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Conformational Aspects of Polypeptide Structure. XXII. Aromatic Side-Chain Effects from Poly-L-*p*-aminophenylalanine and Derivatives*

Murray Goodman and Evaristo Peggion†

ABSTRACT: We synthesized poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine and poly-L-*p*-aminophenylalanine as analogs of poly-L-tyrosine and examined their optical rotatory dispersion (ORD) and circular dichroism (CD). In tetrahydrofuran the ORD and CD of poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine reveals a positive Cotton effect centered at 245 mμ which can be assigned to the $\pi-\pi^*$ electronic transition of *para*-substituted benzene rings existing in a dissymmetric environment; this band disappears in trifluoroacetic acid, a helix-breaking solvent. Poly-L-*p*-aminophenylalanine exists as a random coil between pH 1.08 and 2.56. When the pH rises from 2.56 to 2.78, a sharp change in ORD and CD is observed. Positive Cotton effects at 290 and 245 mμ corresponding to $\pi-\pi^*$ electronic transitions of aromatic side-chain amino groups appear. This can be explained by a coil–helix transition in this pH region, even though only one-half the amino groups are deprotonated, as can be seen by the molar extinction coefficient (ϵ 9000) at 250 mμ. The negative Cotton effect (trough, 230 mμ) in the ORD further suggests a right-handed helix at pH 2.78 or higher.

These results are comparable in the acidic pH range to those found for poly-L-tyrosine in the basic pH range except that soluble poly-L-*p*-aminophenylalanine of high purity and molecular weight can be prepared more easily.

Optical rotatory dispersion (ORD) (Urnes and Doty, 1960; Yang, 1961) and circular dichroism (CD) (Holzwarth and Doty, 1965) studies have been used for the conformational analysis of polypeptides with aromatic side chains (Blout, 1962). When the primary helical arrangement of the peptide bonds necessarily imposes a secondary helical arrangement on the aromatic side chains, electronic interactions can occur between side chains and main-chain chromophores, leading to unusual optical rotatory properties. Such effects have been observed for poly-L-tyrosine (Fasman *et al.*, 1964; Beychok and Fasman, 1964; Pao *et al.*, 1965) and poly-L-phenylalanine (Sage and Fasman, 1966; Auer and Doty, 1966). In both cases Cotton effects have been detected in the region of the benzene–chromophore transitions, indicating that the aromatic side-chain residues exist in dissymmetric environments.

Electronic interactions among the side-chain chromophores have been demonstrated somewhat earlier with copolymers containing poly-β-(*p*-nitrobenzyl)-L-aspartate (Goodman *et al.*, 1963) and recently with copolymers containing poly-L-*p*-(phenylazo)phenylalanine (Goodman and Kossoy, 1966). The helical porphyrin-*d*-uroblin (Moscowitz, 1964) and naturally occurring proteins (Myers and Edsall, 1965) have shown analogous effects.

In our present report we describe the synthesis and stereochemical properties of some analogs of poly-L-tyrosine. Poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine and poly-L-*p*-aminophenylalanine were prepared and the ORD and CD measurements of these substances were carried out.

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† NATO Postdoctoral Fellow, 1965–1966. Present address: Università di Padova, Padova, Italy.

