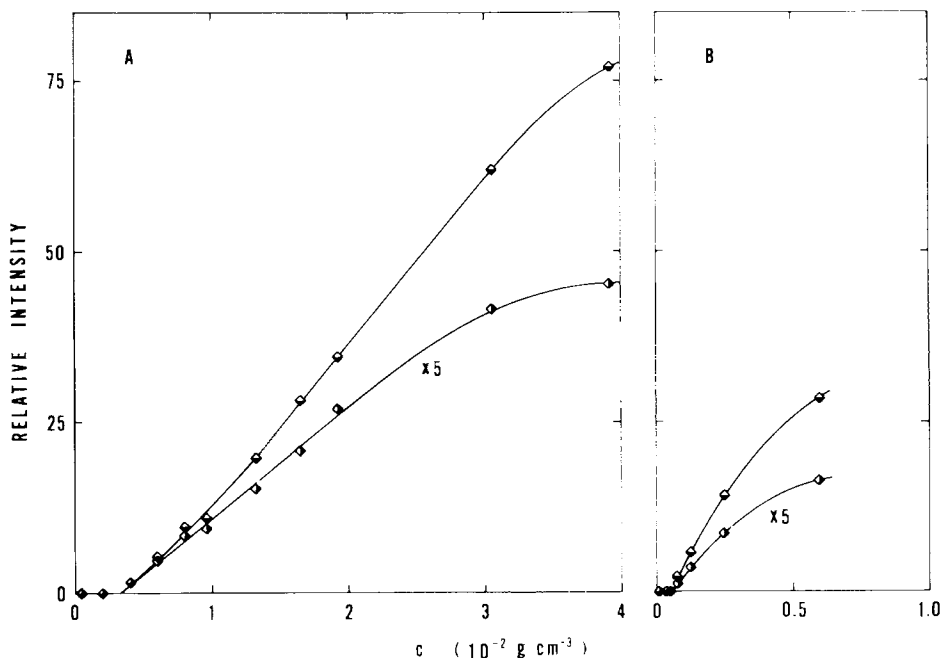
Fig. 6.   Relative intensities of the phenyl CH resonance at 7.11 ppm and the benzyl CH$_2$ resonance at 4.75 ppm as functions of concentration. ◆, phenyl C$H$; ◇, benzyl C$H_2$. Solvent: A, CDCl$_3$; B, CD$_2$ClCD$_2$Cl. The intensities are measured relative to that of the Nps CH resonances at 8.22 ppm. The ×5 for the benzyl CH$_2$ resonances indicates that the ordinate values for the intensity should be ⅕ of those given.

## Temperature Dependence of the Spectra of the Hexapeptide

The nmr spectra of the hexapeptide at 0.077 × 10$^{-2}$g cm$^{-3}$ in CD$_2$ClCD$_2$Cl at different temperatures are shown in Fig. 7. This concentration of the hexapeptide is just above the critical micelle concentration at 25°C. The proton resonances of the restricted phenyl CH group at 7.14 ppm weakens with the rise of temperature and disappears above 35°C. Simultaneously, the fine structure of the resonances becomes manifest. This indicates that micelles are disrupted by elevating temperature. It can be stated that the critical micelle concentration of the hexapeptide is higher at higher temperatures.

