Conformational Studies on Poly-L-glutamic Acid and Copolymers of L-Glutamic Acid and L-Phenylalanine*

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ABSTRACT: Conformational studies have been performed on poly-L-glutamic acid and copolymers of L-glutamic acid and L-phenylalanine as a function of pH and temperature. The α-helical content of a random copolymer of L-Glu (76 mole %) and L-Phe (24 mole %) was compared to the homopolymer. At pH 5.2, $\mu = 0.2$, and 20° the copolymer possessed a higher α-helical content (as measured by optical rotatory dispersion). Upon heating the homopolymer to 60° the α-helical structure was nearly completely lost whereas at 80° the copolymer still maintained some α-helical structure.

Titration of the γ-carboxyl side chains gave an apparent pK_a of 4.94 for the homopolymer as compared to 5.22 for the copolymer. The larger helical content and greater stability of the copolymer is attributed to α-helix stabilization brought about by aromatic hydrophobic side-chain interactions. The optical rotatory dispersion and circular dichroism spectra of a block of α-helical poly-L-phenylalanine, solubilized between DL-Glu blocks, were measured at slightly alkaline pH. Positive [m'] values were observed over the spectral range 225–300 mμ. Four small Cotton effects were seen: a negative one in the wavelength region associated with the α-helical polypeptide backbone and three Cotton effects in the region of the benzene chromophore transitions. A negative ellipticity band was noted in a spectral region associated with the α helix, thereby showing that poly-L-Phe has the right-handed α-helical conformation.

Interactions between amino acid side chains have been shown to be of importance in stabilizing the native conformation of proteins (Kendrew, 1962; Tanford, 1962; Scheraga, 1963) and in influencing the type of ordered conformation attainable by homopoly-α-amino acids (Blout *et al.*, 1960; Bloom *et al.*, 1962; Blout, 1962). With those homopolymers which are able to form α helical structures (*e.g.*, poly-L-Glu, poly-L-Lys, poly-L-Leu, etc.) the conformational stability depends, in a large measure, on hydrophobic side-chain interactions (Fasman, 1962; Gratzer and Doty, 1963; Bixon *et al.*, 1963). Many of these studies were performed in nonaqueous media. There is, however, a paucity of such information in aqueous media.

The heat stability of α-helical structures of poly-L-glutamic acid (PGA)[1] and random copolymers of L-Glu and L-Leu in aqueous solutions has been studied by Fasman and co-workers (Fasman *et al.*, 1964b). The α-helical content of PGA in aqueous solutions (pH 4.9–5.2) showed a continuous decrease as the temperature was raised. The copolymers, under identical conditions, were more heat stable than the homopolymer, and if the L-leucine content was high enough (33 mole %) the helical content was seen to continually increase as the temperature was raised. The greater helical stability of the copolymer was attributed to leucyl–leucyl hydrophobic side-chain interactions. The inverse temperature effect was explained on the basis of a model suggested by Kauzmann (Kauzmann, 1959) and formulated theoretically by Scheraga (Scheraga, 1963). The transition from the random coil to α helix of a polypeptide is accompanied by a transfer of hydrophobic side chains from a relatively aqueous environment to a more nonpolar one (in which hydrophobic side chains are in intimate contact with one another). For aliphatic side chains such a transition would be endothermic on the basis of solubility studies on hydrocarbons in water (see Kauzmann, 1959). Hence, a rise in temperature would encourage the transfer of ali-

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[1] Abbreviations used in this work: PGA, poly-L-glutamic acid; ord, optical rotatory dispersion; copoly(L-Glu:L-Phe), random copolymer of L-Glu and L-Phe; cd, circular dichroism; BLG-NCA, γ-benzyl-L-glutamate-*N*-carboxyanhydride; L-Phe-NCA, L-phenylalanine-*N*-carboxyanhydride; block copoly(DL-Glu)-(L-Phe)(DL-Glu), a sequence of DL-Glu followed by a sequence of L-Phe, followed by another sequence of DL-Glu.

TABLE I: Polymers and Copolymers of L-Glutamic Acid and L-Phenylalanine.

Polymer	Preparation	Composition of Polymer	A/I[a]	Initiator	Composition in Mole Ratios Based on: NCA Addition	Composition in Mole Ratios Based on: Amino Acid[d] Analysis	$[\eta]^{0.2M\,NaC}_{pH\ 7.0}$
1	GF-32	Poly-L-Glu, Na salt					1.31
2	GF-11-478-18	Copoly(L-Glu:L-Phe)[b]	50	$NaOCH_3$	75:25	76:24	0.7
3	GF-11-317-20	Copoly(L-Glu:L-Phe)[b]	50	$NaOCH_3$	90:10		1.0
4	GF-11-323-22	Copoly(L-Glu:L-Phe)[b]	50	$NaOCH_3$	80:20		0.73
5	GF-14-98-19	Copoly(DL-Glu)(L-Phe)(DL-Glu)[c]	20	Hexylamine	40:20:40	41.8:16.5:41.8	0.18
6	GF-14-138-11	Copoly(DL-Glu)(L-Phe)(DL-Glu)[c]	100	Hexylamine	40:20:40	41.3:15.5:41.3	0.37
7	GF-14-166-17	Copoly(DL-Glu)(L-Phe)(DL-Glu)[c]	150	Hexylamine	40:20:40	41.8:16.5:41.8	0.46

[a] A/I, anhydride to initiator ratio. [b] Random copolymer. [c] Block copolymer. [d] Amino acid analysis performed by the method of Spackman *et al.* (1958).

phatic side chains from a polar to a nonpolar environment, explaining the temperature inversion noted by Fasman *et al.* (1964b).

In contrast to this, aromatic hydrocarbons show either a ΔH° of zero or slightly exothermic values for transfer from an aqueous to an aromatic hydrocarbon environment. On the basis of the Kauzmann model a copolymer of an aromatic amino acid and L-glutamic acid would (1) be more α helical than PGA, (2) be more temperature stable, and (3) not show the temperature inversion phenomenon.

To test these speculations the heat stability of copolymers of L-phenylalanine and L-glutamic acid have been studied and are reported herein. Presented are ord[1] studies of PGA and a random copolymer of 24 mole % L-phenylalanine and 76 mole % L-glutamic acid [copoly(L-Glu:L-Phe) (76:24)][1] at pH 5.2 and 7.5, $\mu = 0.2$. A comparison of the α-helical content of PGA and the copoly(L-Glu:L-Phe) (76:24) as calculated by $[m']_{233}$, $-b_0$, and the method of Shechter and Blout (1964) is also given.

In conjunction with these studies the ord and cd[1] spectrum of α-helical poly-L-phenylalanine was examined in aqueous solution. To accomplish this, the block copolymer sandwich technique of Gratzer and Doty (1963) was utilized.

