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The Structure of Copolymers of L-Proline with L-Glutamic Acid in Aqueous Solutions

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Three copolymers of L-proline and L-glutamic acid were synthesized, and their equilibrium conformation in an aqueous solution was studied in relation to their composition. The molar ratios of the proline to the glutamic acid residues in the copolymers were about 1:3, 1:1, and 8:1. All the copolymers were soluble in water, as were poly-α,L-glutamic acid and poly-L-proline II. The conformational change was caused by the pH effect on proline-poor copolymers. Evidence was obtained for the possibility of the absence of such a helix as a right-handed poly-L-proline-I-helix in the copolymers examined. The conformation of Copoly-8:1 was not affected by the pH and was identified as the poly-L-proline-II-helix.

Both poly-L-proline (PLP)[1] and poly-α, L-glutamic acid[2] (PLGA) are water-soluble, and their conformations in water have been investigated in great detail. PLP exists in the PLP-I-helix[3] after being isolated from the polymerization medium, but if it is dissolved in water it changes into a left-handed PLP-II-helix[4] with a threefold screw-axis, after a gradual *trans*-conformation from PLP-I to PLP-II.[5] PLGA assumes an α-helix form at the pH of acid or at a low ionization of side-chain carboxyl groups, but is randomly coiled at a neutral or alkaline pH.[6]

The copolymers of L-proline and L-glutamic acid can be effected to be soluble in water, and can be expected to be suitable for the elucidation of the relative stabilities of those various conformations assumed by each residue in relation to its environment. The water-solubility of the copolymers permits the measurment of the circular dichroism (CD) and the optical rotatory dispersion (ORD) in the far-ultraviolet region; these measurements will give us important informations about the conformational stability. The composition of the copolymers will influence the environment of each residue and, consequently, change the conformation of each residue from that found when the residue is located in a long sequence of the same residue, *i. e.*, in a homo-polymer. Also, the change in pH will affect their conformation. These various parameters of the water-soluble copolymers can afford a greater variety of information than can those of such water-insoluble copolymers as copolymers with γ-benzyl-L-glutamate (γBLG).[7,8]

The copolymers of L-proline and L-glutamic acid have been prepared, and their ORD in the visible region and their viscosities have been measured as a function of the pH. These properties of the copolymers rich in glutamic-acid residues have shown the presence of a transition due to pH change similar to that observed for PLGA. The CD and ORD values in the far-ultraviolet region have also been measured at pH's on both sides of the copolymer transitions.

Experimental

Materials. The γBLG was synthesized from L-glutamic acid and benzyl alcohol in 60% H_2SO_4. The *N*-carboxyanhydrides (NCA's) of γBLG and L-proline were synthesized by the methods of Blout *et al.*[9] and Kurtz *et al.*[10] respectively. Polymerization was performed in nitrobenzene with a total NCA con-

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2) G. D. Fasman, "Poly-α-amino Acids," ed. by G. D. Fasman, Marcel Dekker, Inc., New York (1967), p. 539.

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

4) P. Cowan and S. McGain, *Nature*, **176**, 501 (1955).

5) W. F. Harrington and M. Sela, *Biochim. Biophys. Acta*, **27**, 24 (1958).

6) P. Doty, A. Wada, J. T. Yang and E. R. Blout, *J. Polymer Sci.*, **23**, 851 (1957).

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

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

9) E. R. Blout, R. H. Karlson, P. Doty and B. Hargitay, *J. Am. Chem. Soc.*, **76**, 4492 (1954).

10) J. Kurtz, A. Berger and E. Katchalski, "Recent Advances in Gelatin and Glue Research," ed. by G. Stainby, Pergamon Press, London (1958), p. 131.

TABLE 1. PROPERTIES OF POLYPEPTIDES

Polypeptide	Residue mole fraction of L-proline		Intrinsic viscosity pH *ca.* 7 in 0.1 M NaCl at 25°C	Molecular weight
	at polymerization	from amino acid analysis		
PLP	1	—	0.24 dl/g	6.6×10^{3}*
Copoly-8:1	0.75	0.89	0.12	3.3×10^{3}*
Copoly-1:1	0.50	0.55	0.11	—
Copoly-1:3	0.25	0.26	0.14	—
PLGA	0	—	0.46	2.0×10^{4}**

* Obtained from sedimentation equilibrium.