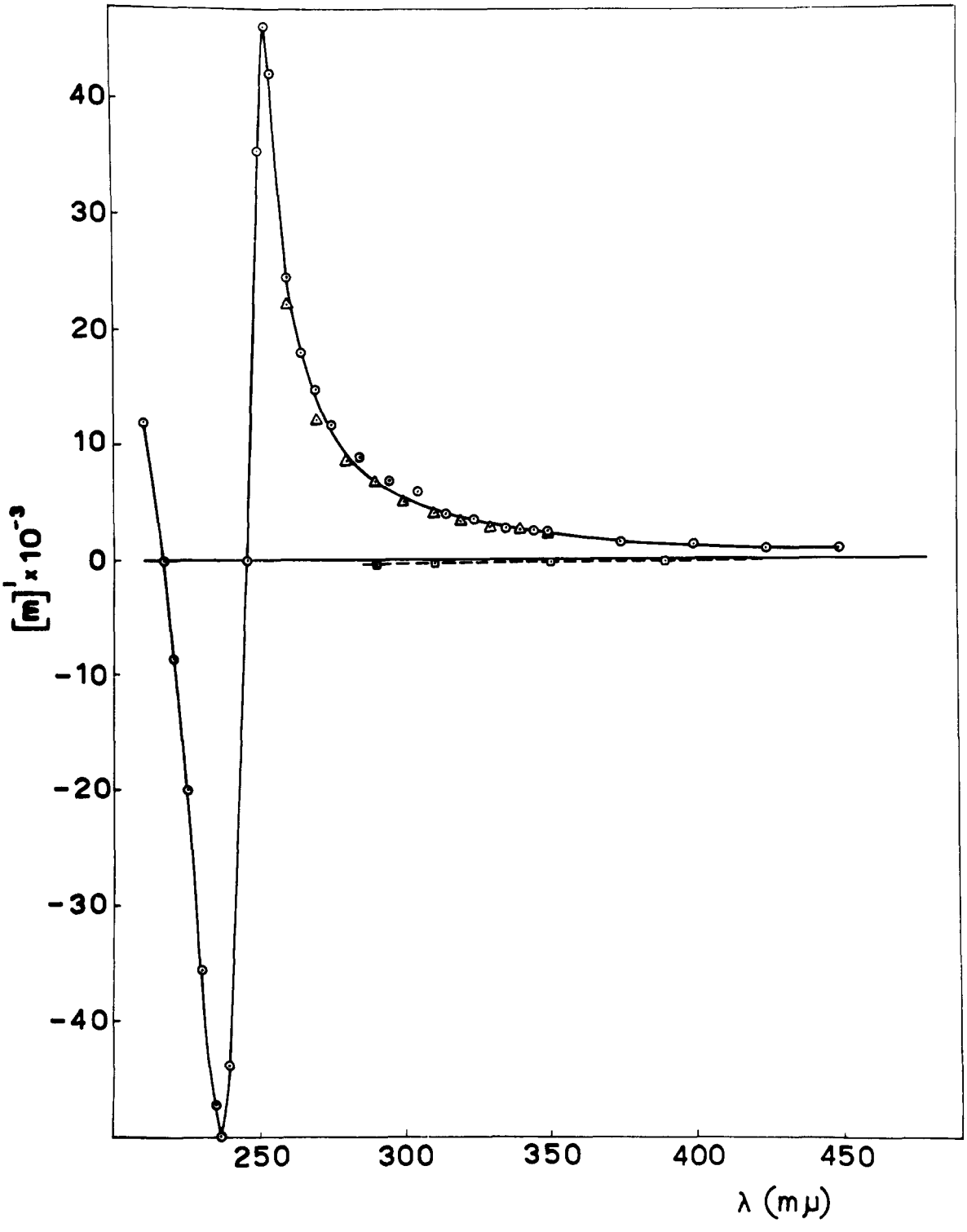
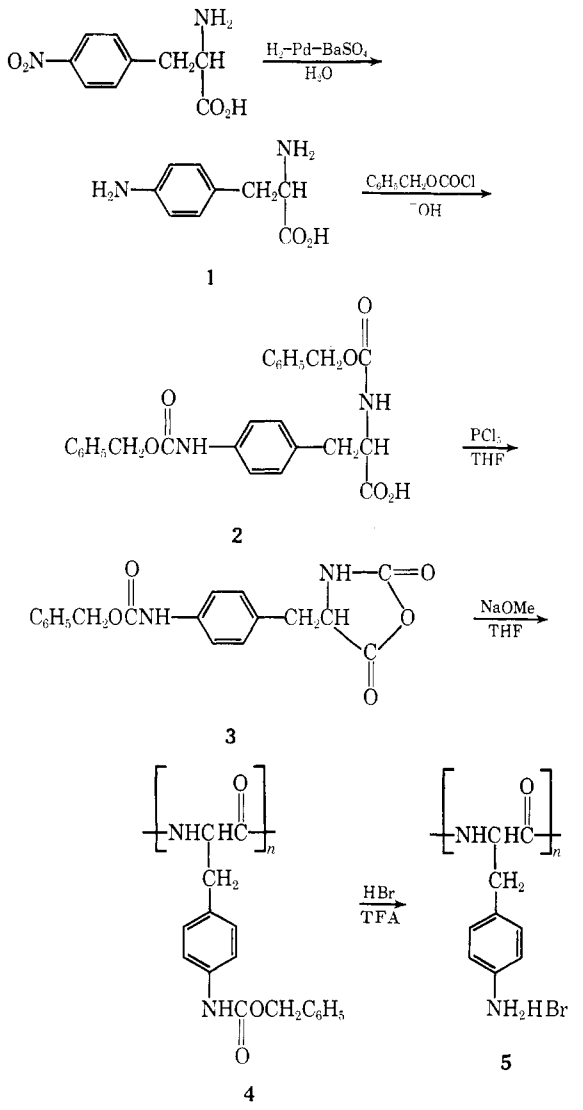


FIGURE 1: Optical rotatory dispersion of poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine in tetrahydrofuran (———) and in trifluoroacetic acid (– – – –).

