BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 43 3054—3063 (1970)

A Nuclear Magnetic Resonance Study of Copolypeptides of L-Proline with γ-Benzyl-L-glutamate*1,1)

Hirofumi Okabayashi,*2 Rume Sakai and Toshizo Isemura

Division of Physical Chemistry, Institute for Protein Research, Osaka University, Kita-ku, Osaka

(Received April 4, 1970)

The nuclear magnetic resonance spectra of the copolymers of L-proline with γ-benzyl-L-glutamate were measured in tetradeuteroacetic acid, deuterochloroform, a mixed solvent (CD_3COOD : $CDCl_3$=4 : 1), and trifluoroacetic acid. The time dependence of the α-CH resonance peaks of copoly-(3 : 1)-(L-proline, γ-benzyl-L-glutamate) and copoly-(1 : 1)-(L-proline, γ-benzyl-L-glutamate) was observed. The NMR spectra of copoly-(1 : 3)-(L-proline, γ-benzyl-L-glutamate) showed no time dependence which can be ascribed to the conformational change in tetradeuteroacetic acid and the mixed solvent. From these experimental facts we concluded that copoly-(3 : 1)-(L-proline, γ-benzyl-L-glutamate) and copoly-(1 : 1)-(L-proline, γ-benzyl-L-glutamate) can assume a poly-L-proline-like helical structure, while copoly-(1 : 3)-(L-proline, γ-benzyl-L-glutamate) can not. Moreover, the ratios of the γ-benzyl-L-glutamate residues which were incorporated into the stable poly-L-proline-II-like helical structure was estimated in copoly-(3 : 1)-(L-proline, γ-benzyl-L-glutamate) and copoly-(1 : 1)-(L-proline, γ-benzyl-L-glutamate).

Poly-L-proline can assume two different conformations, namely, the forms I and II. The structure of poly-L-proline II has been determined by the X-ray diffraction method by Cowan and McGavin[2] and Sasisekharan,[3] who found that the II form has a left-handed helix and that the peptide bonds are in a *trans* configuration. Traub and Shmueli[4] have also found by the X-ray diffraction method that the I form assumes a right-handed helix and that each peptide bond is in a *cis* configuration.

The poly-L-proline takes the I form immediately after the sample is dissolved in organic acids, water, and *m*-cresol. However, it is eventually transformed into the II form.

The first NMR spectra of the poly-L-prolines I and II were reported by Okabayashi, Sakai, and Isemura,[5–7] who observed a marked difference in the magnetic shielding values of the α-CH resonance peaks of the two polymers. The α-CH peaks of the I form appeared at the shielding values of 5.5 ppm in the mixed solvent (deuterochloroform : tetradeuteroacetic acid=8 : 1) and 5.4 ppm in tetradeuteroacetic acid, while that of the poly-L-proline II appeared at 5.2 ppm in tetradeuteroacetic acid. The marked shift of the α-CH resonance peak has been ascribed to the differences in the conformations of the two polymers. During the course of the I→II transition of poly-L-proline in tetradeuteroacetic acid, the two α-CH resonance peaks were observed at 5.2 and 5.4 ppm. However, when the spectrum was measured immediately after the sample has been dissolved in tetradeuteroacetic acid as rapidly as possible, the only α-CH resonance peak of the I form appeared at the magnetic shielding value of 5.4 ppm. The appearance of the α-CH resonance peak of the II form at 5.2 ppm and a gradual increase in the intensity of the resonance peak with the time was observed, whereas the α-CH resonance peak of the I form showed an accompanying decrease in intensity with the time (see Fig. 1).

*1 This paper was presented at the 8th Symposium on Biophysics of the Biophysical Society of Japan, October, 1969, and at the Symposium on Molecular Structures of the Chemical Society of Japan, October, 1969.

*2 Present address: Department of Industrial Chemistry, Nagoya Institute of Technology, Gokiso, Showa-ku, Nagoya.

1) H. Okabayashi and T. Isemura, Abstracts for Symposium on Molecular Structures (October, 1969), p. 199.

2) P. Cowan and S. McGavin, *Nature*, **176**, 501 (1955).

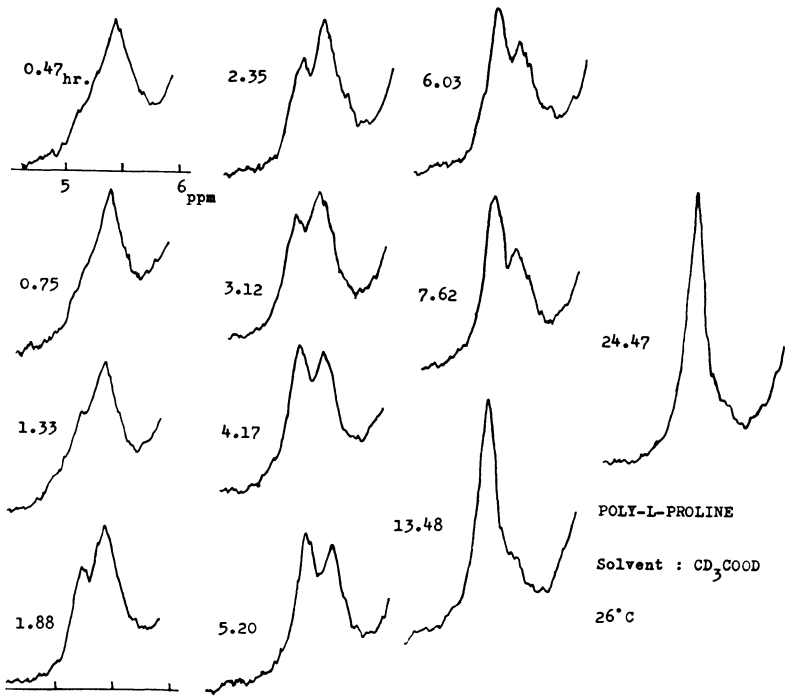
3) V. Sasisekharan, *Acta Cryst.*, **12**, 897 (1959).