Materials and Methods

Optical Rotatory Dispersion. Measurements of the optical rotation were made with a Bendix Ericcson Polarimatic 62 automatic recording spectropolarimeter, with a Sargent Model SR recorder. Further details of the use of this instrument are given by Fasman *et al.* (1964c). The ord data are expressed in terms of $[m']_\lambda$, the reduced mean residue rotation (Fasman, 1963), defined as $[m']_\lambda = [\alpha]_\lambda \times (mrw/100) \times (3/n^2 + 2)$, where $[\alpha]_\lambda$ is the specific rotation at wavelength λ *mrw* is the mean residue weight, and n is the refractive index of the solvent.

Absorbance Measurements. Absorbance was measured on a Cary Model 14 spectrophotometer.

pH Measurement. Measurements of pH were made with a Radiometer 25 SE pH meter, Copenhagen, Denmark, and are accurate to ± 0.01 pH unit.

γ-Benzyl-L-glutamate-N-carboxyanhydride (BLG-NCA).[1] BLG-NCA was prepared by a modification of published procedures (Blout and Karlson, 1956).

L-Phenylalanyl-N-carboxyanhydride (L-Phe-NCA).[1] L-Phenylalanine was suspended in dry dioxane (200 ml) (Fieser, 1941). Dry phosgene was passed through the mixture for 2–3 hr at 65° with rigorous exclusion of moisture. Excess phosgene was removed from the resulting clear solution by a stream of dry nitrogen (1 hr) and the solvent was evaporated off under reduced pressure at 40°. The resulting oil was dissolved in 50 ml of $CHCl_3$, and 50 ml of *n*-hexane was added to opalescence. The solution was filtered through Celite and stored at −20°. The resulting amorphous solid was dissolved in ethyl acetate (150 ml), and *n*-hexane was added in 40-ml portions up to 1000 ml. L-Phe-NCA crystallized out on standing at −20°. Further purification was obtained by recrystallization from ethyl acetate (150 ml) and *n*-hexane (900 ml added in several portions). The crystals were filtered and dried *in vacuo* for 3 hr at −20°; yield 5.5 g (47.5%), mp 84°, lit. 97° (Hanby, 1956). *Anal.* Calcd for $C_{10}H_9NO_3$: C, 62.81; H, 5.28; N, 7.33. Found: C, 62.6; H, 5.0; N, 7.4.

Copoly(L-glutamic acid:L-phenylalanine) (76:24) [Copoly(L-Glu:L-Phe)[1] *(76:24)].* L-Phe-NCA (0.1009 g, 5.28×10^{-4} mole) was dissolved in benzene (10 ml) and BLG-NCA (0.416 g, 15.83×10^{-4} mole) was dissolved in benzene (42 ml). Sodium methoxide

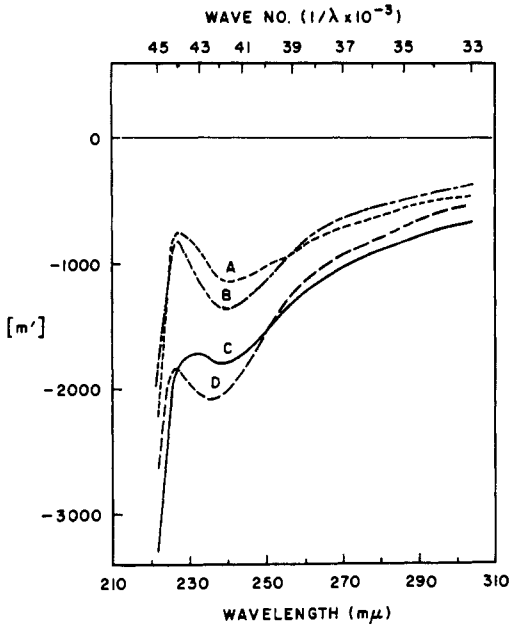

FIGURE 1: The optical rotatory dispersion of copoly(L-Glu:L-Phe) (76:24) and poly-L-glutamic acid, at pH 7.5, μ = 0.2, as a function of temperature. Copoly(L-Glu:L-Phe): curve A, - - -, 20°; curve B, — - —, 79°. Poly-L-glutamic acid: curve C, ———, 20°; curve D, – – –, 90°. Concentrations of 0.1–0.25 mg/ml were used. Path length = 1 cm.

(0.115 ml, 0.368 N $NaOCH_3$; A/I = 50) was added with stirring to the combined NCA solutions, and the reaction mixture was allowed to stand for 24 hr. Anhydrous hydrogen chloride was then bubbled through the solution until saturation (5 min) (with drying tubes on the inlet and outlet tubes of the reaction mixture). Anhydrous hydrogen bromide was then bubbled through the solution, causing immediate precipitation. This treatment was continued for 20 min and the suspension was allowed to stand overnight. Excess HBr and the solvent were removed *in vacuo* (water aspirator). The polymer was extracted with anhydrous ether for 2 hr and dried under high vacuum (1 mm) for 2 hr at 65°; yield 0.248 g (white powder), 58.5%, $[\eta]^{0.2MNaCl}_{pH\ 6.85}$ = 0.7. The specific viscosity of the blocked precursor was 0.77 (0.2% in dichloroacetic acid). The composition of the polymer was determined by amino acid analysis using the method of Spackman *et al.* (1958) and found to contain 76 mole % glutamic acid and 24 mole % phenylalanine (see Table I for experimental details). The molecular weight as determined from approach to sedimentation equilibrium (Schachman, 1959) was approximately 16,000. The copolymer was readily soluble in neutral solution but precipitated out when the pH was lowered to 4.8 or below.

The other random copolymers were prepared in a similar manner, varying the ratios of the *N*-carboxyanhydrides as indicated in Table I.

Block Copolymer of DL-*Glutamic Acid and* L-*Phenylalanine* (DL-*Glu*)(L-*Phe*)(DL-*Glu*)[1] (*40:20:40*). γ-Benzyl-DL-glutamate-NCA[2] (0.240 g, 9.13 × 10^{-4} mole) was dissolved in dry benzene (24 ml, 1% solution). Polymerization was initiated by the addition of hexylamine (0.152 ml, 0.302 N hexylamine in benzene, A/I = 20). After 24 hr L-Phe-NCA (0.087 g, 4.57 × 10^{-4} mole) dissolved in benzene (9 ml) was added with stirring to the polymerization mixture. After another 24 hr γ-benzyl-DL-glutamate-NCA (0.24 g, 9.13 × 10^{-4} mole) dissolved in dry benzene (24 ml) was added with stirring to the viscous solution, and the polymerization was allowed to proceed for 24 hr. The polymer was debenzylated and treated in the same manner as copoly(L-Glu:L-Phe) (76:24) above, giving 0.264 g (56.5% yield); $[\eta]^{0.2MNaCl}_{pH\ 6.95}$ = 0.165. The specific viscosity of the blocked polymer was 0.175 (0.2% in dichloroacetic acid). The other block copolymers were prepared in a similar manner, varying the A/I as indicated in Table I.