Results and Discussion

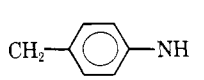
Synthesis. The synthesis of the desired polypeptides is outlined in Scheme I. The amino acid, L-*p*-aminophenylalanine (**1**), was prepared by catalytic hydrogenation of L-*p*-nitrophenylalanine according to the procedure of Bergmann (1952). *N*-Carbobenzoxylation of the amino acid **1** gave the dicarbobenzoxy derivative **2** which was then treated with phosphorous pentachloride in order to prepare the corresponding α-

SCHEME I



amino acid *N*-carboxyanhydride (NCA) **3** (Katchalsky and Sela, 1953; Sela and Katchalsky, 1954).[1,2] NCA **3** was polymerized in tetrahydrofuran with sodium methoxide as initiator (Blout and Karlson, 1956) to obtain poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine (**4**). Subsequent removal of the N^{ω}-carbobenzoxy groups from the polypeptide **4** by the hydrogen bromide–trifluoroacetic acid method (Boissonnas *et al.*, 1960) gave poly-L-*p*-aminophenylalanine (**5**) as the hydrobromide salt.

Conformational Studies of Poly-N^{ω}-carbobenzoxy-L-p-aminophenylalanine. The ORD curves of poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine in tetrahydrofuran and in trifluoroacetic acid are shown in Figure 1. In tetrahydrofuran, the polypeptide exhibits a positive Cotton effect with a strong peak at 253 mμ, followed by a deep trough at 237 mμ; the crossover point lies at 247 mμ. The beginning of a second positive Cotton effect appears below 220 mμ, but is not clearly evident from the ORD curve. The CD of the polypeptide in tetrahydrofuran was measured in order to resolve possible overlapping of Cotton effects in this region. The CD curve (Figure 2) reveals at least four dichroism bands in the wavelength interval 250–200 mμ. The first dichroism band is strongly positive and is centered at 245 mμ. On the basis of ultraviolet absorption spectra of N-substituted toluidines of the type **6** (Grammaticakis, 1951), this ellipticity band at 245 mμ can be assigned to the primary $\pi-\pi^*$ electronic transitions



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of the *para*-substituted benzene rings. Significantly, contributions of the benzene rings of the N^{ω}-carbobenzoxy groups, which should appear in the 260–250-

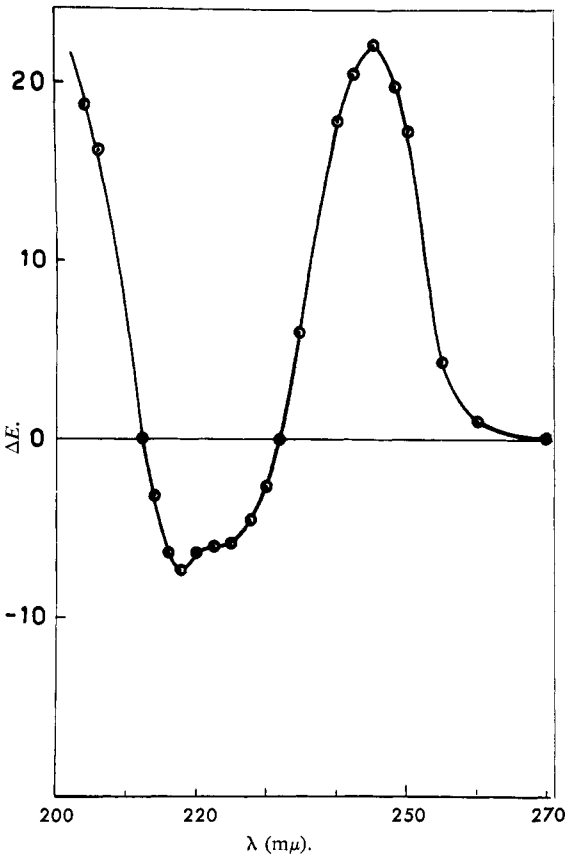


FIGURE 2: Circular dichroism of poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine in tetrahydrofuran.

[1] Abbreviations used: NCA, *N*-carboxyanhydride; THF, tetrahydrofuran; TFA, trifluoroacetic acid.

[2] NCA **3** is a highly crystalline, high-melting compound. It is stable over long periods of time in air and, therefore, can be easily purified. This is in sharp contrast to the instability and difficulty of purification of *o*-carbobenzoxy-L-tyrosine NCA.