4) W. Traub and U. Shmueli, "Aspects of Protein Structure," ed. by G. N. Ramachandran, Academic Press, New York (1963), p. 81.

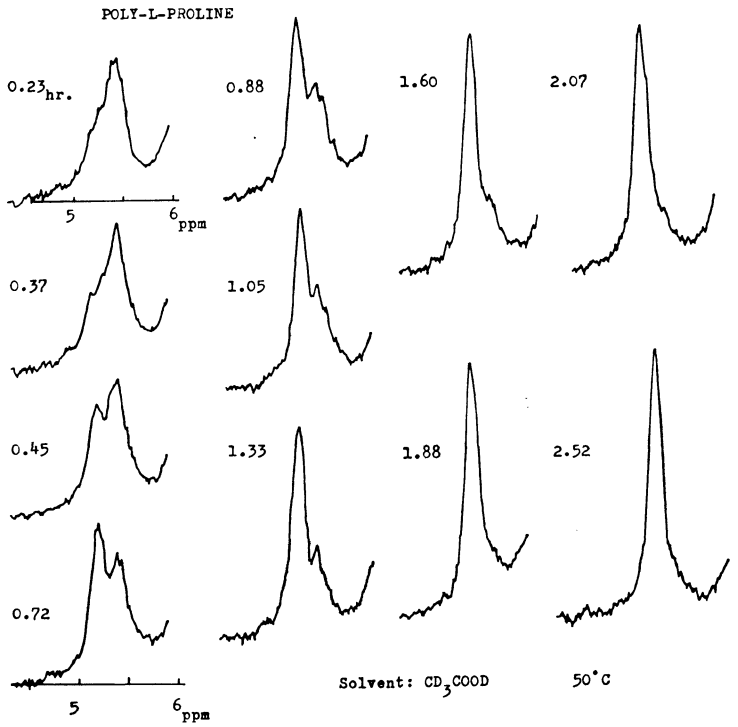
5) H. Okabayashi, R. Sakai and T. Isemura, Abstract IV, at the 21st Annual Meeting of the Chemical Society of Japan, April, 1968, p. 2685.

6) H. Okabayashi, R. Sakai and T. Isemura, "Abstract", presented at the Symposium on High Polymers of the Chemical Society of Japan, October, 1968, p. 721.

7) R. Sakai, H. Okabayashi and T. Isemura, This Bulletin, **42**, 3028 (1969).



(a) at 26°C



(b) at 50°C

Fig. 1. The time dependence of the NMR spectra of poly-L-proline.

If the temperature is raised, such a transition progresses rapidly. From the ratios of the areas of the α-CH peaks of the I form to those of the II form, the percentages of the I form (or of the II form) during the transition were estimated. The results showed that the α-CH resonance peak of the I form disappeared in thirty hours at 26°C and that the I→II transition was completed within three hours at 50°C. The I→II transition can be concluded to proceed more rapidly than indicated by the measurement by means of the optical rotations.[6]

These studies have now been extended to copolypeptides of L-proline with γ-benzyl-L-glutamate.

In this work the NMR spectra of copoly-(3 : 1)-(L-proline, γ-benzyl-L-glutamate),*3 copoly-(1 : 1)-(L-proline, γ-benzyl-L-glutamate),*3 and copoly-(1 : 3)-(L-proline, γ-benzyl-L-glutamate)*3 were measured and compared with those of the poly-L-prolines I and II and poly-γ-benzyl-L-glutamate.

The steric structures of the copolymers of L-proline with γ-benzyl-L-glutamate in organic solvents have been investigated by Sakai, Ikeda, and Isemura[8] using the methods of the optical rotatory dispersion and the infrared absorption spectra. The results may be summarized as follows.

In 1,2-dichloroethane, which stabilizes the poly-L-proline-I helix and the α-helix of poly-γ-benzyl-L-glutamate, proline residues assume the *cis*-configuration if the copolymers have a proline content of more than 50%. On the other hand, in the copolymers with proline contents of less than 50%, the proline residues tend to take the *trans*-configuration. In an α-helicogenic solvent such as *m*-cresol, the proline residues of the copolymers have the *trans*-configuration, and most benzyl glutamate residues are in random coils in dichloroacetic acid.

Experimental

These copolypeptides, copoly-(3 : 1)-(L-proline, γ-benzyl-L-glutamate), copoly-(1 : 1)-(L-proline, γ-benzyl-L-glutamate), and copoly(1 : 3)-(L-proline, γ-benzyl-L-glutamate), which has been synthesized by one of the present authors, were used in these studies. Table 1 shows the residue mole fractions of the L-proline of the copolymers.[9]

Table 1. Residue mole fraction of L-proline in the copolymers

	A	B	C
Copoly-(3:1)	0.75	0.767	0.756
Copoly-(1:1)	0.50	0.535	0.528
Copoly-(1:3)	0.24	0.274	0.260

A : polymerization ratio,
B : fraction of proline determined from amino acid analysis
C : from absorbance of benzyl residues.