** Deduced from the relationship between molecular weight and intrinsic viscosity on PLGA reported by Wada.[12]

centration of 4%, and was initiated by sodium methoxide at molar ratio of the NCA's to the initiator of 100. The mixing ratios of NCA's are shown in Table 1. After the polymerization had been allowed to go on about a week, anhydrous hydrogen bromide was bubbled through all the solutions except for that of PLP. Each solution became turbid or a precipitate separated. After 24 hr standing, the excess hydrogen bromide was removed from the solutions by passing a stream of dry air through the solutions. The polymers were then isolated by the addition of ether to the solution, followed by centrifugation. They were exhaustively washed with anhydrous ether and acetone alternatively and dried *in vacuo*. All the isolated polymers except for the Copoly-8:1 were stored in a desiccator over silica gel. The compositions of the copolymers were determined by amino-acid analysis. PLP, PLGA, and three copolymers were prepared in this way; some of their properties are shown in Table 1. Copoly-8:1 has a very different proline content from that of the mixed ratio of NCA, although the other two copolymers have almost the same ratio. A polymer sample prepared in a proline to glutamate ratio of 3:1 became black and sticky within a few days after isolation. The black material (6 g) was dissolved in water, decolorized by Norit, and filtered, and then acetone was added to separate the purified polymer. It was washed with anhydrous acetone several times and designated as Copoly-8:1 (1.8 g). It seems likely that fractionation according to composition occurred during the above additional procedure.

The ORD of PLP and the copolymers changed with time after they have been dissolved in 0.1 M sodium chloride or sodium fluoride. All the physical measurements described below were made on solutions after the mutarotation had ceased. The mutarotation was complete within 24 hr for the copolymers, while it took 7 days for PLP.

Amino-acid Analysis. The copolymers were hydrolysed at 110°C for 20 hr with twice-distilled hydrogen chloride. Amino-acid analysis was performed on a Beckman Spinco Model 120 amino-acid analyzer.

Molecular Weight. The molecular weights of PLP and Copoly-8:1 were determined by the method of sedimentation equilibrium performed in a Spinco E Ultracentrifuge at 26°C for PLP and at 31°C for Copoly-8:1, using a temperature control unit. To correlate the concentration with the fringe scale, a filled Epon double sector synthetic boundary cell was used. The solvent used was a 0.1 M sodium chloride solution. The partial specific volume of the two polymers was assumed to be 0.758 m*l*/g.[11] Equilibrium patterns for both polymers showed that they were relatively homogeneous in molecular weight. The weight-average molecular weight of PLGA was determined from its intrinsic viscosity by using the molecular weight-viscosity relationship provided by the data of Wada.[12] These results are also included in Table 1.

Viscosity. The viscosity was measured using an Ubbelohde-type viscometer, with a flow time of 239 sec for a 0.1 M sodium chloride solution at 25±0.01°C.

The ORD in the Visible Region. The ORD in the visible region was measured on a Perkin Elmer Model 141 spectrophotoelectric polarimeter. The measurement was made on a 0.5% solution at 25°C. The data were analyzed in the way described in previous paper.[8] The results followed the Moffitt-Yang equation.[13]

The CD and ORD in the Far Ultraviolet Region. The CD and ORD curves in the far ultraviolet region were recorded on a JASCO Model ORD/UV-5 spectropolarimeter, equipped with a circular dichroism attachment, at room temperature. For both measurements, 0.1 g/d*l* polymer solutions in 0.1 M sodium fluoride were placed in cells of with light paths of 0.2, 1, or 2 mm, depending on the wavelength region scanned. The CD spectra are shown in terms of the residue ellipticity, $[\theta]$:

$$[\theta]=3300(\varepsilon_{\mathrm{L}}-\varepsilon_{\mathrm{R}}),$$

where $(\varepsilon_{\mathrm{L}}-\varepsilon_{\mathrm{R}})$ is the difference between the residue mole extinction coefficients of the left and right circularly-polarized lights. The ORD spectra are shown in terms of the residue rotation, [m]:

$$[\mathrm{m}]=\frac{M_{\mathrm{R}}}{100}[\alpha],$$

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[11] I. Z. Steinberg, W. F. Harrington, A. Berger, M. Sela and E. Katchalski, *J. Am. Chem. Soc.*, **82**, 5263 (1960).