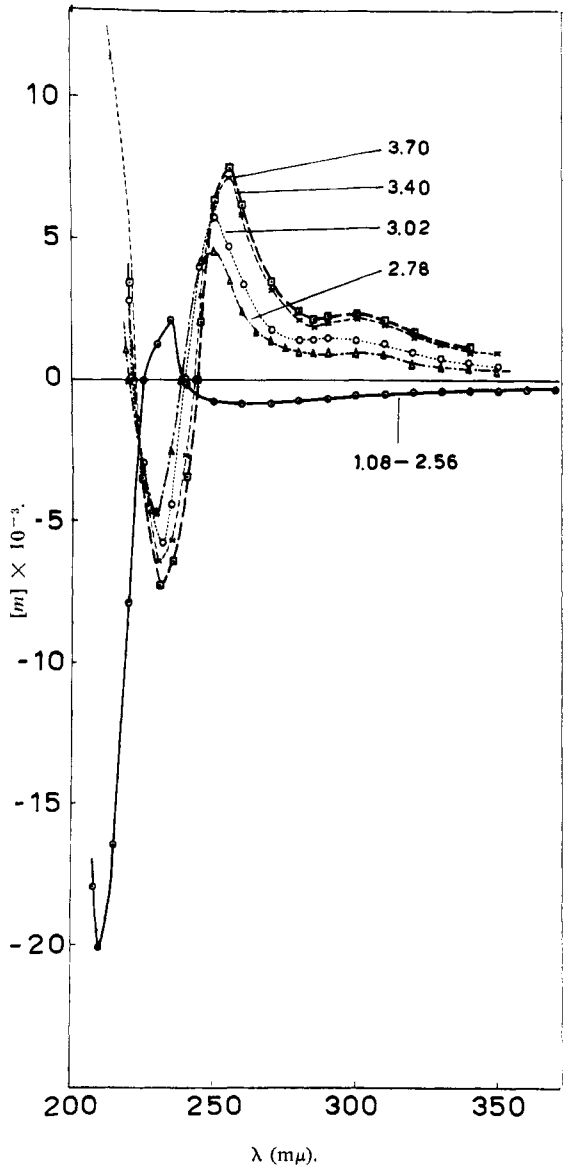


FIGURE 3: Optical rotatory dispersion of poly-L-*p*-aminophenylalanine as a function of pH in aqueous media. pH 1.08–2.56 (———), pH 2.78 (— · —), pH 3.02 (· · · · · ·), pH 3.40 (– – – –), and pH 3.70 (— —) as indicated.

mμ region, have not been detected; such contributions are either too small or entirely absent. When trifluoroacetic acid is used as the solvent, the band at 245 mμ disappears. These results suggest that in the helix-supporting solvent, tetrahydrofuran, the phenyl groups near the backbone of the main chain exist in a dissymmetric environment; in trifluoroacetic acid the polypeptide exists as a random coil and the dissymmetry is lost.

Supporting evidence that the polypeptide exists in helical conformations is obtained from the CD data below 240 mμ. In this region, there are two distinct, overlapping negative bands located at 225 and 217 mμ; also the beginning of a strong positive band in the region of 200 mμ can be detected. The negative ellipticity band at 225 mμ appears to be the n–π^* peptide transition associated with the α-helical conformation of polypeptides and the negative band at 217 mμ and the positive band below 200 mμ are assigned, respectively, to the parallel-polarized and perpendicular-polarized π–π^* exciton transitions of the peptide groups (Holzwarth and Doty, 1965). In this case the three dichroism bands are in approximately the same positions and have the same signs and magnitudes as the experimental and calculated bands of poly-γ-methyl-L-glutamate in a right-handed α-helix conformation (Holzwarth and Doty, 1965).

On the basis of the analogies between our data and the data of Holzwarth and Doty (1965), we conclude that poly-*N*-carbobenzoxy-L-*p*-aminophenylalanine in tetrahydrofuran is a right-handed α helix and that side-chain–main-chain and/or side-chain–side-chain interactions can be observed in this conformation.

Conformational Studies of Poly-L-p-aminophenylalanine. The ORD curves of poly-L-*p*-aminophenylalanine as a function of pH in aqueous media are shown in Figure 3. In the pH range 1.08–2.56, a weak peak at 235 mμ followed by a deep trough at 210 mμ is observed. However, when the pH is increased from 2.56 to 2.78, a sharp change in the ORD spectra occurs; two peaks at 300 and 250 mμ and a trough at 230 mμ appear. Only small changes are observed on further increase of the pH and at pH higher than 3.85 the polypeptide precipitates. At a pH greater than 2.78 the peak at 250 mμ shifts to 255 mμ and the absolute values of the optical rotations at the peaks and troughs increase to a small extent.