TABLE II: $[m']_{233}$ and $[m']_{trough}$ *vs.* Composition of L-Glutamic Acid–L-Phenylalanine Copolymers at pH 7.5, μ = 0.2, Temperature 20°.

Copolymer Composition (% Phe:% Glu)		$[m']_{233}$	$[m']_{trough}$
0	100	−1725°	−1800°[a]
10	90	−1500°	−1740°
20	80	−1200°	−1350°
24	76	−910°	−1150°[b]

[a] 238 mμ. [b] 239 mμ.

Poly-L-glutamic Acid (PGA).[1] This polymer was prepared according to the method of Idelson and Blout (1958). The viscosity of the polymer was $[\eta]^{0.2MNaCl}_{pH\ 7.0}$ = 1.31. The molecular weight was estimated to be 58,000 using the above viscosity and the calibration curve of Idelson and Blout (1958).

Buffers. Two buffers were used in this study. The pH 5.20 buffer was cacodylate buffer prepared by adjusting the pH of 10 ml of 1 M cacodylic acid to 5.2 with 1 M NaOH and adjusting the volume to 50 ml with 0.2 M NaCl. The ionic strength of this buffer was 0.19–0.20. It was chosen because it did not absorb appreciably in the ultraviolet range. Phosphate buffer pH 7.5 was prepared by mixing μ = 0.2 solutions of NaH_2PO_4 and Na_2HPO_4 in the correct proportions.

Preparation of PGA and Copoly(L-*Glu*:L-*Phe*) (*76:24*) *Samples for Ord Studies.* Stock solutions of PGA and the copolymer were prepared by dissolving approximately 1 mg of polymer/ml of 0.2 M NaOH. Both polymers were instantly soluble. The alkaline solutions were neutralized to pH 7.5 with 0.2 M HCl, added slowly with mixing. These stock solutions were analyzed

[2] Prepared as γ-benzyl-L-glutamate-*N*-carboxyanhydride (Blout and Karlson, 1956).

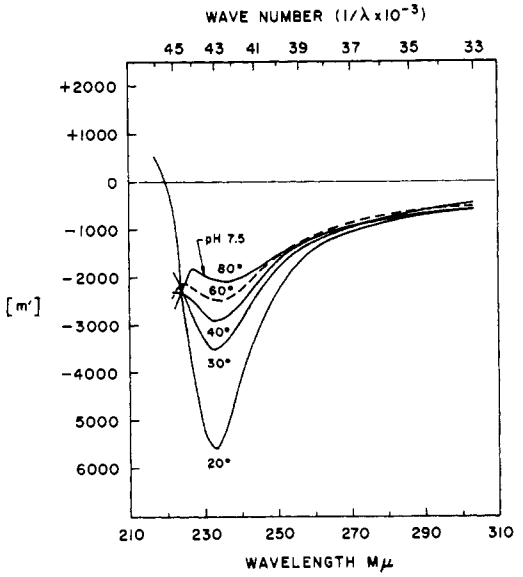

FIGURE 2: The optical rotatory dispersion of poly-L-glutamic acid at pH 5.20, μ = 0.2, as a function of temperature. Temperature as indicated. Concentration of polymer is 0.08–0.1 mg/ml. Path length of cell is 1 cm.

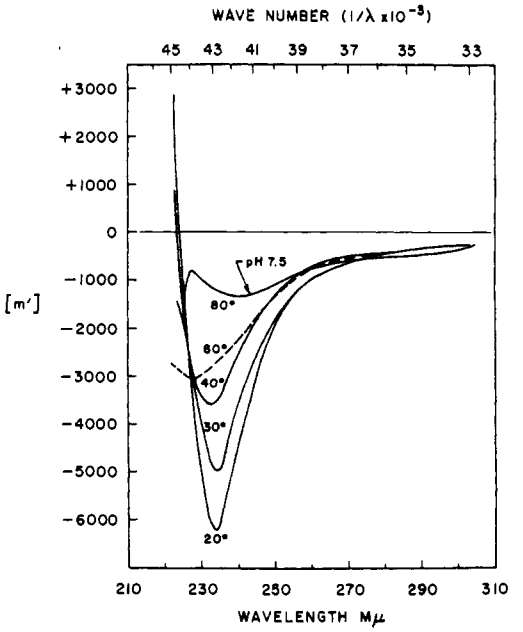

FIGURE 3: The optical rotatory dispersion of copoly(L-Glu:L-Phe) (76:24) at pH 5.2, μ = 0.2, as a function of temperature. Concentration of polymer is 0.08–0.1 mg/ml. Path length of cell is 1 cm.

for nitrogen by a modified Nessler analysis (Lang, 1958) and usually contained approximately 50 μg of nitrogen/ml. This corresponds to approximately 0.5 mg of polymer/ml. The solutions were kept refrigerated and never used more than 2–3 weeks after preparation. For ord studies a 0.4–1-ml sample of stock solution was measured out. To this was added 0.2 ml of the appropriate buffer (pH 5.20 cacodylate or pH 7.50 phosphate) and enough 0.2 M NaCl to make the total volume 2 ml. The concentrations were 0.08–0.25 mg of polymer/ml. After stirring at room temperature for 1 hr the sample was transferred to a jacketed 1-cm quartz polarimeter cell. The sample was allowed to equilibrate at the desired temperature for 0.5 hr before measurements were taken. The pH of the solution was checked before and after the ord measurements. After the measurements were made samples were analyzed for nitrogen by Nessler analysis to make sure no precipitation had occurred and that the concentration was the same as calculated from the stock solution. With copolymer samples prepared at neutral pH a further check on the concentration was made by measuring the OD at 258. The $E^{1\%}_{258}$ 4.71 for the copoly(L-Glu:L-Phe) (76:24) was determined.

Preparation of Block Copolymers for Ord Studies. Samples of approximately 10 mg of the block copolymers, (DL-Glu)(L-Phe)(DL-Glu), were weighed out and dissolved in ≃2 ml of 0.2 M NaOH. After stirring at room temperature for 30 min the pH was brought to 8.5–9.0 with approximately 2 ml of 0.2 M HCl. With block copolymers 6 and 7 the solutions were centrifuged at 34,000 *g* for 20 min and the small precipitate discarded. With the block copolymer 5 this step was unnecessary because all of the sample was soluble. The above procedure was used because direct solution of the polymers at pH 8.5–9.0 was very slow. The concentration of each sample was determined by Nessler nitrogen analysis. The ord curves of the samples were run at room temperature (23–24°). To obtain a more extended ord spectrum the path length of the cell was varied (1.0-, 0.5-, and 0.1-cm cells were used) rather than dilution of the sample. In overlapping regions the [α] values were independent of the optical path length.