[12] A. Wada, *Mol. Phys.*, **3**, 409 (1960).

[13] W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci.*, **42**, 596 (1956).

where M_R is the residue weight and $[\alpha]$, the specific rotation. To reduced $[\theta]$ and [m] to the residue basis for copolymers, the mean residue weight calculated from the composition of the copolymer was used.

Results

The ORD of a solution of each polymer in 0.1 M sodium chloride was observed at various pH's, and the parameters, a_0 and b_0, of Moffitt-Yang's equation were calculated for each solution. The pH dependence of these parameters for each polymer is shown in Fig. 1.

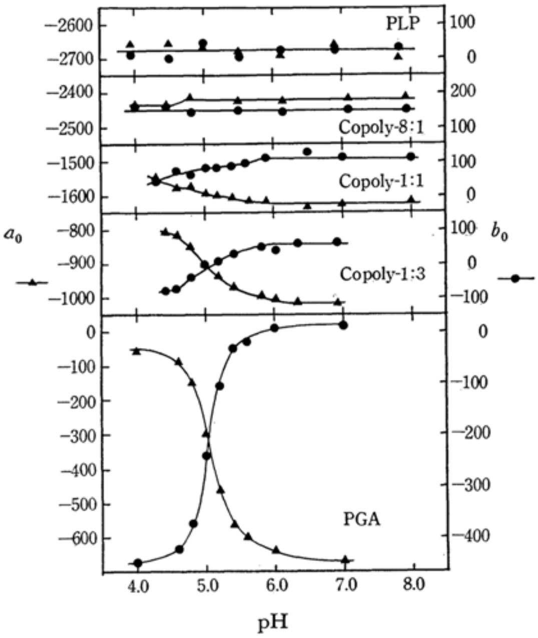

Fig. 1. The effect of pH on the a_0 and b_0 for each polymer in 0.1 M sodium chloride.

It is evident that PLP has no pH dependence of the ORD, while the largest pH dependence is observed for PLGA, which undergoes a helix-coil transition around pH 5.0, as has previously been shown by Doty *et al.*[6] Although a trivial difference in a_0 was observed for the Copoly-8 : 1, no corresponding difference in b_0 as the pH of the solution was observed. The other copolymers have a narrow pH region in which both a_0 and b_0 change with the pH. As the pH increases from 4.3 to 6, the a_0 value decreases and the b_0 value increases. As compared with that of PLGA, it is likely that the pH region observed for the copolymers represents the region where they undergo a conformational change caused by the ionization of side-chain carboxyls of the glutamic-acid residues. The differences in the two parameters, a_0 and b_0, in the two extrema are smaller as the proline content of the copolymer is higher. For example, the b_0 values of PLGA, Copoly-1:3, and Copoly-1:1 are −450°, −77°, and 44° at pH 4.3, while they are 10°, 60°, and 113° at a neutral pH. This is shown in Fig. 2, which clearly indicates that the differences in both a_0 and b_0, especially in the latter, decrease sharply with the proline content, more than expected.

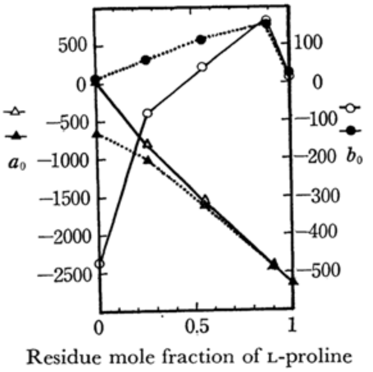

Fig. 2. The plots of a_0 and b_0 at pH 4.0—4.3 or pH 7 against residue mole fraction of L-proline.
—△— —○— pH 4.0—4.3
···▲··· ···●··· pH 7

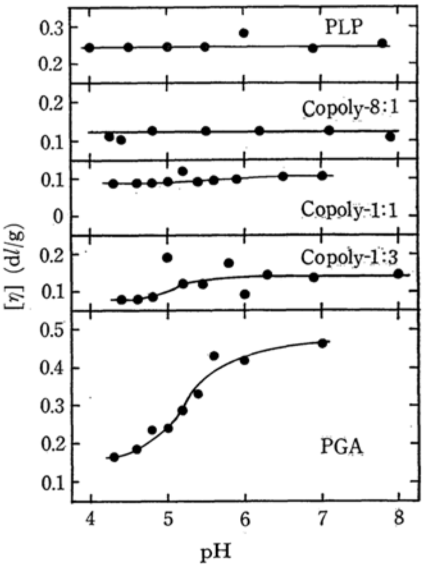

Fig. 3. The effect of pH on intrinsic viscosity.