Similar results have been observed for poly-L-tyrosine (Fasman *et al.*, 1964). At pH 11.2, poly-L-tyrosine was found to exhibit multiple Cotton effects with peaks at 286 and 254 mμ, and a trough at 238 mμ. Upon further ionization of the phenolic hydroxyls of poly-L-tyrosine the positive peak of the Cotton effect at 286 mμ vanishes, the peak at 254 mμ diminishes, and simultaneously the trough at 238 mμ decreases. These results were attributed to an α-helix to random coil transition, although the magnitude and extent of changes in the ORD and CD spectra are small. With our polymer (Figure 3) the changes are large in the ORD between pH 2.56 and 2.78. We are able to confirm this sharp transition in the ultraviolet spectrum of the polypeptide (Figure 4). At pH 2.78 and higher, two distinct absorption peaks appear at 290 and 240 mμ. Further increase of the pH to 3.7 results only in increasing absorbance of these maxima. A plot of absorbance at 240 mμ *vs.* pH (Figure 5) markedly shows the sharp transition between pH 2.56 and 2.78.

The CD of the polypeptide as a function of pH shows the same results (Figure 6) observed in the ORD and ultraviolet measurements. At pH 1.08 a weak positive ellipticity band at 220 mμ is observed. At pH values greater than 2.78 four dichroism bands centered at 290, 245, 223, and 200 mμ are present in the wavelength interval 300–200 mμ.

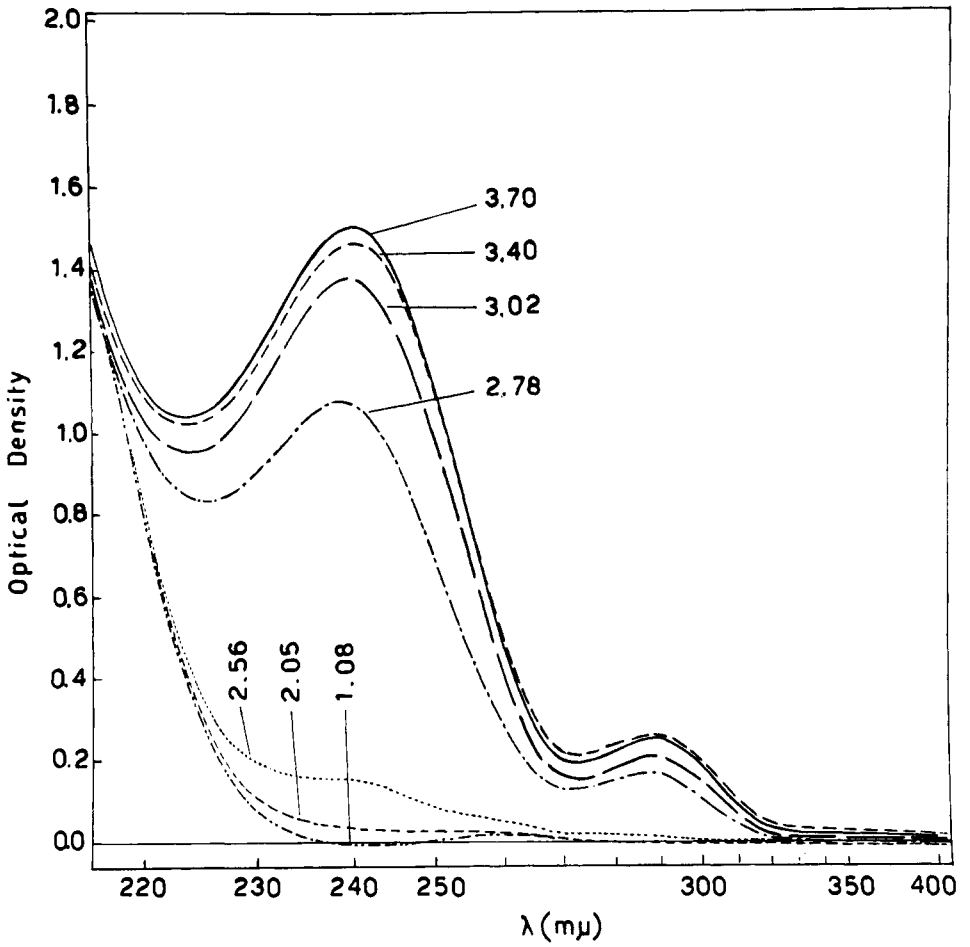
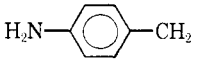


FIGURE 4: Ultraviolet absorption spectra of poly-L-*p*-aminophenylalanine as a function of pH in aqueous media. pH 1.08 (— · · —), pH 2.05 (– – – –), pH 2.56 (· · · · · ·), pH 2.78 (– · – ·), pH 3.02 (— —), pH 3.40 (- - - - - -), and pH 3.70 (———) as indicated.