These copolypeptides were synthesized by the copolymerization of *N*-carboxy anhydrides of L-proline and γ-benzyl-L-glutamate in nitrobenzene.

The deuterochloroform (isotopic purity: 99%), tetradeuteroacetic acid (isotopic purity: 99%), trifluoroacetic acid, and tetramethylsilane for spectroscopy were purchased from Merck, Ltd; they were used without further purification.

All the proton magnetic resonance spectra were measured at 100 MHz with the use of a Japan Electron Optics Laboratory (Jeolco), model JNM-4H-100, NMR spectrometer.

A 5% solution of the copolypeptides was used at 26°C in all the experiments.

Results and Discussion

Figure 2 shows the NMR spectra of poly-L-prolines I and II, which were reported in our previous papers.[6,7] All the resonance peaks are fully developed, and their relative areas are proportional to the numbers of protons at the different sites in the polymers. In tetradeuteroacetic

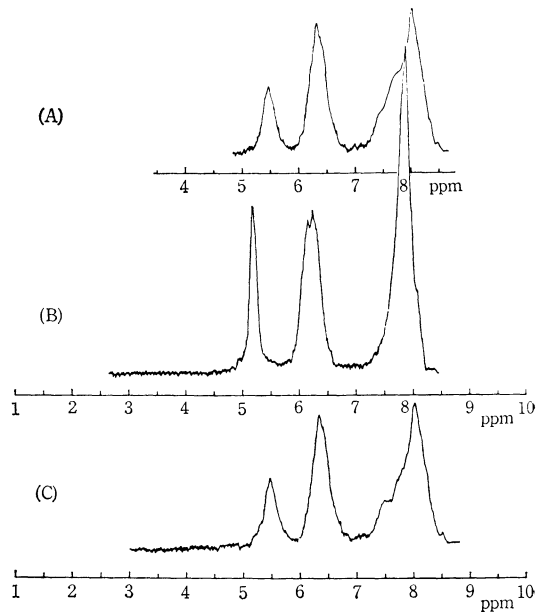


Fig. 2. The NMR spectra of poly-L-prolines I and II in three solvents at 26°C.
(A) Polyproline I
Solvent: CD_3COOD Reference: TMS
(B) Poly-L-proline II
Solvent: CD_3COOD Reference: TMS
(C) Poly-L-proline I
Solvent: $CDCl_3$: CD_3COOD=8 : 1

*3 Abbreviations used: Copoly-(3 : 1), copoly-(3 : 1)-(L-proline, γ-benzyl-L-glutamate); Copoly-(1 : 1), copoly-(1 : 1)-(L-proline, γ-benzyl-L-glutamate); Copoly-(1 : 3), copoly-(1 : 3)-(L-proline, γ-benzyl-L-glutamate).

8) R. Sakai, S. Ikeda and T. Isemura, This Bulletin, **42**, 1332 (1969).

9) R. Sakai, S. Ikeda and T. Isemura, This Bulletin, **39**, 2308 (1966).

acid the α-CH resonance peak of the I form completely disappeared at 26°C in thirty hours, thereafter only the α-CH resonance peak of the II form was observed, at 5.2 ppm. At 45°C the α-CH peak of the I form disappeared within five hours. During the I→II transition of poly-L-proline, no line broadening or decrease in area was observed.

Figure 3 shows the NMR spectra of poly-γ-benzyl-L-glutamate and those of poly-L-proline in trifluoroacetic acid. Table 2 gives the resonance positions of the NMR spectra of two poly-L-prolines and poly-γ-benzyl-L-glutamate. Bovey's data[10] are also listed in this table.

It is of interest to ascertain whether the changes in the α-CH resonance peak found in the NMR spectra of poly-L-proline can be observed in the copolypeptides of L-proline with γ-benzyl-L-glutamate.

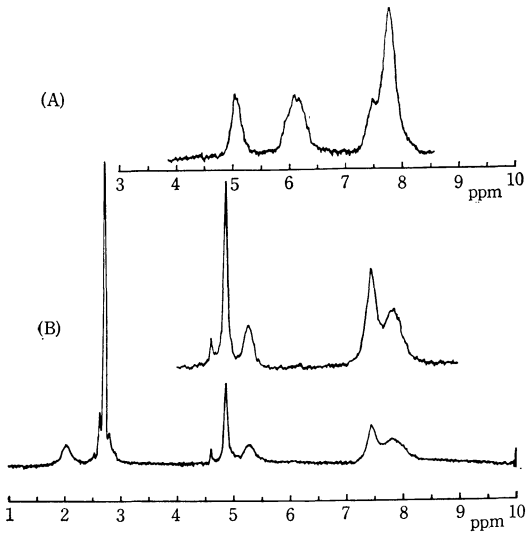


Fig. 3. The NMR spectra of poly-γ-benzyl-L-glutamate and poly-L-proline in trifluoroacetic acid at 26°C.
(A) Poly-L-proline
Solvent: CF_3COOH Reference: TMS
(B) Poly-γ-benzyl-L-glutamate
Solvent: CF_3COOH Reference: TMS

TABLE 2. NUCLEAR MAGNETIC RESONANCE SPECTRA OF POLY-L-PROLINE AND POLY-γ-BENZYL-L-GLUTAMATE AT 26°C

(a) Poly-L-prolines I and II

τ-values relative to internal reference				
CF_3-COOH	CD_3COOD		mixed solvent	assignments
	I*	II	I	
5.05	5.40	5.23	5.50	α-CH
6.15	6.30	6.25	6.30	δ-CH_2
7.50, 7.79	8.0	7.90	7.80, 8.0	β, γ-CH_2

(b) Poly-γ-benzyl-L-glutamate

τ-values relative to internal reference		
CF_3COOH	Bovey's data (CF_3COOH)	assignments
2.05	1.84	NH
2.73	2.54	phenyl group
4.88	4.74	benzylic methylene
5.27	4.98	α-CH
7.45, 7.85	7.50	β, γ-CH_2

Internal reference: TMS
Mixed solvent: (CD_3COOD: $CHCl_3$=4: 1)
* The NMR spectra of the I form in tetradeuteroacetic acid were measured as soon as the sample was completely dissolved.