The effect of the pH on the intrinsic viscosity of each polymer in 0.1 M sodium chloride was studied as an alternative method to check the conformational change, the results are shown in Fig. 3. The results are compatible with those to be expected from ORD. The intrinsic viscosity of PLP and Copoly-8:1 are independent of the pH, while a slight pH dependence is observed for Copoly-1:1. Although the viscosity of Copoly-1:3 around the transition pH was variable, it was constant below pH 4.6 and above pH 6.3. The intrinsic viscosity is higher at a neutral pH than at an acid pH; this finding is opposite to that reported by Doty *et al.*[6] The difference seems to come from the difference in the molecular weight or in the solvent used in the experiments. The inflection point, *i. e.*, the transition pH, on the [η]-pH curve was, at pH 5.3, slightly higher than that obtained from the ORD.

It is now generally accepted that PLP in an aqueous solution has a form-II helix after mutarotation has ceased; the present ORD and viscosity measurements of PLP at different pH values show that the same helical conformation exists in the pH region between 4 and 8. The helix-coil transition of PLGA is induced by the change in pH. PLGA is in the α-helix conformation at an acid pH, while it is randomly coiled at a neutral pH. The ORD and viscosity profiles against the pH imply that at pH 4.3 the copolymers assume definite conformations characteristic to each of them which differ from those at pH 7.

The CD and ORD, in the far ultraviolet region, of polymers at different pH's are shown in Figs. 4—7. The positions and magnitudes of the dichroic bands are given in Table 2, while the peaks and troughs of the Cotton effect are shown in Table 3.

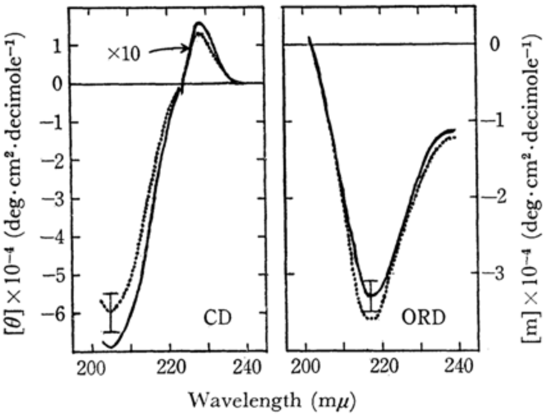

Fig. 4. The CD and ORD spectra of PLP and Copoly-8:1.
—— PLP
…… Copoly-8:1

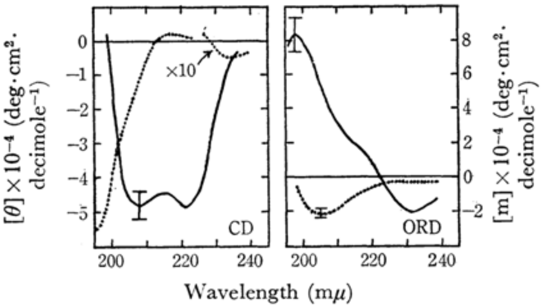

Fig. 5. The CD and ORD spectra of PLGA at acidic pH and neutral pH.
—— pH 4.5
…… pH 7

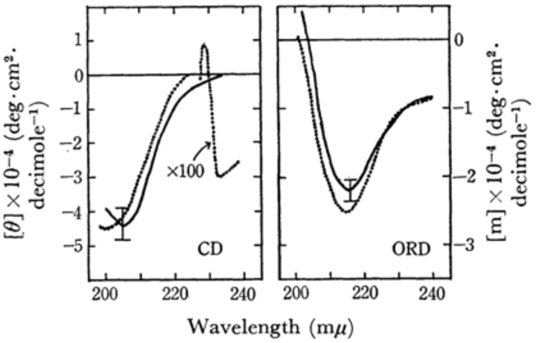

Fig. 6. The CD and ORD spectra of Copoly-1:1 at an acidic pH and a neutral pH.
—— pH 4.3
…… pH 7