As shown in Fig. 8, chemical shifts of the NH and α-CH resonances change linearly with temperature in the low- and high-temperature regions, respectively, and a transition takes place between 25 and 30°C for all the resonances except for an α-CH resonance. The NH resonances of Glu$^2$ to Glu$^6$ and ethylamide exhibit a linear temperature dependence, shifting upfield at higher temperatures. The α-CH resonances of Glu$^2$ to Glu$^6$, except for one (Glu$^3$), show weaker temperature dependence, but they shift downfield with rising temperature. At low temperatures the NH resonance of Glu$^1$ is not distinguishable from
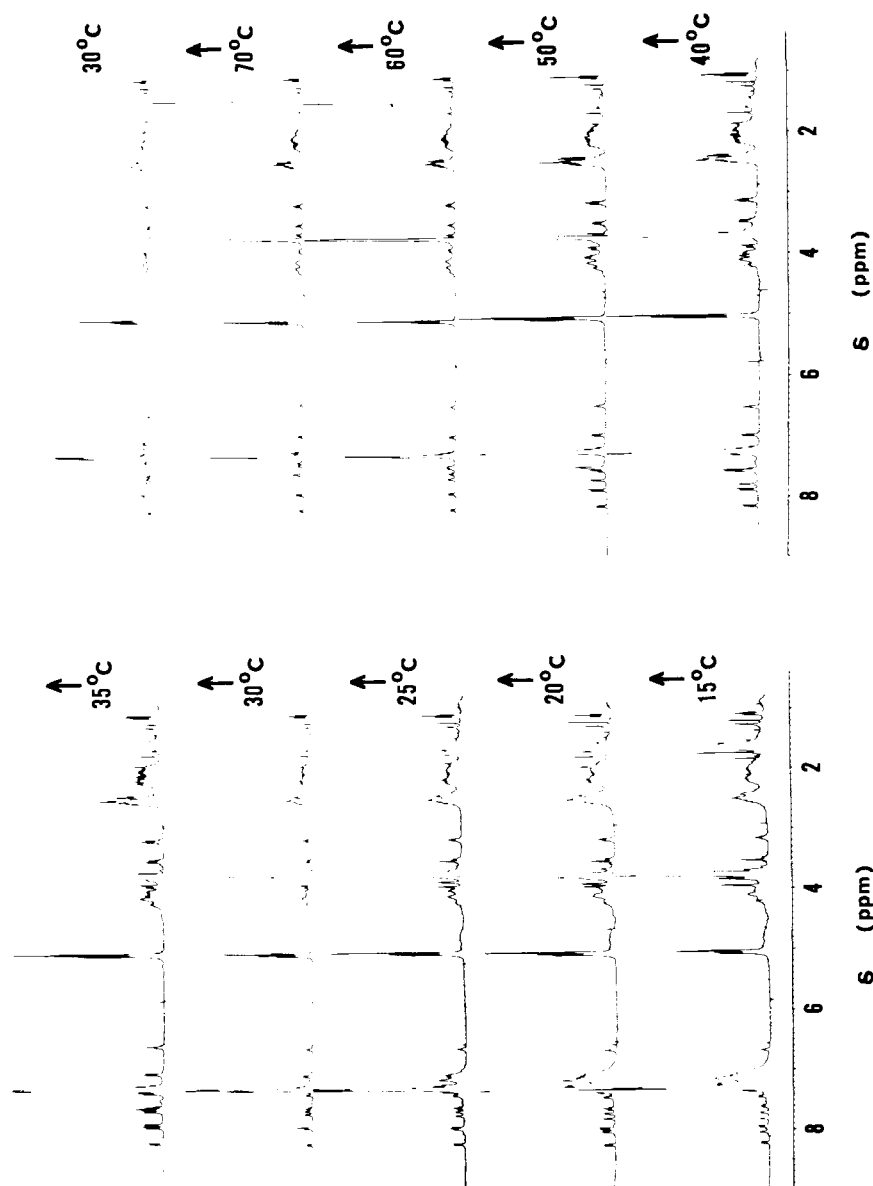
Fig. 7. The nmr spectra of the hexapeptide in $CD_2ClCD_2Cl$ at different temperatures. Concentration: $0.077 \times 10^{-2}$g cm$^{-3}$. Temperature was raised from 15 to 70°C stepwise and finally cooled to 30°C.
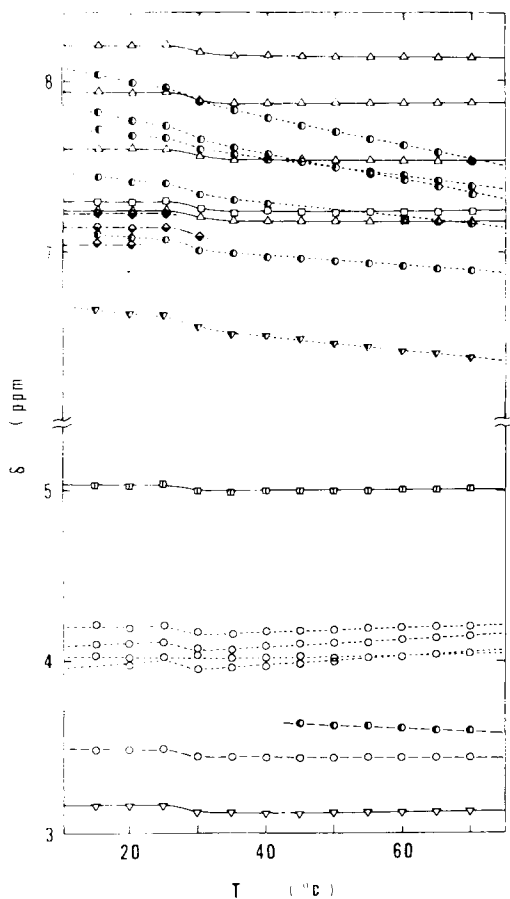
Fig. 8.  Chemical shifts of various resonances of the hexapeptide in $CD_2ClCD_2Cl$ as functions of temperature. Concentration: $0.077 \times 10^{-2} g\ cm^{-3}$. ($\triangle$), Nps C$H$; ($\bullet$), N$H$; ($\square$) and ($\blacklozenge$), phenyl C$H$; ($\blacktriangledown$), N$H$C$_2$H$_5$; ($\boxdot$), benzyl C$H_2$; $\bigcirc$, $\alpha$-C$H$; ($\triangledown$), NHC$H_2$CH$_3$.