On the basis of ultraviolet spectra of *para*-substituted aromatic amines of the type 7 (Doub and Vandenbelt, 1947), the bands at 290 and 245 mμ can be assigned



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to the π–π^* electronic transitions of the deprotonated form of the aromatic side-chain amino groups. However, we believe that the simple deprotonation of side-chain amino groups cannot be entirely responsible for the dramatic change observed in the narrow pH range 2.56–2.78. In fact, according to the reported ultraviolet spectrum of *p*-toluidine (Doub and Vandenbelt, 1947), the extinction coefficient (ϵ) of the primary band at 245 mμ should be approximately 9000; however, a comparable value can be obtained from the ORD and ultraviolet data of the polypeptide only when just one-half of the amino groups are assumed to be protonated. If deprotonation were the only phenomenon occurring with increase of pH from 2.56 to 2.78, a linear change in ultraviolet, ORD, and CD spectra would be observed. On the basis of these considerations, we suggest that a coil–helix transition must occur in this pH range.

Our experimental results for the polypeptide between pH 1.08 and 2.56 can be explained on the basis of calculated and experimental ORD and CD spectra of random polypeptides. Holzwarth and Doty (1965) have shown that random polypeptides exhibit weak positive dichroism near 220 mμ and strong negative dichroism near 200 mμ corresponding to the n–π^* and π–π^* peptide transitions, respectively. At pH values below 2.56 we observed the weak dichroism band (Figure 6) at 220 mμ associated with the n–π^* transition, but we were unable to perform measurements at wavelengths lower than 215 mμ. However, the presence of a deep trough at 210 mμ in the ORD spectrum (Figure 3) and the observed negative optical rotation in the visible wavelength region suggest the presence of a strong negative dichroism band in the 200-mμ region. Therefore, we conclude that at pH

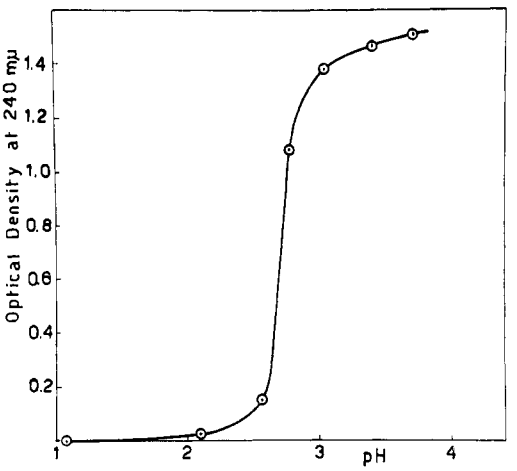

FIGURE 5: Optical density of poly-L-*p*-aminophenylalanine as a function of pH in aqueous media at 240 mμ. Concentration is 0.1%. The cell path is 0.5 mm.

2.56 and lower, poly-L-*p*-aminophenylalanine is a random coil.

The interpretation of our experimental results at pH values higher than 2.78 appears to be much more complicated. As previously mentioned, the CD spectrum (Figure 6) shows at least four ellipticity bands in the wavelength region 300–200 mμ. As already suggested, the positive bands at 290 and 245 mμ can be assigned to the π–π^* electronic transitions of the deprotonated, aromatic side-chain amino groups. In order to interpret the remaining bands, we compared our CD data with the data of Beychok and Fasman (1964) for poly-L-tyrosine in an α-helix conformation.

A negative dichroic band at 223 mμ with $\Delta E \simeq -4.5$ is observed for helical poly-L-tyrosine; in the same position, poly-L-*p*-aminophenylalanine exhibits a much weaker negative band. Beychok and Fasman (1964) did not report measurements in the 200-mμ wavelength region where theoretical calculations of Pao *et al.* (1965) predict strong positive dichroism for helical poly-L-tyrosine. We believe that Pao's quantum mechanical treatment can be applied to poly-L-*p*-aminophenylalanine. At pH 2.78 and higher, the beginning of a strong positive dichroic band below 210 mμ can be observed.

From these results, it appears that definite conclusions concerning all contribution interactions at pH values higher than 2.78 cannot be reached. We can assign a right-handed α helix to poly-L-*p*-aminophenylalanine based on the negative Cotton effect (trough ~230 mμ) observed in the ORD spectrum. Since Cotton effects are present in the absorption region of the aromatic side-chain groups, we conclude that these chromophores are in a dissymmetric environment. Since approximately one-half of the side-chain amino groups are still protonated, the side chains probably contribute to the stability of the helix through side-chain–main-chain and side-chain–side-chain interactions. The task of analyzing these interactions is formidable since we must consider effects from amino aromatic groups to the main chain, protonated amino aromatic groups to the main chain, amino aromatic groups to each other, amino aromatic groups to protonated amino aromatic groups, and protonated amino aromatic groups to each other. From our work to date, we cannot distinguish or separate these interactions into their component parts. However, we are commencing to prepare a series of copolymers of L-*p*-aminophenylalanine with noninteracting comonomers. We will attempt to unravel some of these side-chain effects by a statistical analysis of the magnitude and positions of the side-chain Cotton effects (Bradley *et al.*, 1966). Of course, a more fundamental interpretation must await a quantum mechanical treatment of the problem as commenced by Pao *et al.* (1965).