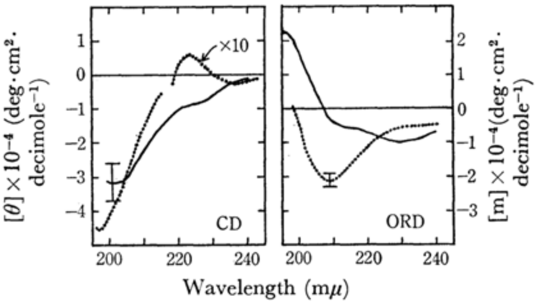

Fig. 7. The CD and ORD spectra of Copoly-1:3 at an acidic pH and a neutral pH.
—— pH 4.3
…… pH 7

Table 2. CD bands

Polypeptide	pH 4.3—4.5		pH 7	
	λ	[θ]	λ	[θ]
PLP	205	-7×10^4	205	-7×10^4
	228	1.6×10^3	228	1.6×10^3
Copoly-8:1	205	-6×10^4	205	-6×10^3
	228	1.3×10^3	228	1.3×10^3
Copoly-1:1	205	-4.4×10^4	201	-4.5×10^4
			228	90
			233	-3×10^2
Copoly-1:3	201	-3.2×10^4	~197	-4.6×10^4
			223	6×10^2
			236	-3×10^2
PLGA	208	-4.3×10^4	~196	-5.5×10^4
	221	-4.4×10^4	216	2×10^3
			234	5×10^2

λ: Wavelength (mμ)
[θ]: Residue ellipticity (deg·cm^2·decimole^{-1})

TABLE 3. THE PEAKS AND TROUGH OF COTTON EFFECTS

Polypeptide	pH 4.3—4.5		pH 7	
	λ	[m]	λ	[m]
PLP	217	-3.3×10^4	217	-3.3×10^4
Copoly-8:1	217	-3.6×10^4	217	-3.6×10^4
Copoly-1:1	216	-2.2×10^4	215	-2.5×10^4
Copoly-1:3	~196	-2.2×10^4	209	-2.1×10^4
	229	-1.0×10^4		
PLGA	198	8.4×10^4	206	-2.2×10^4
	231	-2.1×10^4		

λ: Wavelength (mμ)
[m]: Residue rotation (deg·cm^2·decimole^{-1})

The CD and ORD spectra of PLP and Copoly-8:1 in Fig. 4 are not influenced by the pH, as was to be expected from the results on ORD in the visible region and from the viscosity studies. The spectra of PLP are almost identical with those reported by Timasheff *et al.*14) and by Bovey and Hood.15) As is shown in Fig. 5, the CD of PLGA at pH 4.3 has two negative dichroic bands, while at a neutral pH it has a strong negative band, together with a weak positive and a trivial negative band. The spectra are almost identical with those previously reported.16) The Cotton effects of PLGA at two pH's (shown in Fig. 5) are also similar to those already found by other workers.17) It may be noted in Fig. 6 that Copoly-1:1 at an acid pH is associated with a single negative dichroic band similar to PLP, but it does not have any dichroic band near 228 mμ. The CD spectra of Copoly-1:1 change when the pH is raised by the shift of the negative dichroic band to a shorter wavelength and the appearance of weak dichroic bands at longer wavelengths. This change in CD is not clearly manifested in the ORD curve. The CD of Copoly-1:3 does markedly change with the pH, as is shown in Fig. 7. There are two plateau regions, around 220 and 202 mμ, at an acid pH; these regions may correspond to the negative bands of the PLGA α-helix, but the CD pattern at a neutral pH is completely different. The CD curve of Copoly-1:3 at a neutral pH is similar to that of PLGA at a neutral pH, but it is more depressed by the contribution of the proline residue. The same tendency is observed from the ORD curves in Fig. 7.