Titration of PGA and Copoly(L-Glu:L-Phe) (76:24). Stock solutions containing 0.5 mg/ml of PGA and copoly(L-Glu:L-Phe) (76:24) were prepared in CO_2-free 0.2 M NaCl adjusted to pH 9.0 with 0.2 M NaOH. Samples were measured out (3 ml), the pH was adjusted to 9.5, and titration was done manually. A microburet (Manostat Corp.) was used to add the titrant (0.2 M HCl). PGA was soluble down to pH 3.5. The copolymer became opalescent at pH 4.9 and was extensively precipitated by pH 3.5. Re-solution of the precipitated copolymer by addition of 0.2 M NaOH was not instantaneous. Because precipitation occurred and was not instantaneously reversible titration curves starting from the acid region were not attempted. Samples of PGA and copolymer were done in triplicate and triplicate blanks were done with each titration.

Results

Ord Studies on PGA and Copoly(L-Glu:L-Phe) (76:24) at pH 7.5. The ord spectra of PGA and copoly(L-

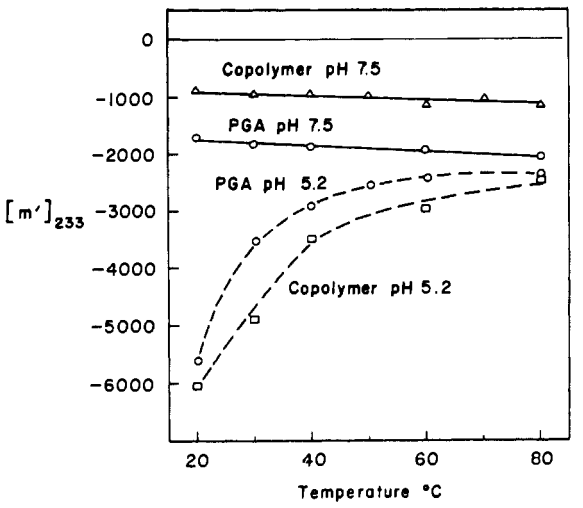

FIGURE 4: The temperature stability of PGA and copoly(L-Glu:L-Phe) (76:24). $[m']_{233}$ *vs.* temperature. Copolymer, –△–△–, pH 7.5; ⊡ – – ⊡, pH 5.2; PGA, ⊙–⊙–⊙, pH 7.5; O – – O, pH 5.2.

Glu:L-Phe) (76:24) at pH 7.5, μ = 0.2, at 20 and 80° are shown in Figure 1. At neutral pH PGA has been shown to be devoid of α-helical content (Doty *et al.*, 1957). It can be seen (Figure 1) that there is a slight but definite trough in the ord of PGA with a minimum at 238 mμ (curve C). As the temperature was raised the trough deepened with the $[m']_{238}$ values becoming progressively more negative (curve D) (−1800° at 20° *vs.* −2040° at 80°). The position of this small trough is neither at the wavelength associated with helical polypeptides (233 mμ) nor at the trough associated with random-coiled polypeptides (204 mμ) (Simmons *et al.*, 1961; Blout *et al.*, 1962). This Cotton effect may be a reflection of a structure which is neither a random coil nor an α helix but perhaps the partially oriented extended form. PGA, at pH 7.5, probably exists extensively in an extended form due to electrostatic repulsion of the ionized carboxyl side chains. The spectra of the copolymer at pH 7.5 were similar to the homopolymer. However, the presence of L-Phe in the copolymer tended to decrease the magnitude of $[m']$ over the entire spectrum. In order to examine the effect of L-Phe incorporation on the ord of nonhelical PGA, random copolymers were prepared in which the ratio of L-Phe to L-Glu was varied (see Table I). The ord spectra of these copolymers were measured. Table II shows the relationship between $[m']_{233}$, $[m']_{\text{trough}}$ and composition of a number of different copolymers of L-Phe and L-Glu. The value of $[m']_{233}$ was progressively smaller in magnitude as the mole per cent of L-Phe was increased. The temperature stability of copoly(L-Glu:L-Phe) (76:24) was essentially the same as PGA as judged by the change in $[m']_{233}$ (Figure 1, curves A and B).

Ord Studies of PGA and Copolymer at pH 5.2. Studies performed with PGA and copoly(L-Glu:L-Phe) (76:24) at pH 5.2, μ = 0.2, as a function of temperature are shown in Figures 2 and 3, respectively. The minimum of the trough for PGA at 20° was at 233 mμ ($[m']_{233}$ = −5600°), while that for the copolymer at 20° was at 235 mμ ($[m']_{235}$ = −6250°). The magnitude of the trough of the Cotton effect of both the homopolymer and copolymer is seen to diminish as the temperature was raised. This is interpreted as a temperature melt out of the α-helical regions in the two polymers. If one assumes that the value of $[m']_{233}$ = −1800° for PGA (Simmons *et al.*, 1961; Blout *et al.*, 1962), and $[m']_{235}$ = −910° for the copolymer at pH 7.5 (Table II) represents the nonhelical forms, then it is seen that the copolymer possessed a higher helical content at 80° than PGA at 60°. This is shown more emphatically in Figure 4 where the $[m']_{233}$ is plotted against temperature for both polymers at pH 7.5 and pH 5.2. The value of $[m']_{233}^{60°}$ of PGA was only 500° more negative at pH 5.2 than at pH 7.5, while the value of $[m']_{235}^{60°}$ for the copolymer was 1900° more negative at pH 5.2 than at pH 7.5. Another major difference in the behavior of PGA and the copolymer was found in the reversibility of the temperature effects. The changes in ord of PGA as a function of temperature were entirely reversible and essentially instantaneous. Although the changes in ORD of the copolymer on raising the temperature were instantaneous, they were not reversible on bringing the temperature back to 20°. On increasing the temperature from 22.5 to 60° a decrease in $[m']_{235}$ of 630° was noted for the copolymer. On lowering the temperature back to 22.5° the copolymer regained only 20% of this change in $[m']_{235}$, as shown in Table III. The heat-treated copolymer could be brought back to its original state by first raising the pH of the solution to 7.5 at room temperature, followed by lowering the pH back to 5.2 (Table III). The irreversibility did not appear to be due to a small amount of precipitation of the copolymer on heating. A heated sample centri-

TABLE III: Temperature Reversibility Studies of Copoly(L-Glu:L-Phe) (76:24) at pH 5.22, μ = 0.20.