Figures 4, 5, and 6 show the NMR spectra of copoly-(3 : 1), copoly-(1 : 1) and copoly-(1 : 3) in tetradeuteroacetic acid, deuterochloroform, and a mixed solvent ($CDCl_3$: CD_3COOD=8 : 1). The assignments of the resonance peaks are given in Table 3.

In Fig. 4 especially, the NMR spectra of the copolypeptides in deuterochloroform and tetradeuteroacetic acid are remarkable. In deuterochloroform an α-CH resonance peak of the copoly-

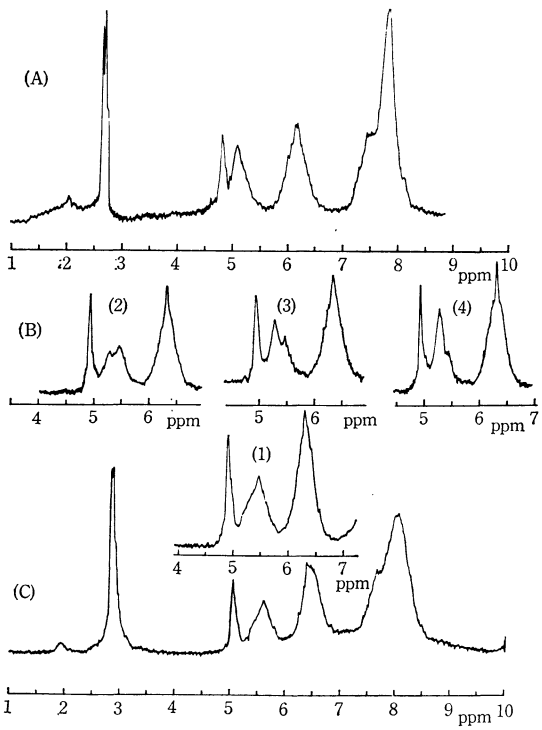


Fig. 4. The NMR spectra of copoly-(3:1) at 26°C.
(A) Solvent: CF_3COOH
(B) Solvent: CD_3COOD
(1) after 10 min (2) after 30 min
(3) after 90 min (4) after 24 hr
(C) Solvent: $CDCl_3$

10) F. A. Bovey, G. V. D. Tiers and G. Filipovich, *J. Polym. Sci.*, **38**, 73 (1959).

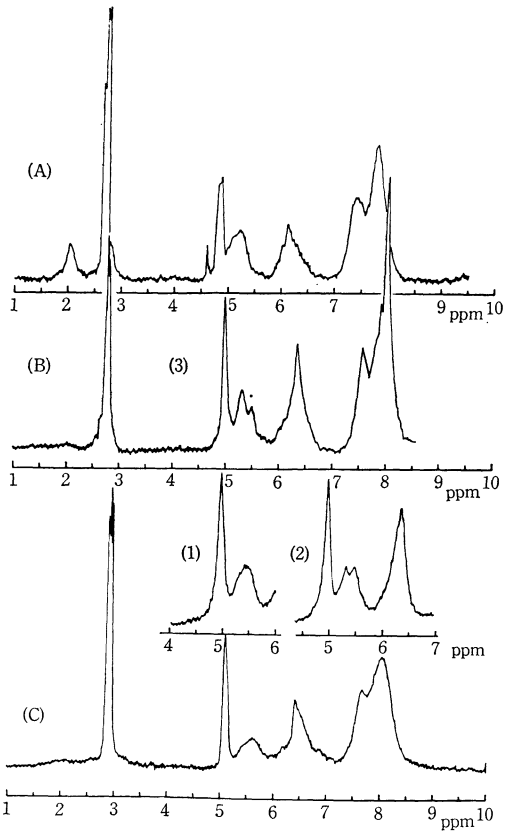


Fig. 5. The NMR spectra of copoly-(1 : 1) at 26°C.
(A) Solvent: CF_3COOH
(B) Solvent: CD_3COOD
(1) after 8 min (2) after 20 min
(3) after 24.85 hr
(C) Solvent: $CDCl_3$

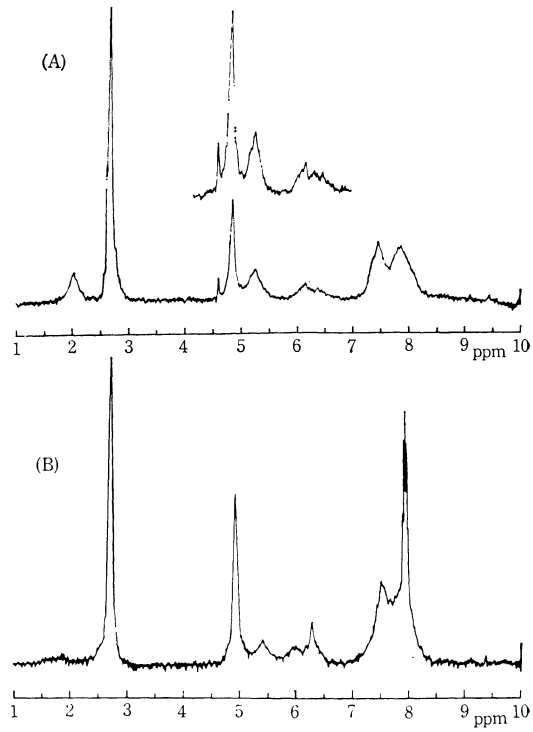


Fig. 6. The NMR spectra of copoly-(1 : 3) at 26°C.
(A) Solvent: CF_3COOH Reference: TMS
(B) Solvent: CD_3COOD : $CDCl_3$=4 : 1
Reference: TMS