the strong resonance of solvent contaminant. The nature of the transition around 25 – 30°C must be related to some change in the $\sigma$-conformation caused by the disruption of micelles, since the new resonances of side chains also disappear above this temperature region. It is observed that chemical shifts of NH resonances of Glu[4] and Glu[5] and of $\alpha$-CH resonances of Glu[3] and Glu[4] exchange their orders from higher to lower fields at 50 and 60°C, respectively.

The temperature coefficients of the NH and $\alpha$-CH resonances are summarized in Table II. They are nearly equal above and below the transition temperature. The low-temperature coefficient of NH resonance indicates the hydrogen-bond-free state, while its high value corresponds to the weakly hydrogen-bonded state.[3,16] Two of the NH resonances at the lowest fields show a stronger temperature-dependent

TABLE II
Temperature Coefficients of Chemical Shifts of the NH and $\alpha$-CH Resonances of the
Hexapeptide in $CD_2ClCD_2Cl$[a]

| | $\delta$ at 25°C (ppm) | $d\delta/dT$ (ppm K$^{-1}$) |
|---|---|---|
| NH resonances | | |
| Glu$^3$ | 7.97 | −0.008 |
| Glu$^4$ | 7.74 | −0.008 |
| Glu$^5$ | 7.67 | −0.005 |
| Glu$^6$ | 7.40 | −0.004 |
| Glu$^2$ | 7.07 | −0.003 |
| Glu$^1$ | 3.68 | −0.002 |
| Ethylamide | 6.63 | −0.004 |
| $\alpha$-CH resonances | | |
| Glu$^2$ | 4.21 | 0.002 |
| Glu$^6$ Glu$^5$ | 4.11 | 0.002 |
| Glu$^3$ | 4.02 | 0.001 |
| Glu$^4$ | 3.98 | 0.003 |
| Glu$^1$ | 3.49 | 0.000 |

[a] Concentration: $0.077 \times 10^{-2}$g cm$^{-3}$.

upfield shift than the others. These two NH groups assigned to Glu$^3$ and Glu$^4$ must participate in intramolecular hydrogen bonding, as will be shown later.