Conditions	Temp of Ord Measurement (°C)	$[m']_{235}$
No pretreatment	22.5	−4725°
	60	−3095°
Heated to 60° 1 hr and left overnight at room temperature	22.5	−3430°
Heated to 60° 1 hr, pH raised to 7.5, pH lowered to 5.22, and left overnight at room temperature	22.5	−4640°

TABLE IV: Optical Rotatory Dispersion Parameters of PGA and Copoly(L-Glu:L-Phe) (76:24).

Polymer	pH	μ	Temp (°C)	b_0[a] Value	b_0[a] % Helix	$[m']_{233}$[b] Value	$[m']_{233}$[b] % Helix	$A(\alpha,\rho)_{225}$[c] Value	$A(\alpha,\rho)_{225}$[c] % Helix	$A(\alpha,\rho)_{198}$[c] Value	$A(\alpha,\rho)_{198}$[c] % Helix
PGA	5.2	0.2	20	−130	30	−5610	27	−557	25	+137	24
PGA	5.2	0.2	40	−37	16	−2902	8	−326	13	−212	15
PGA	5.2	0.2	60	−19	13	−2444	5	−278	11	−262	13
PGA	5.2	0.2	80	−16	13	−2349	4	−292	12	−178	16
PGA	4.9	≃0	24	−540	92	−13,500	82	−1532	74	+2032	76
PGA	4.37	≃0	20	−590	100	−16,000	100	−2153	105	+2825	98
PGA	7.5	0.2	20	+70	0	−1750	0	−56	0	−870	−3
PGA	7.5	0.2	80	0	10	−2065	2	−194	6	−446	8
Copolymer	5.2	0.2	20	−210	48	−6180	50	−696	32	+866	44
Copolymer	5.2	0.2	30	−198	46	−4883	38	−643	29	+773	42
Copolymer	5.2	0.2	40	−129	33	−3471	30	−357	15	+225	27
Copolymer	5.2	0.2	60	−70	22	−2978	20	−348	14	+193	26
Copolymer	5.2	0.2	80	*d*	*d*	−2349	14	*d*	*d*	*d*	*d*
Copolymer	7.5	0.2	20	+50	0	−910	0	0	0	−680	2
Copolymer	7.5	0.2	80	−6	10	−1159	2	−214	8	−169	15

[a] Calculated from data in the wavelength region 333–263 mμ, using $\lambda_0 = 216$. b_0 for helix = +70, b_0 for 100% helix = −590 for PGA. For the copolymer, 0% helix b_0 = +50, and 100% helix b_0 = −490°. [b] For PGA $[m']_{233}$ = −1800 for 0% helix; $[m']_{233}$ = −16,000° for 100% helix. For copolymer $[m']_{235}$ = −910 for 0% helix; $[m']_{235}$ = −11,300 for 100% helix. [c] Shechter and Blout (1964). [d] The data did not give a straight line plot.

fuged and treated as above gave the same value of $[m']_{235}$ as the unheated sample (Table III). The possible significance of the irreversible temperature change will be discussed subsequently.

In Table IV are shown the values of $[m']_{233}$ and b_0 values obtained from $[m']$ values in the range 303–263 mμ for PGA and the copolymer. A value of $\lambda_0 = 216$ mμ was used to calculate b_0. This was used because this λ_0 value gave the best straight line Moffitt–Yang plots (Moffitt and Yang, 1956) in the 303–263 mμ range with PGA. Also included in Table IV are the above data treated according to the method of Shechter and Blout (1964; Shechter *et al.*, 1964). To estimate the percent α helix of PGA from $[m']_{233}$ a value of $[m']_{233} = -1800°$ for 0% helix and $[m']_{233} = -16{,}000°$ for 100% helix was used. The latter value was obtained from ord studies of PGA at pH 4.37 and low ionic strength (samples dissolved in distilled water and pH adjusted to the desired value). This value agrees very well with those obtained by Yang and McCabe (1965). For the copolymer the value of $[m']_{235} = -910°$ was chosen to represent 0% α helix. This was the measured value at pH 7.5, 20° (Table IV, Figure 1). The value of $[m']_{235} = -11{,}300°$ was chosen to represent a 100% helix. This is a calculated value obtained from ord spectra of 100% α-helical PGA and 100% helical poly-L-Phe (see Discussion). With PGA, values of $b_0 = +70°$ and $b_0 = -590°$ were chosen to represent 0 and 100% α helix, respectively (assuming $\lambda_0 = 216$ mμ). For the copolymer values of $b_0 = +50°$ and $b_0 = -490°$ were chosen to be 0 and 100% α helix, respectively (assuming $\lambda_0 = 216$ mμ). Also included in Table IV are the data treated according to the method of Shechter and Blout (1964).

Titration of Copoly(L-Glu:L-Phe) (76:24) and PGA. Electrostatic charge repulsion is responsible for the α helix → random coil transition of PGA as the pH is raised from acidic to neutral values (Doty *et al.*, 1957; Fasman *et al.*, 1964b). At the same pH (*e.g.*, pH 5.2) one would expect that PGA would have a higher charge density than the copolymer (assuming that the pK_a of the glutamyl residues is the same in both polymers). A plausible explanation for the greater helical stability of the copolymer at pH 5.2 is that it might thus merely be due to a dilution of charge effect rather than a hydrophobic side-chain interaction. To test this one must first determine the effect of phenylalanine incorporation on the pK_a of the glutamyl residues. pH titrations were performed on both PGA and the copolymer. The titration curves obtained, plotted as H^+ bound per glutamyl residue, are shown in Figure 5. The pK_a found for PGA was 4.94, while that for the copolymer was 5.22. To obtain the intrinsic pK of the carboxyl groups a plot of α, the degree of dissociation, *vs.* pH + log $[(1 - \alpha)/\alpha]$ was made for the polymers (Figure 6). At low values of α a straight line should be observed which extrapolates to the value of pK_{int} at $\alpha = 0$. This was obtained for PGA, and the pK_{int} calculated was 4.2. The copolymer data did not fit on a straight line and an extrapolation of the data to $\alpha = 0$ was impossible. Nagasawa and Holtzer (1964) noted a similar behavior for PGA at very low values of α. This be-

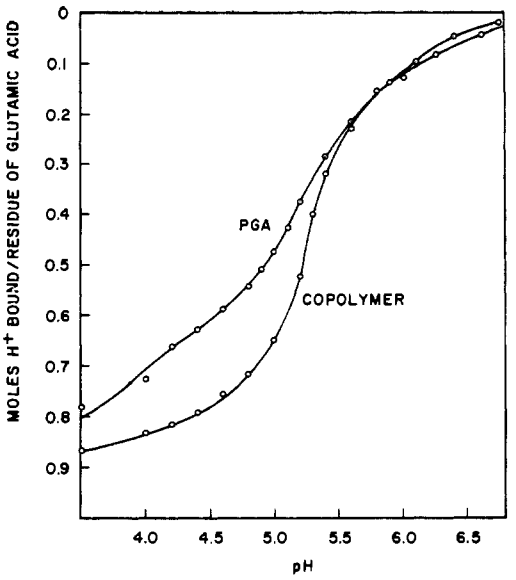

FIGURE 5: Titration curves of PGA and copoly(L-Glu:L-Phe) (76:24) at μ = 0.2. pH *vs.* H^+ bound/residue of glutamic acid.

havior was attributed to aggregation of PGA at these α values. A similar explanation could be applicable to the titration of the copolymer since precipitation occurred below pH 4.8. Because of this result the question of whether the greater helical stability of the copolymer was due to dilution of charge effects or hydrophobic interactions was not answered unequivocally by this experiment (however, see Discussion).