(3 : 1) consists of only one component, which shows the agreement between the position of the α-CH resonance peak of the L-proline residues and that of the γ-benzyl-L-glutamate residues. Poly-γ-benzyl-L-glutamate in deuterochloroform has a right-handed α-helical structure, while the conformation of poly-L-proline in the solvent is considered to be a poly-L-proline-I-like helix, since the value of $[\alpha]_{546}$ is +30° in the mixed solvent (CD_3COOD : $CDCl_3$= 1 : 8).

Figure 4 (B) shows the NMR spectra of the copoly-(3 : 1) in the tetradeuteroacetic acid solution. By measuring the spectra immediately after the sample has been completely dissolved as rapidly as possible, the symmetrical α-CH resonance peak was observed. The magnetic shielding value, 5.45 ppm, of the α-CH resonance peak is almost the same as that of the NMR spectra of poly-L-proline I in tetradeuteroacetic acid. After thirty minutes, the NMR spectrum of the copoly-(3 : 1) gives two components of the α-CH resonance peak at 5.2 and 5.45 ppm. The component of 5.2 ppm is in accord with the α-CH peak of the poly-L-proline II in the position. Considering the α-CH peak at 5.2 ppm, the copolypeptide can be said to assume the structure of the poly-L-proline II. The resonance peak at 5.2 ppm increases in intensity with the time, while the peak at 5.45 ppm decreases in intensity with the time, as in the case of the I→II transition of poly-L-proline. Such behavior of the α-CH resonance peak of copoly-(3 : 1) clearly shows that the copolypeptide has the poly-L-proline-like structure. In the NMR spectra of the copoly-(3 : 1), no line broadening or loss of the peak area of the α-CH resonance was observed during the poly-L-proline-I-like→II transition.

In Fig. 5 the α-CH resonance peak of the copoly-(1 : 1) shows a transition of poly-L-proline I→II similar to that in the case of the copoly-(3 : 1). This fact also suggests that the copoly-(1 : 1) partially assumes a helical structure as does poly-L-proline. In the α-CH resonance of the copoly-(1 : 1), a shoulder at 5.2 ppm was observed, with a main peak of the α-CH resonance at 5.4 ppm in tetra-

TABLE 3. NUCLEAR MAGNETIC RESONANCE τ-VALUES FOR COPOLYPEPTIDES OF L-PROLINE WITH γ-BENZYL-L-GLUTAMATE AT 26°C

(a) Copoly-(3:1)-(L-proline, γ-benzyl-L-glutamate)

τ-Values relative to internal reference			assignments
CF_3COOH	CD_3COOD	$CDCl_3$	
—	—	1.95	NH of γ-benzyl-L-glutamate
2.70	2.65	2.88	phenyl group
4.82	4.90	5.06	benzylic methylene
5.10	5.45* 5.40** 5.23	5.60	α-CH
6.51	6.30	6.45	δ-CH_2 of prolyl residues
7.50 7.83	7.52 7.99	7.70 8.05	β, γ-CH_2 of γ-benzyl-L-glutamate and prolyl residues

* Obtained at ten minutes after the dissolution of the sample was completed.
** Obtained at 1.5 hr after the sample was completely dissolved.

(b) Copoly-(1:1)-(L-proline, γ-benzyl-L-glutamate)

τ-Values relative to internal reference			assignments
CF_3-COOH	CD_3-COOD	$CDCl_3$	
2.05	—	—	NH of γ-benzyl-L-glutamate
2.72	2.70	2.91	phenyl group
4.85	4.92	5.08	benzylic methylene
5.20	5.25 5.40	5.60	α-CH
6.15	6.32	6.40	δ-CH_2 of L-proline residues
7.40 7.80	7.56 8.03	7.65 8.03	β, γ-CH_2 of γ-benzyl-L-glutamate and L-proline residues

(c) Copoly-(1:3)-(L-prolin, γ-benzyl-L-glutamate)

τ-Values relative to internal reference		assignments
CF_3COOH	mixed solvent	
2.05		NH of γ-benzyl-L-glutamate
2.75	2.72	phenyl group
4.86	4.92	benzylic methylene
5.28	5.40	α-CH
6.2	6.30	δ-CH_2 of L-proline residues
7.45 7.85	7.52 7.95	β, γ-CH_2 of γ-benzyl-L-glutamate and L-proline residues

Internal reference: TMS
Mixed solvent: (CD_3COOD: $CDCl_3$=4 : 1)

deuteroacetic acid. In deuterochloroform, a shoulder at 5.4 ppm was also found, with a main peak of the α-CH resonance at 5.6 ppm. The shoulder at 5.2 ppm may be interpreted as a function of the helical lengths of the copoly-(1 : 1), in which the short chains of the L-proline residues take a poly-L-proline II-like helical structure, judging from the results of our NMR study[11].