A similar temperature-induced conformational change is observed for the hexapeptide at $0.96 \times 10^{-2}$g cm$^{-3}$ in CDCl$_3$. Resonances at 7.11 and 4.75 ppm for the restricted side chains weaken with a rise of temperature from 23 to 48°C, but they do not disappear even at 48°C. The NH resonances show chemical shifts upfield with rising temperature, as in $CD_2ClCD_2Cl$. Three of the NH resonances at lower fields shift markedly, while the NH proton of Glu$^1$ shifts less. The three NH groups assigned to Glu$^3$ to Glu$^5$ are supposed to be intramolecularly hydrogen-bonded, as will also be shown below. Four $\alpha$-CH resonances of Glu$^2$ to Glu$^6$ shift downfield with rising temperature, as in $CD_2ClCD_2Cl$.

## DISCUSSION

### Structure and Association of the Tetrapeptide

#### Concentration Dependence of the NH and $\alpha$-CH Resonances

At infinite dilution the tetrapeptide is the the $\sigma$-conformation and molecularly dispersed in solution. With increasing concentration, the residues of the $\sigma$-form are gradually transformed into those of the $\beta$-form, and the tetrapeptide molecules are intermolecularly associ-

ated. The intermolecular aggregates of the tetrapeptide consist of the β-structure of the out-of-register type or the imperfect β-conformation.[9]

The behavior of chemical shifts of the NH resonances of $Glu^2$ to $Glu^4$, appearing around 7.1–7.8 ppm, as well as that of $Glu^1$ around 3.6 – 4.0 ppm, may reflect the states of each NH group in the σ-β conformational change and, therefore, the aggregation states of the molecules.

The downfield shift of the NH resonance on increased concentration was also observed for $N$-methoxyethoxyethoxyacetyl-tri- and tetra-L-alanine ethyl esters,[1] $t$-butyloxycarbonyl-tri- and tetra-L-isoleucine,[1] -L-norvaline,[2] or oligo-L-methionine[3] methyl ester in $CDCl_3$. A similar downfield shift of the NH protons was also found on lowering the temperature of these oligopeptides in $CDCl_3$. All these observations were attributed to the formation of increasing amounts of intermolecular hydrogen bonds and association.

The downfield shift of the α-CH resonances with increased concentration occurs almost parallel to that of the NH resonances, and it can be observed more clearly. It may also reflect the states of residues, possibly of C=O groups, in the σ-β conformational change and, therefore, in the aggregated states of the molecules.

There seem to be subtle differences in the feature of sigmoidal changes of the NH and α-CH resonances with concentration, but the observed data are not sufficiently accurate to permit making such a distinction. Thus, we regard all these sigmoidal changes as occurring in the same manner, so that we may consider that they reflect the aggregated states of the tetrapeptide molecules in solution.

We postulate that each of NH and α-CH resonances has chemical shifts characteristic of the aggregated states of the molecule: $\delta_1$ in the unassociated molecules or the monomer, and $\delta_i$ in the aggregate consisting of $i$ molecules or the $i$-mer. For simplicity, the aggregates are assumed to be monodisperse. If the weight fraction of the $i$-mer is represented by $w_i$, the observed chemical shift at a given concentration, $c$ (g cm$^{-3}$), should have a value

$$\delta = (1 - w_i)\,\delta_1 + w_i\delta_i \tag{1}$$

The monomer, $A$, and the $i$-mer, $A_i$, are in equilibrium,

$$i\,A \rightleftarrows A_i \tag{2}$$

for which the law of mass action is given by

$$\frac{w_i}{c^{i-1}(1 - w_i)^i} = K_i' \tag{3}$$

where $K_i'$ is the association constant.

Equation (1) indicates that $\delta$ varies linearly with $w_i$; accordingly, following results on the aggregation of the tetrapeptide may be qualitatively derived from Eq. (3). For $i = 2$, no sigmoidal change of $\delta$ against $c$ is reproduced, but $\delta$ should change concave downward against $c$, if $\delta_i - \delta_1 > 0$. Only if $i$ is larger than 2 a sigmoidal change of $\delta$ with $c$ can be expected, but $i$ should not be so large as to give a critical concentration.