Ord Studies on Block Copoly(DL-*Glu*)(L-*Phe*)(DL-*Glu*). In conjunction with the studies on the randomly copolymerized polymer [copoly(L-Glu:L-Phe) (76:24)] it was of interest to measure the ord of poly-L-phenylalanine under conditions where poly-L-phenylalanine was essentially α helical, thus allowing for aromatic–aromatic side-chain interactions. Because poly-L-phenylalanine was not water soluble, solubilizing blocks of poly-DL-glutamic acid were sandwiched about a central block of poly-L-phenylalanine. Ord studies were carried out on the block copolymer with the assumption that any rotatory properties of the block copolymer were attributable solely to the central poly-L-Phe block. Three such block copolymers were studied. These copolymers were examined in order to evaluate the role of the size of the central poly-L-Phe block on the conformation. The block copolymer (5) had an estimated length of ten residues while the copolymer (7) had an average chain length of 75 residues. These are listed in Table I with some of their properties. When examined in the ultracentrifuge at pH 8.5 and μ = 0.05 both block copolymers 5 and 7 appeared to be highly aggregated and polydisperse (polymer 6 was not studied). In addition to more heavily sedimenting material a component with a sedimentation constant of $s_{20,w}$ 5.18 was noted with block copolymer 5 (this run was done with a polymer concentration of $\simeq$8 mg/ml). A synthetic boundary run performed on the same material showed a peak with a sedimentation constant of about $s_{20,w}$ 5.2 and one of about equal size which did not appreciably move away from the position of the solvent–solution boundary (33,450 rpm for 3 hr). A peak with a sedimentation constant of $s_{20,w}$ 3.2 (8 mg of polymer/ml) was noted with the larger block copolymer 7 together with a large amount of faster sedimenting material run under the same conditions as above. At the ionic strength and pH used in both the ultracentrifugal studies and the ord studies the block copolymers were highly aggregated, probably due to a salting out effect. The presence of salt lessens the intermolecular electrostatic repulsion of the DL-glutamate side chains. As a result hydrophobic interactions between the central poly-L-Phe blocks cause molecular aggregation. The ord curve of a block of poly-L-phenylalanine is shown in Figure 7 together with the ord curve of L-phenylalanine. The ord curves of the block copolymers 5, 6, and 7 were indistinguishable within the experimental error ($\pm$5–10% of [m'] values in the range 400–225 mμ). Whereas the ord curves of the copolymers were practically identical, the difference between these curves and that found for the L-phenylalanine monomer is striking. The monomer had small negative [m'] values over the wavelength range 400–260 mμ and gave a simple dispersion curve. (Since the completion of this work Moscowitz *et al.* (1965) have shown that extremely small Cotton effects can be observed for L-Phe if optimum conditions are used.) Block poly-L-phenylalanine, on the other hand, gave positive [m'] values of larger magnitude over the entire 400–225 mμ range studied and has a complex dispersion curve. A trough was noted at 240 mμ with a value of [m'] of 450 to 650°. There were also three small additional peaks at 266.5, 259, and 254.5 mμ. These peaks occurred in the region of the four maxima in the ultraviolet absorption spectrum of L-phenylalanine, namely 268, 263, 258, and 259 mμ. It is therefore possible that in the ord curves these are peaks of Cotton effects due to phenyl aromatic side chains being oriented by the α-helical polypeptide conformation. The concentrations of copolymer used in this study were 1.5–2.5 mg/ml, corresponding to 0.3–0.5 mg of poly-L-Phe/ml. In the spectral region 255–225 mμ, the [m'] values were independent of path length (1 or 0.1 cm). The instrument was not sensitive enough to measure the three small maxima when a 0.1-cm path length cell was used. The trough at 240 mμ had a positive sign and was located in the region usually associated with an α-helical polypeptide Cotton effect. However, with nonaromatic right-handed helical polypeptides negative [m'] values are usually found (Simmons *et al.*, 1961). Therefore, because of the difference in the sign of this trough one could not unequivocally state that the block poly-L-Phe helix was right handed. In order to resolve this difficulty circular dichroism, studies were performed on one of the block copolymers, 5. We are indebted to Dr. Sherman Beychok for performing these studies. The cd results are shown in Figure 8. A negative ellipticity band centered

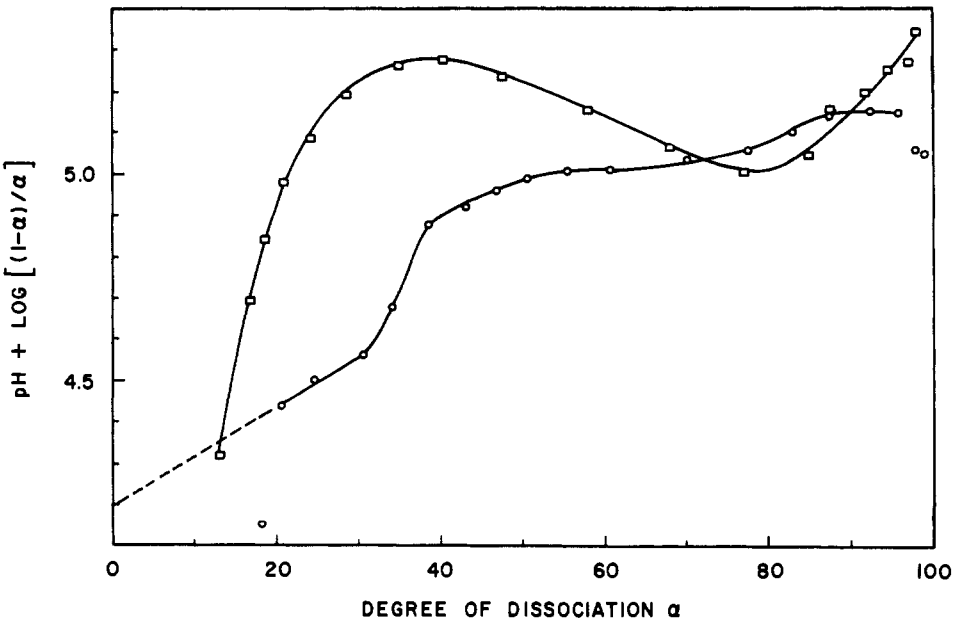

FIGURE 6: Data of Figure 5 plotted as pH + log $[(1 - \alpha)/\alpha]$ *vs.* α for PGA, O–O–O, copoly(L-Glu:L-Phe) (76:24), □–□–□.

at 228 mμ was found, which indicated that block poly-L-phenylalanine had a right-handed α-helical conformation.