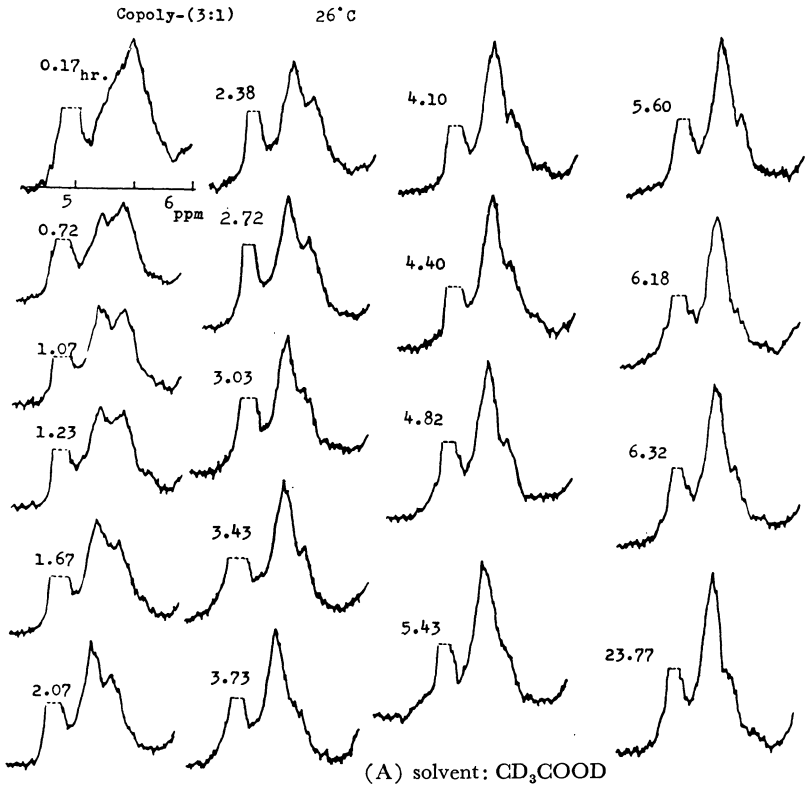
Figure 6 shows the NMR spectra of the copoly-(1: 3) obtained by using a mixed solvent (CD_3-$COOD$: $CDCl_3$=4 : 1) solution, since the copolypeptide can not be dissolved in tetradeuteroacetic acid. Although the α-CH resonance peak of the copoly-(1: 3) appears at the magnetic shielding value of 5.4 ppm, a value which agrees with the position of the α-CH resonance of poly-L-proline I in tetradeuteroacetic acid solution, the α-CH resonance peak of this copolymers can probably be ascribed to that of γ-benzyl-L-glutamate residues, which are contained in ratios of 76% within the copoly- (1 : 3).

However, the poly-L-proline I→II transition could not be observed in the NMR spectra of copoly-(1 : 3). This result suggests that copoly-(1: 3) can not assume a poly-L-proline-like helical structure. In this copolymer, there are scattered L-proline residues or short chains of L-proline which can not assume a I poly-L-proline-like structure, even the benzyl-L-glutamate assumes a right-handed helix. The shoulder which was observed around 5.2 ppm, with the main peak of the α-CH resonance at 5.4 ppm, should be ascribed to the short chain of L-proline residues.[11]

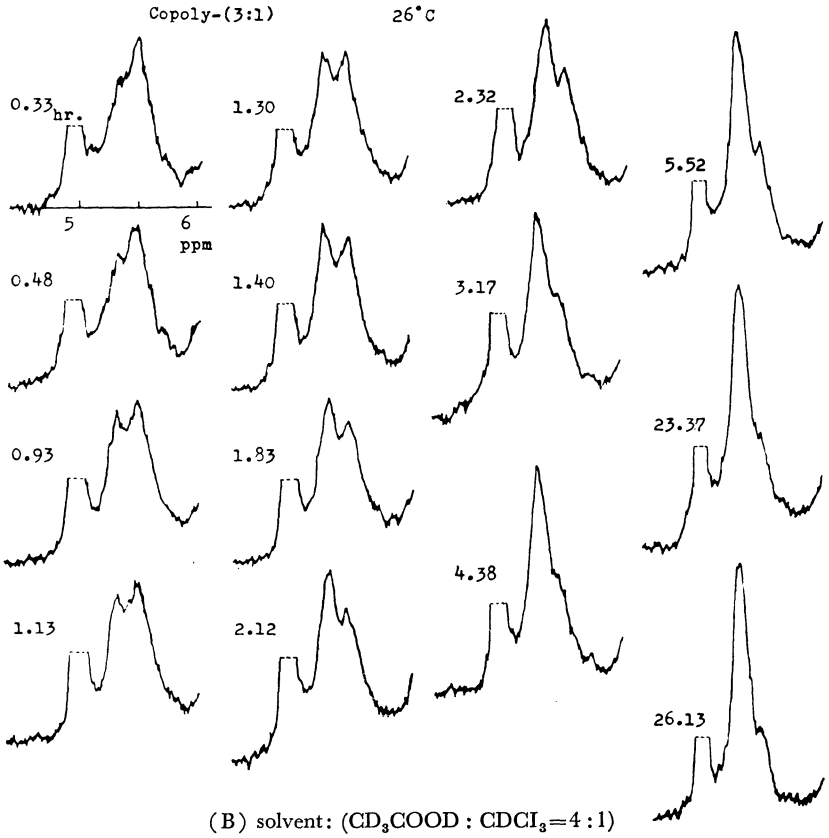
Further considerations, however, of the NMR spectra of the copoly-(3 : 1) and the copoly-(1 : 1) in tetradeuteroacetic acid are needed; they will be of great importance in ascertaining the ratios of the incorporation of γ-benzyl-L-glutamate residues into the stable II poly-L-proline-like helical structure. The α-CH resonance peak of the two copolypeptides should also be investigated in more detail.