In the previous work on light scattering,[10] we have found that the average aggregaation number of aggregates, excluding monomer, is 23 for the tetrapeptide in ethylene dichloride. Furthermore, we know a series of solvent species favoring both $\beta$-form formation and aggregation for low-molecular-weight poly($\gamma$-benzyl-L-glutamate)[17] and the hexapeptide.[7] That is, chloroform is a slightly weaker solvent than dioxane, and dioxane is a solvent of about one-third as strong as ethylene dichloride. We may then imagine that the average aggregate size of the tetrapeptide is less than 8 in chloroform.

Actual calculation of the chemical shifts as functions of concentration was performed by means of a computer program[18] for the association–dissociation equilibrium, Eq. (2), using data on all the $\alpha$-CH resonances observed in CDCl$_3$. The calculation was performed for Eq. (3) with $i = 2 - 15$, and the results showed that only the cases with $i = 3 - 7$ hold for the observed data within a deviation of less than 2.5%, giving appropriate values of association constants. We can thus conclude that aggregates having a size less than about 8, excluding dimer, are formed by the tetrapeptide in CDCl$_3$. This is in good agreement with our expectation. The derived range of $i$ values indicates some polydispersity of the aggregates.

In CD$_2$ClCD$_2$Cl, similar aggregation takes place more extensively at lower concentrations, because the chemical shifts of the NH and $\alpha$-CH resonances are more sensitive to the concentration, as seen in Fig. 3. This is in accord with our previous light-scattering results,[10] that the aggregates having size about 23 are formed by the tetrapeptide in ethylene dichloride.

### Structure of the $\beta$-Conformation of the Tetrapeptide

In previous ir spectral studies,[9] we have deduced that residues in the $\sigma$-form are either hydrogen-bond-free or intramolecularly hydrogen-bonded. The former residue will be solvated and disordered, while the latter is a part of the $2_7$-helix or the $2_7$-ribbon.[19,20] The $2_7$-ribbon structure was originally proposed by Pysh and Toniolo[2] as one of the possible structures of tri- and tetrapeptides of L-norvaline in CDCl$_3$, and more intensively by Ribeiro et al.[3,4] for oligopeptides of L-methionine and $\gamma$-ethyl-L-glutamate, both in CDCl$_3$.

Infrared spectral analysis has shown[9] that in chloroform four residues in the tetrapeptide molecule form two rings of the $2_7$-ribbon and

one open form at infinite dilution, while in ethylene dichloride they form only one ring of the $2_7$-ribbon and two open forms at infinite dilution. Figure 9 shows such structures of the tetrapeptide in chloroform and ethylene dichloride. If the intramolecular hydrogen bond causes a downfield shift of the NH and $\alpha$-CH resonances, their chemical shifts in $CDCl_3$ located at lower fields than those in $CD_2ClCD_2Cl$ (Table I) are also consistent with these structures of the tetrapeptide.

The chemical shift of the NH resonance of Glu[1] changes less sensitively with concentration. Thus, the NH groups of Glu[1] must be involved in the strong hydrogen bonding with the nitro group of Nps moiety, as previously suggested.[9]

At infinite dilution, the NH group of Glu[2] is hydrogen-bond-free, a the $2_7$-ring is formed by the hydrogen bond between the NH group of Glu[3] and the C=O group of Glu[1]. Then, the NH resonance of Glu[2] appears at the highest field among the three residues, Glu[2] to Glu[4]. The NH group of Glu[4] and the C=O group of Glu[2] in $CDCl_3$ also form a $2_7$-ring at infinite dilution, leaving the NH group of ethylamide hydrogen-bond-free. However, they do not form the $2_7$-ring in $CD_2ClCD_2Cl$.

The intermolecular hydrogen bond can be formed most easily in $CDCl_3$ between the NH group of ethylamide of a molecule and the C=O group of Glu[4] of another molecule as the concentration is increased. Such a structure that leads to the multimer or aggregate formation is illustrated in Fig. 10(a) in a planar projection. For a residue to be converted from the $\sigma$- to the $\beta$-form, one of the $2_7$-rings of a molecule must be opened and the intermolecular hydrogen bonds must link both the NH and C=O groups with the counterparts of the other molecules. One of such conversions of a residue may be accomplished by opening a $2_7$-ring and forming an intermolecular hydrogen bond of the NH group of Glu[4], as shown in Fig. 10(b).