Experimental Section

Melting points are uncorrected and were taken using a Kofler hot stage. Elemental analyses were performed by Instituto Di Chimica Organica Dell'Universita, Padova, Italy. Optical rotations were measured with a Rudolph Model 80 photoelectric polarimeter. L-*p*-Nitrophenylalanine was obtained from the Cyclo Chemical Corp. and was used without further purification. All solvents used were of reagent grade and were used without further purification.

Optical Rotatory Dispersion. Measurements of the optical rotation were taken on a Cary 60 spectropolarimeter. The instrument is estimated to read ±0.0002° in the wavelength range 600–185 mμ. The measurements were carried out in 0.1-, 0.2-, and 2-mm cylindrical cells.[3]

Circular Dichroism. Measurements of the circular dichroism were carried out with a Jasco UV-CD-ORD-5 spectropolarimeter using the same type of cells employed in the ORD measurements above.

Ultraviolet Absorbance. The ultraviolet measurements were performed using a Perkin-Elmer Model 350 double-beam spectrophotometer. The concentration used was 0.1% in a 0.5-mm cell.

Preparation of Poly-L-p-aminophenylalanine Solutions for ORD, CD, and Ultraviolet Measurements. The polypeptide was weighed out in a 10-ml volumetric flask (*e.g.*, 1.5 mg) and approximately 6 ml of water was added. The solutions were titrated with sodium hydroxide (0.1 N) solution and the pH was measured simultaneously with a Radiometer automatic titrator type TTT-1C coupled to the Titragraph. The volume was then built-up to the 10-ml mark with distilled water.

Viscometry. Intrinsic viscosities, [η], were determined using Ubbelohde viscometers with solvent flow times greater than 100 sec. All measurements were made at 25 ± 0.1°.

[3] Optical Cell Co., Brentwood, Md.

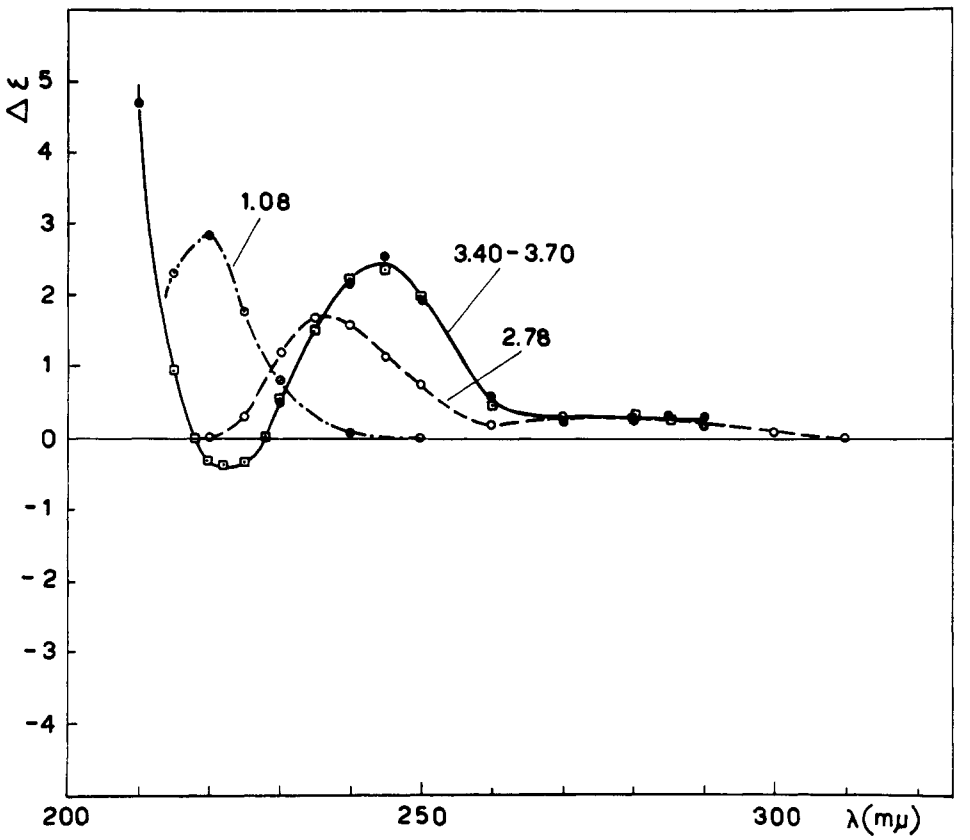

FIGURE 6: Circular dichroism of poly-L-*p*-aminophenylalanine as a function of pH in aqueous media. pH 1.08 (— · —), pH 2.78 (– – – –), and pH 3.40–3.70 (———) as indicated.