In Table V are shown the b_0 values calculated from Figure 7 together with the same data treated by the Shechter–Blout method. The value of $b_0 = -130$ to $-160°$ for the helical block poly-L-Phe agrees very well with a value of $b_0 = -120°$ obtained by H. Auer and P. Doty (private communication). It is obvious that the calculated values of the per cent α helix obtained by b_0 and the Shechter–Blout treatment do not agree, nor are the values of H_{225} and H_{193} of the latter treatment self-consistent.

Discussion

In the introduction a model was presented, based on the work of Kauzmann and Scheraga which assigned an appreciable role to hydrophobic side-chain interactions in the stabilization of α-helical structures. Several speculations about the effect of aromatic side-chain interactions on α-helical stability were made which will now be evaluated.

The random copolymer of L-Glu and L-Phe [copoly-(L-Glu:L-Phe) (76:24)] had a higher helical content at 20° than did PGA as measured by $[m']_{233}$ and b_0. Even more striking was the greater heat stability found for the copolymer. Auer (1964) has also studied block copolymers of (DL-Glu)(L-Phe)(DL-Glu) in aqueous solution, and his work agrees with the work presented here. He has shown that the α-helical poly-L-Phe block was more stable than PGA by the much slower deuterium exchange of the backbone N–H in the poly-L-Phe and by the greater heat stability of block poly-L-Phe (as measured by the effect of temperature on $[\alpha]$). From the data of Table III, values of $\Delta H°$ (coil → helix) could be calculated for PGA and the copolymer. It was found that $\Delta H°$ over the temperature range 20–40° for PGA at pH 5.2, $\mu = 0.2$, was −14.4 kcal/mole while that for the copolymer was −7.8 kcal/mole. Némethy and Scheraga (1962) have calculated that $\Delta H°$ for the formation of a phenylalanine–phenylalanine hydrophobic bond is about +0.8 kcal/mole. Kresheck and Benjamin (1964) conclude, on the basis of heats of solution of phenylalanine, that hydrophobic interactions of the side chains is very likely. The more positive $\Delta H°$ of the copolymer could be explained on the basis on such interactions.

The higher helical content and heat stability of the copoly(L-Glu:L-Phe) (76:24) could have been due to a smaller charge density in the copolymer as compared to PGA. Attempts were made to examine this possibility by titration studies. From the titration curve of PGA ($\mu = 0.2$) a pK_a of 4.94 and a pK_{int} of 4.2 were obtained for the carboxyl side chains, in agreement with published values (Wada, 1960; Applequist and Breslow, 1963; Nagasawa and Holtzer, 1964).

The pK_a for the carboxyl groups of the copolymer was 5.22, almost 0.3 pH unit higher than that for PGA. If dilution of charge were the only factor of importance one would have expected a lower pK_a for the copolymer than for PGA. However, the aggregation of the copolymer does not allow one to use this information as unequivocal evidence against the dilution of charge effect hypothesis. The studies of Auer (1964) have indicated that block poly-L-Phe has a greater intrinsic α-helix stability than does PGA. It is very probable that a large part of the greater heat stability of copoly-

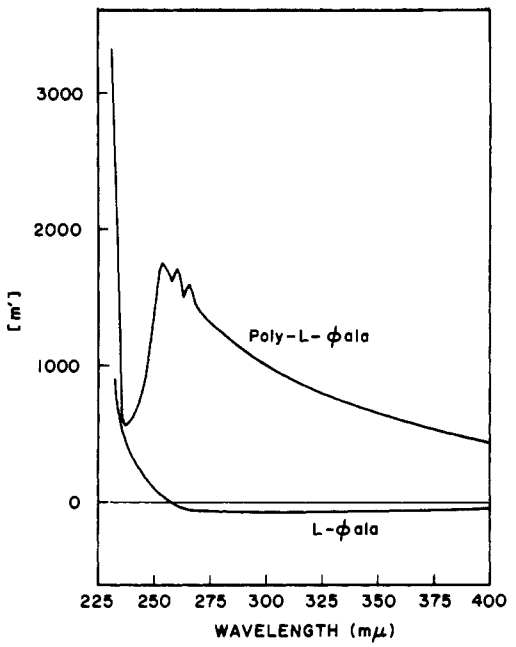

FIGURE 7: The optical rotatory dispersion curves of block copoly(DL-Glu)(L-Phe)(DL-Glu) (40:20:40) and L-phenylalanine at pH 8.5, $\mu = 0.2$. Concentration, 2.8 mg/ml of copolymer which equals 0.5 mg/ml of L-Phe. L-Phe concentration is 4.2×10^{-2} M. Path length, 1- and 0.1-cm cells used.

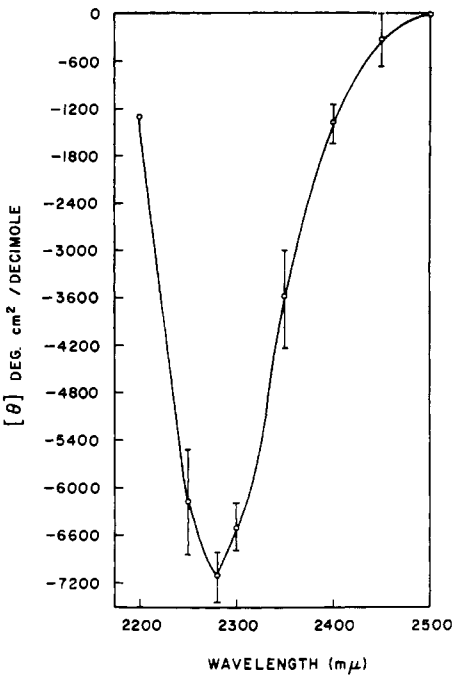

FIGURE 8: Circular dichroism spectra of copoly(DL-Glu)(L-Phe)(DL-Glu) (40:20:40) at pH 9.0 in 0.2 M NaCl. Concentration, 1 mg/ml. Path length, 1 mm.