In $CD_2ClCD_2Cl$ the tetrapeptide molecules also exhibit similar concentration dependence of the NH and $\alpha$-CH resonances of all the residues. Because the number of $2_7$-rings of the tetrapeptide in $CD_2ClCD_2Cl$ is smaller, as compared with that in $CDCl_3$, the NH group of Glu[4] residues should be hydrogen-bond-free at infinite dilution, so that the conversion of Glu[4] from the $\sigma$- to the $\beta$-form can be accomplished more easily. The structure formed in this way will be similar to that shown in Fig. 10(b).

A more perfect $\beta$-conformation of the tetrapeptide will be formed in only very small amounts, in both $CDCl_3$ and $CD_2ClCD_2Cl$, since the content of $\beta$-structure is as low as 24% at $3 \times 10^{-2}$g cm$^{-3}$ and 50% at $2 \times 10^{-2}$g cm$^{-3}$, respectively.

The CH resonances of Nps group change only slightly and linearly upfield with increasing concentration, indicating that the Nps group is not influenced by the conformational change and the intermolecular association of the tetrapeptide.
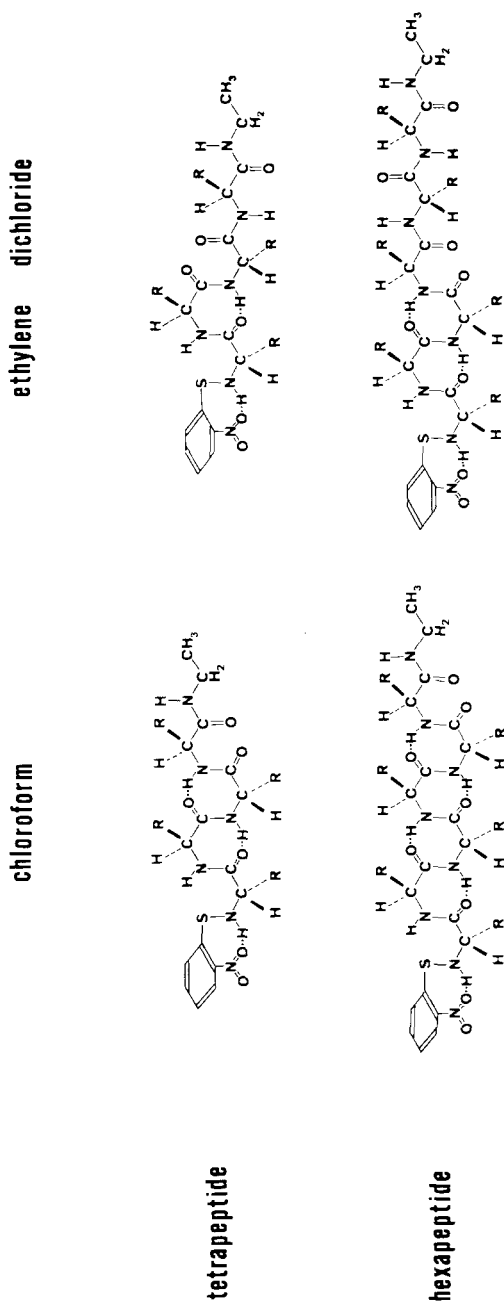
Fig. 9. Structure of the unassociated molecules of the tetrapeptide and hexapeptide in chloroform and ethylene dichloride. The tetrapeptide is exclusively in the σ-conformation at infinite dilution, while the hexapeptide is in the σ-conformation below certain critical concentrations.
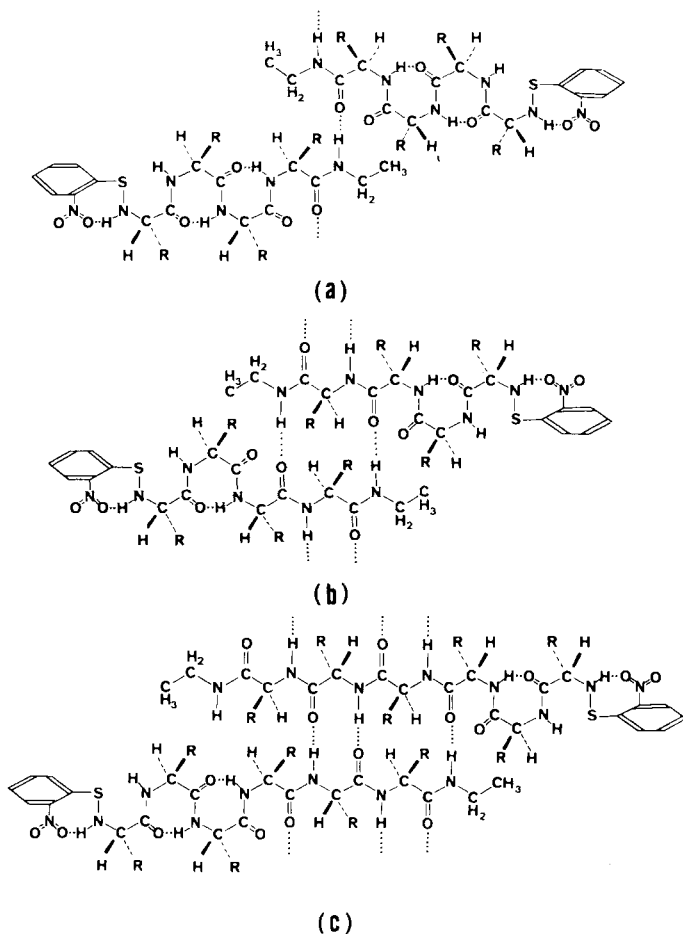
**(a)**