Discussion

The pH-induced conformational transitions are clearly seen in Copoly-1:3 and Copoly-1:1 as well as in PLGA in Figs. 1 and 3. It is well known that PLGA is a random coil if the side-chain carboxyl groups are highly ionized, while it assumes an α-helix if they are not. The stable form of PLP in 0.1M sodium chloride is a PLP-II-helix, a short sequence of proline residues also tends to assume the *trans*-configuration of the peptide bonds, as in the PLP-II-helix.8) The peptide bonds of the L-proline residues in the copolymers containing a small quantity of L-proline residues would, therefore, assume the *trans*-configuration in 0.1M sodium chloride. Since the PLP-II-helix has a zero b_0 value,5) a zero b_0 value can be assigned to a proline residue in the *trans*-configuration. Consequently, the b_0 value of the copolymers can be used as a measure of the conformation of the glutamic-acid residue. As is shown in Fig. 2, the observed difference in the b_0 value of each copolymer between acid and neutral pH values is smaller in magnitude than that to be expected if a linear relation between the difference and the glutamic-acid content is assumed. One of the reasons for the deviation is perhaps the effect of the chain length on the b_0 value. Proline residues cut up the segment composed of glutamic-acid residues. Goodman *et al.*18) have reported that the b_0 value of the oligo-γ-methyl-L-glutamates in helicogenic solvents abruptly decreases in absolute value with a decrease in the polymerization degree. This may be because the α-helix is unstable in the oligomers or because a considerably long series of residues is necessary to produce a large negative b_0 value for the α-helix, even though oligomers can assume a perfect α-helix. The same reasons are appropriate in explaining the b_0 value of copolymers. Another reason may be that glutamic-acid residues in the copolymers take a definite conformation, *e. g.*, a left-handed helix. Even at higher pH values the copolymers can assume the same conformation as that in an acid pH, because the electric repulsion between glutamic-acid residues is more reduced than that in PLGA.

It would be interesting to ascertain whether or not these copolymers assume a definite conformation under the conditions described above. This question can be partly answered by considering the ORD and CD results in the far ultraviolet region. The results for Copoly-8:1 are very similar to those of PLP, as is shown in Fig. 4. If Copoly-8:1 contains some parts of left-handed α-helices or random coils, the CD spectra or ORD will be different from those of PLP by the inverted CD

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14) S. N. Timasheff, H. R. Townend, L. Stevens, M. J. Gorbunoff and T. F. Kumonski, "Conformation of Biopolymers," Vol. 1, ed. by G. N. Ramachandran, Academic Press, London (1967).

15) F. A. Bovey and E. P. Hood, *J. Am. Chem. Soc.*, **88**, 2326 (1966).

16) G. Holzwarth and P. Doty, *ibid.*, **87**, 218 (1965).

17) E. R. Blout, I. Schmier, N. S. Simmons, *ibid.*, **84**, 3193 (1962)

18) M. Goodman, I. Listowski and E. E. Schmitt, *ibid.*, **84**, 1296 (1962).

or ORD of PLGA at an acid pH or the CD or ORD at a neutral pH. The CD and ORD of Copoly-8:1 show this tendency only a very little. Therefore, the conformation of the copolymers is probably the same as that of the PLP-II-helix.

This inference is also supported by the following facts. The axial ratio, as calculated from the intrinsic viscosity by Simha's equation[19] assuming a partial specific volume and no hydration, 11, is nearly equal to that calculated from the molecular weight on the assumption that the conformation of the copolymers is the same as that of the PLP-II-helix, 13. The CD spectra and ORD of Copoly-1:1 at an acid pH reflect those of PLP to some extent. For example, they have a negative dichroic band in the CD spectra at 205 mμ, the same wavelength as in PLP. The situation is similar in ORD. At a neutral pH, the parts rich in glutamic-acid residues in the copolymers will be in random coils, because the CD pattern is like that of PLGA at a neutral pH, although the CD band around 220 mμ shifts towards a longer wavelength, possibly because of the CD arising from proline residues. The CD and ORD of Copoly-1:3 at an acid or neutral pH reflect those of PLGA, as is shown in Fig. 7. Some parts in the copolymer assume the α-helix at an acid pH, but they are randomly coiled at a neutral pH.

The above discussion makes it clear that glutamic-acid residues are folded with proline residues into a PLP-II type helix when the proline residue content in the copolymers is high. The positive b_0 values of the copolymers, as is shown in Fig. 1, do not always imply the left-handed α-helix; they might come from such a helix of glutamic-acid residues as the PLP-II-helix.

The presence of no PLP-I-helix-type helix was detected in any of our experiments.

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[19] R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).