Figure 7 also shows the time dependence of the α-CH resonance peak of the copoly-(3 : 1) and the copoly-(1 : 1) in tetradeuteroacetic acid and the mixed solvent at 26°C. The changes in intensity in the two components of the α-CH resonance peaks of the two copolypeptides seem to stop within five hours, and the resonance peak at 5.45 ppm remains unchanged. This fixing of the site of the α-CH resonance peaks can be ascribed to γ-benzyl-L-glutamate residues which were not incorporated into the poly-L-proline-like helical structure. Assuming that the conformation of the γ-benzyl-L-glutamate residues is independent of that of the L-proline residues in the copoly-(1 : 1), the two components of the α-CH resonance peak can be

11) H. Okabayashi and T. Isemura, This Bulletin, **43**, 359 (1970).



(A) solvent: CD_3COOD



(B) solvent: (CD_3COOD : $CDCl_3$=4 : 1)

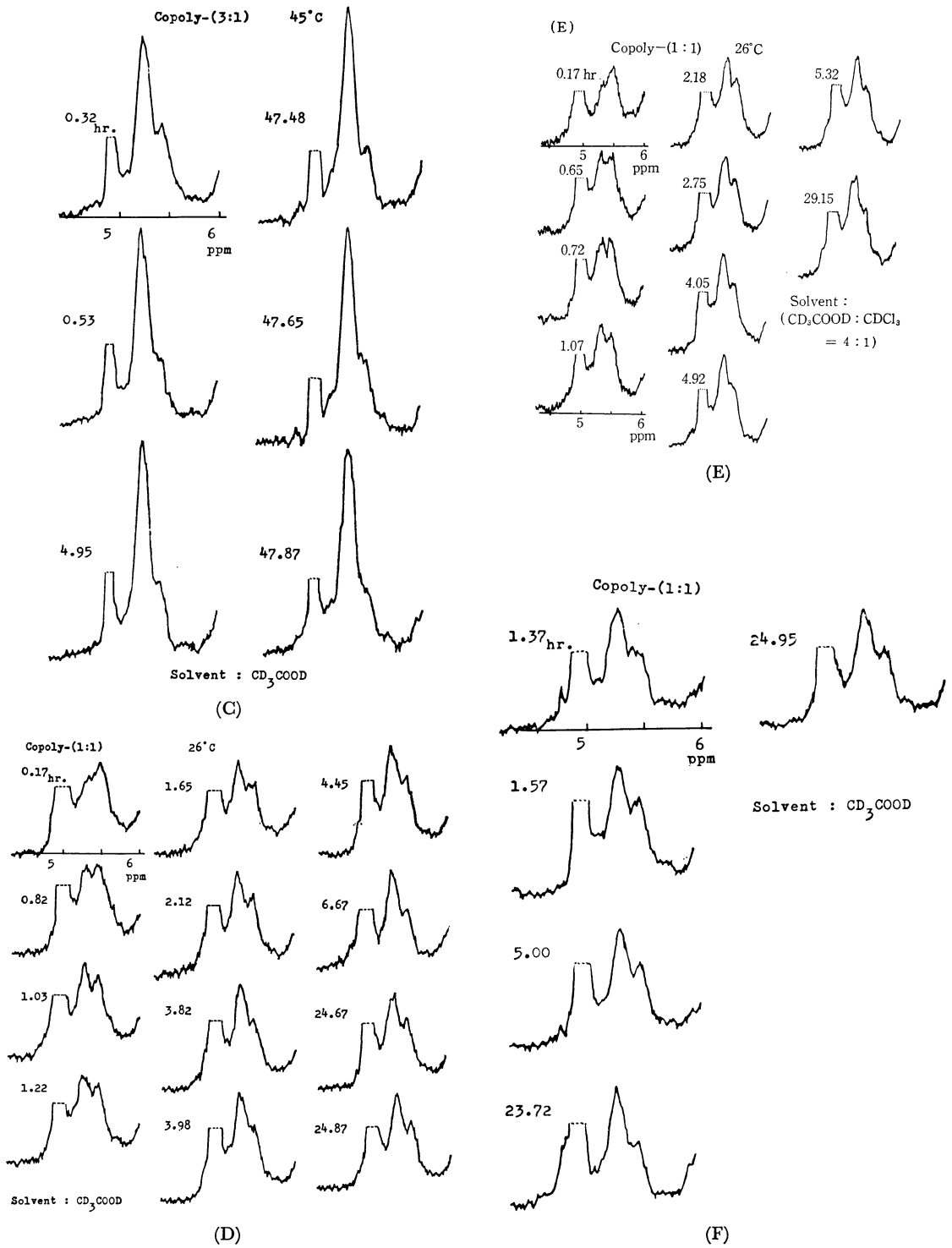
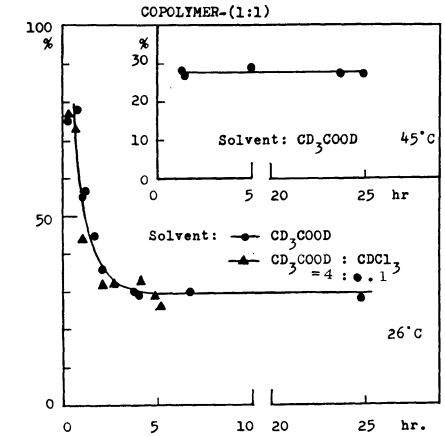
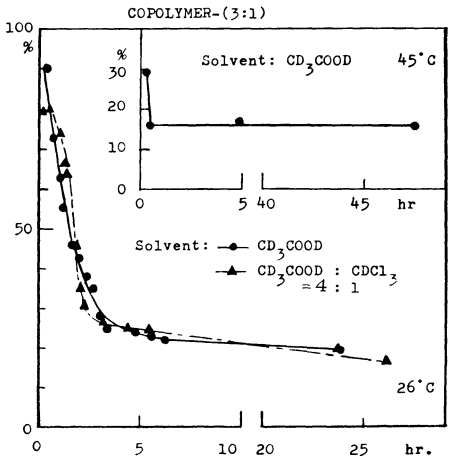