**(b)**



**(c)**

Fig. 10. Some possible structures of associated molecules of the oligopeptides. (a), (b): Tetrapeptide in the $\beta$-conformation. (c) Hexapeptide in the $\beta$-structure of the out-of-register type.

## Structure and Association of the Hexapeptide

According to ir spectral studies,[6,9] the hexapeptide molecules are in the $\sigma$-conformation in chloroform and ethylene dichloride below the critical concentrations, where all the residues are in the $\sigma$-form. The $\sigma$-conformation of the hexapeptide consists of four rings of the $2_7$-ribbon and one open form in chloroform and of two rings and three open forms in ethylene dichloride. The NH group of Glu$^1$ is involved in the hydrogen bonding with the nitro group of Nps moiety, and those of Glu$^2$ and of ethylamide would be hydrogen-bond-free, as suggested by the location of their resonances at the highest fields.

The high negative temperature coefficients of the two NH resonances at the lowest fields indicate that they are intramolecularly

hydrogen-bonded in $CD_2ClCD_2Cl$, indicating intramolecular hydrogen bonding of the NH groups of Glu[3] and Glu[4]. Similarly, the higher negative temperature coefficients are observed for three NH resonances in $CDCl_3$, which supports the intramolecular hydrogen bonds of NH groups of Glu[3] to Glu[5]. Thus, the intramolecular hydrogen bonds are more readily formed with residues closer to the N-terminal, except for Glu[2].

Figure 9 illustrates the structures of the hexapeptide molecules in chloroform and ethylene dichloride that are in the unassociated state or in the σ-conformation. As shown in Table I, chemical shifts of the NH and α-CH resonances in $CDCl_3$ are located at lower fields than those in $CD_2ClCD_2Cl$. Since the intramolecular hydrogen bond would cause downfield shift of these resonances, our observations are in agreement with the structures in Fig. 9.

The hexapeptide molecules are transformed from the σ- to the β-conformation with increasing concentration above the critical concentration; the residues are converted from the σ- to the β-form by forming intermolecular hydrogen bonds, and micelles are formed in both chloroform and ethylene dichloride.