(L-Glu:L-Phe) (76:24) as compared with PGA is not due to a dilution of charge but rather to the nature of the side chains. The greater stability of the copolymer may be attributed in a large part to side-chain interactions of the benzyl side chains and to possible interactions of the aromatic side chains with the backbone and hydrophobic regions of the glutamyl side chains.

Upon heating the copolymer at pH 5.2, a loss in α-helical content was indicated by a decrease in the magnitude of $[m']_{235}$. This loss in conformation was not completely reversible simply by lowering the temperature. It could be reversed by first raising the pH to 7.5 and then lowering the pH back to pH 5.2. This irreversibility is similar to that noted by Fasman *et al.* (1964a) for poly-L-tyrosine upon ionization. It was shown by these authors that, to obtain the original structure, a special procedure had to be followed, which permitted the aromatic rings the opportunity to realign in a manner which allowed the backbone polypeptide to assume a helical conformation. Both of these phenomena (the heat irreversibility of the phenylalanine copolymer and the pH irreversibility of poly-L-tyrosine) probably reflect steric hindrance of the bulky aromatic residues during the process of helical formation. Once the copolymer has become random this allows for aromatic side-chain interactions which are not necessarily broken upon cooling. This results in the prevention of the reformation of the helical structure.

The temperature inversion was not seen with the copolymer of L-Glu and L-Phe as was noted for copolymers of L-Glu and L-Leu (Fasman *et al.*, 1964b). This fits well into the hypothesis of Kauzmann (1959) and Scheraga (1963). However, it was impossible to examine copolymers containing more than 24 mole % of L-Phe because of solubility problems. It is possible that with higher L-Phe contents temperature inversion might be seen.

The choice of a method for estimating per cent α helix, for copolymers containing aromatic residues, by ord is a difficult one. It is clear that, by using either the Moffitt–Yang or the Shechter–Blout treatment, results are obtained which are neither consistent with themselves (Shechter–Blout) nor with each other. It is the

TABLE V: Optical Rotatory Parameters of Block Copolymers of (DL-Glu)(L-Phe)(DL-Glu).

		Shechter–Blout			
			% Helix		% Helix
Polymer	b_0[a]	$A(\alpha,\rho)_{225}$	H_{225}	$A(\alpha,\rho)_{193}$	H_{193}
5	−160°	0	−3	1443	60
6	−185°	0	−3	1462	61
7	−130°	0	−3	1340	57

[a] Calculated using $\lambda_0 = 216$ mμ in the spectral range 263–400 mμ.

feeling of the present authors that $[m']_{233-235}$ offers the best estimate, and the reason will be discussed below.

Because complete precipitation of the copoly(L-Glu:-L-Phe) (76:24) occurred below pH 4.8, a value of $[m']_{235}$ for a 100% α-helical content was not measurable in aqueous solution. To estimate this value, the value of $[m']_{233} = 16{,}000°$ and $[m']_{235} = +600°$ were taken as representing 100% helical PGA and the block poly-L-Phe, respectively. Using these values and the known composition of the copolymer, the calculated value of $[m']_{233} = -11{,}300°$ was obtained which represents the value expected for the 100% helical conformation (assuming that the optical rotatory contribution of each amino acid to the ord of the copolymer was in direct proportion to its molar content). It is evident that with the presence of aromatic amino acids the interpretation of ord spectra of polypeptides and proteins becomes difficult and complicated. As illustrated here for phenylalanine and demonstrated previously for tyrosine (Fasman *et al.*, 1964b) and tryptophan (Fasman *et al.*, 1965), the parameters usually used to evaluate the helical content (b_0, $[m']_{233}$, $A(\alpha,\rho)_{225}$, etc.) become dependent *not only* on the peptide conformation but also upon the rotatory contribution of these other chromophores. Furthermore, other chromophores can also effect optical activity or contain intrinsic optical rotatory power [*e.g.*, S–S (Iizuka and Yang, 1964; Beychok, 1965), histidine (Beychok *et al.*, 1965)] which can further complicate this problem.

To evaluate the ord contribution, in greater detail, of the phenylalanyl chromophore when incorporated into a polypeptide α-helical conformation, block copolymers sandwiching a sequence of L-Phe between two DL-Glu sequences were studied. The Moffitt–Yang plot of the ord of the block copoly(DL-Glu)(L-Phe)(DL-Glu) in H_2O yielded a b_0 value of -130 to $-160°$. This b_0 value of approximately $-145°$ is considerably different than the value of -630 to $-700°$ obtained for 100% α-helical PGA. These values compare favorably with the value of b_0 of $-120°$ obtained by Auer (1964). The presence of aromatic Cotton effects therefore have a profound effect on the magnitude of one of the "α-helical Cotton effects," causing the values of $[m']$ to be less negative and shifting the trough from 233 mμ toward higher wavelengths, to 235 mμ in this case. Previously it was reported that the trough value for poly-L-tyrosine was observed at 238 mμ (Fasman *et al.*, 1964a). It was shown by cd that this was caused by an overlap of the peptide helical Cotton effect and one of the tyrosyl Cotton effects (Beychok and Fasman, 1964). It is obvious that the use of b_0 and the Shechter–Blout treatment of ord spectra can be seriously questioned for estimating the per cent α helix of polypeptides and proteins containing aromatic amino acids (*e.g.*, poly-L-tyrosine analyzed by the Shechter–Blout equation erroneously indicated a left-handed helix). This latter treatment has been previously challenged (Yang, 1965). The ord spectrum of poly-L-Phe (Figure 7) shows three small Cotton effects in the wavelength region where the benzene chromophore exhibits a number of absorption maxima. These Cotton effects are attributed to the aromatic side chains of poly-L-Phe held in an asymmetric environment by the α-helical backbone, possibly allowing aromatic–aromatic interactions. Moscowitz *et al.* (1964) have observed very small Cotton effects in the spectral region 240–260 mμ with L-phenylalanine. These Cotton effects were not observed in the present studies. However, Moscowitz observed these effects only at a very low concentration (1.26×10^{-3} M). The concentration of L-Phe used in our studies was considerably higher (4.2×10^{-2} M).

A similar but different phenomenon was first reported by Stryer and Blout (1961) who noted an "extrinsic" Cotton effect in the absorption region of acridine orange when absorbed onto α-helical PGA. This dye showed no Cotton effect when bound to the random coil form. Fasman and co-workers (1964b, 1965) have reported similar "intrinsic" Cotton effects with poly-L-tyrosine and poly-L-tryptophan, and several authors have reported similar phenomena in proteins (*e.g.*, Myers and Edsall, 1965; Glazer and Simmons, 1965).

Although serious complications have been shown to arise in the interpretation of the ord spectra of proteins containing aromatic residues, these spectra contain such a wealth of information that further work will undoubtedly shed light on some extremely interesting problems of protein conformation.

Acknowledgment

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