Fig. 7. The time-dependence of the α-CH resonance peak of the copoly-(3 : 1) and the copoly-(1 : 1) in tetradeuteroacetic acid and the mixed solvent (CD_3COOD : $CDCl_3$=4 : 1) at 26°C and 45°C.



(A) copoly-(1 : 1)



(B) copoly-(3 : 1)

Fig. 8. The time-dependence of the ratios of peak area of the α-CH resonance at 5.40_{ppm} to that of all the α-CH resonance peaks in the NMR spectra of copolypeptides at 26°C and 45°C.

expected to have approximately the same intensity after the transition is completed. However, contrary to our expectations, a great difference in the intensities of the two α-CH resonance peaks was observed. These experiments clearly show the incorporation of parts of the γ-benzyl-L-glutamate residues into the stable II poly-L-proline-like helical structure. The measurement of the areas of the two α-CH resonance peaks can be considered to be a method of estimating the content of the II poly-L-proline-like structure of the γ-benzyl-L-glutamate residues, since hardly no line broadening or decrease in the area of the two α-CH resonance peaks in the copoly-(3 : 1) was observed and since the decrease in the area in the copoly-(1 : 1) is invariable, without any line broadening during the transition in either tetradeuteroacetic acid or the mixed solvent.

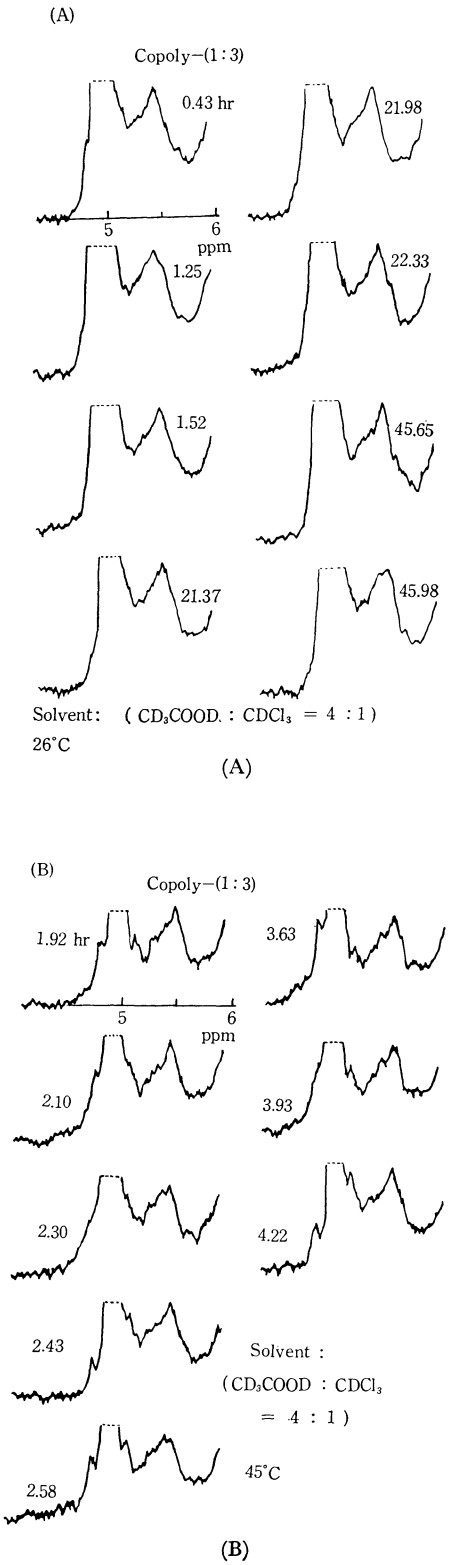


Fig. 9. The time-dependence of the α-CH resonance peak of the copoly-(1 : 3).

Figure 8 shows the time dependence of the ratios of the peak area of the α-CH resonance at 5.45 ppm to that of all the α-CH resonance peaks. These results demonstrate that, in copoly-(3 : 1), 10% of the γ-benzyl-L-glutamate residues were incorporated into the II poly-L-proline-like helical structure of the copolypeptides. Moreover, in copoly-(1 : 1) about 20% of the γ-benzyl-L-glutamate residues were incorporated into the helical structure. The α-CH resonance peak of copoly-(1 : 3) was observed in detail at 26°C and 45°C, as is shown in Fig. 9. However, the transition of copolypeptides such as poly-L-proline was not found. This observation suggests that the poly-L-proline-like structure can not be formed if a sufficient γ-benzyl-L-glutamate ratio exists in the copolypeptide.