Nevertheless, the nmr spectra of the hexapeptide in both $CDCl_3$ and $CD_2ClCD_2Cl$ do not show any concentration dependence of chemical shifts, except for some of the side-chain resonances. The intensities of the NH and α-CH resonances remain nearly constant, or decrease, with increasing concentration, when referred to the intensity of the resonance of contaminant $H_2O$.

With increasing concentration, the intermolecular hydrogen bonds are formed successively from residues closer to the C-terminal. Figure 10(c) illustrates a possible structure of the hexapeptide molecules in the β-conformation, i.e., the β-structure of the out-of-register type.

We may conclude that the nmr spectra of the hexapeptide in both solvents do not reflect the residues of all the molecules in solution but give information only about the unassociated molecules or the σ-conformation. The spectra of the associated molecules in the micelles are not detectable because of the spectral broadening arising from their too rigid structure, which also makes the exchange of residues between the σ- and the β-form difficult or slow. Only the side chains keep some freedom to give observable resonances even in the associated molecules or the β-conformation. Thus, the new resonances associated with the phenyl CH and benzyl $CH_2$ groups appear above the critical concentration, indicating separation of two rotational isomeric states.

The high contents of β-structure of the hexapeptide in chloroform and ethylene dichloride,[6,9] as well as its in-register β structure in ethylene dichloride,[8] are also consistent with our present observations. The concentration independence of chemical shifts of the hexapeptide is in agreement with that observed for the NH and α-CH resonances of t-butyloxycarbonyl-hexa- and hepta-L-methionine methyl esters in $CDCl_3$.[3]

## References

1. Goodman, M., Ueyama, N., Naider, F., & Gilon, C. (1975) *Biopolymers* **14**, 915–925.

2. Pysh, E. & Toniolo, C. (1977) *J. Am. Chem. Soc.* **99**, 6211–6219.

3. Ribeiro, A. A., Goodman, M. & Naider, F. (1979) *Int. J. Pept. Protein Res.* **14**, 414–436.

4. Ribeiro, A. A., Saltman, R. P. & Goodman, M. (1980) *Biopolymers* **19**, 1771–1790.

5. Okahashi, K. & Ikeda, S. (1979) *Int. J. Pept. Protein Res.* **13**, 462–472.

6. Okahashi, K. & Ikeda, S. (1979) *Biopolymers* **18**, 2105–2113.

7. Ikeda, S. & Okahashi, K. (1979) *Biopolymers* **18**, 2115–2126.

8. Imae, T., Okahashi, K. & Ikeda, S. (1981) *Biopolymers* **20**, 2553–2566.

9. Imae, T. & Ikeda, S. (1984) *Biopolymers* **23**, 2573–2586.

10. Imae, T. & Ikeda, S. (1985) *Biopolymers* **24**, 585–599.

11. Idelson, M. & Blout, E. R. (1957) *J. Am. Chem. Soc.* **79**, 3948–3955.

12. Markley, J. L., Meadows, D. H. & Jardetzky, O. (1967) *J. Mol. Biol.* **27**, 25–40.

13. Goodman, M., Verdini, A. S., Toniolo, C., Philips, W. D. & Bovey, F. A. (1969) *Proc. Natl. Acad. Sci. USA* **64**, 444–450.

14. Goodman, M., Ueyama, N. & Naider, F. (1975) *Biopolymers* **14**, 901–914.

15. Naider, F., Ribeiro, A. A. & Goodman, M. (1980) *Biopolymers* **19**, 1791–1799.

16. Stevens, E. S., Sugawara, N., Bonora, G. M. & Toniolo, C. (1980) *J. Am. Chem. Soc.* **102**, 7048–7050.

17. Imae, T. & Ikeda, S. (1973) *Biopolymers* **12**, 1203–1221.

18. Sugeta, H. (1981) *Bull. Chem. Soc. Jpn.* **54**, 3706–3710.

19. Huggins, M. L. (1943) *Chem. Rev.* **32**, 195–218.

20. Bragg, W. L., Kendrew, J. C. & Perutz, M. F. (1950) *Proc. R. Soc. London, Ser. A* **203**, 321–357.