L-*p-Aminophenylalanine* (**1**) was prepared according to the procedure of Bergmann (1952). The amino acid, L-*p*-nitrophenylalanine, was reduced by catalytic hydrogenation in water and the crude material was recrystallized four times from water to obtain the desired product in 82% yield, mp 225–227° (Bergmann (1952) reported mp 254°).

*$N^{\alpha,\omega}$-Dicarbobenzoxy-*L*-p-aminophenylalanine* (**2**). The procedure of Sela and Katchalsky (1954) for the preparation of $N^{\alpha,\omega}$-dicarbobenzoxy-DL-*p*-aminophenylalanine was followed. Carbobenzoxy chloride (25 g, 0.15 mole) and 40 ml of 4 N sodium hydroxide solution were simultaneously added during 1 hr to a vigorously stirred solution of L-*p*-aminophenylalanine (11.5 g, 0.0638 mole) in 60 ml of 2 N sodium hydroxide solution at 0°. The pH of the reaction mixture was maintained between 9 and 11 by adjusting the flow rate of the carbobenzoxy chloride and the sodium hydroxide solution; the sodium salt of the dicarbobenzoxy derivative precipitated during the reaction. After 1 hr 500 ml of water was added and the mixture was stirred at room temperature for 3 hr. The mixture was acidified to pH 4 with 6 N hydrochloric acid solution and the resultant precipitate was collected and dried. The crude product was dissolved in tetrahydrofuran, treated with Norit, and dried over magnesium sulfate. The solvent was removed *in vacuo* and the resultant solid was recrystallized four times from ether. The desired product was obtained in 31.4% (9 g) as a white solid, mp 143°, $[\alpha]_D^{25}$ −5.14° (*c* 2.09, acetone).

Anal. Calcd for $C_{25}H_{24}N_2O_6$: C, 66.96; H, 5.36; N, 6.25. Found: C, 67.17; H, 5.63; N, 6.24.

*N^{ω}-Carbobenzoxy-*L*-p-aminophenylalanine-N^{α}-carboxyanhydride* (**3**). The procedure of Sela and Katchalsky (1954) for the preparation of N^{ω}-carbobenzoxy-DL-*p*-aminophenylalanine-*N*-carboxyanhydride was modified as follows. A solution of $N^{\alpha,\omega}$-dicarbobenzoxy-L-*p*-aminophenylalanine (5 g, 0.011 mole) in 50 ml of tetrahydrofuran was treated with 3 g (0.014 mole) of phosphorous pentachloride (PCl_5). The mixture was shaken for 5 min at room temperature, the residue (PCl_5) was filtered off, and the resultant solution was heated to 50° for 15 min. The product was precipitated by addition of *n*-hexane and dried. Recrystallization two times from ethyl acetate–*n*-hexane afforded 2 g (53.4%) of a white solid, mp 204–205°, $[\alpha]_D^{25}$ −70.1° (*c* 2, tetrahydrofuran).

*Poly-N^{ω}-carbobenzoxy-*L*-p-aminophenylalanine* (**4**). N^{ω}-Carbobenzoxy-L-*p*-aminophenylalanine-N^{α}-carboxyanhydride (2 g, 5.9 mmoles) was dissolved in 100 ml of tetrahydrofuran. The polymerization was initiated with sodium methoxide (1.0 ml, 0.1 N $NaOCH_3$; monomer/initiator 60) and allowed to proceed at room temperature for 4 days. The viscous reaction mixture

was poured into vigorously stirred *n*-hexane. The resultant solid was reprecipitated from tetrahydrofuran with *n*-hexane and dried to obtain 1.6 g (89%) of a white fibrous polymer, $[\eta]_{DCA}$ 0.67.

Anal. Calcd for $C_{17}H_{16}N_2O_3$: C, 68.92; H, 5.40; N, 9.46. Found: C, 68.21; H, 5.55; N, 9.48.

Poly-L-*p-aminophenylalanine* (**5**). Poly-N^{ω}-carbobenzoxy-L-*p*-aminophenylalanine (300 mg, 1 mmole) was dissolved in 20 ml of TFA and dry hydrogen bromide was passed through the clear solution at room temperature for 45 min. The resultant white amorphous precipitate was collected by filtration, washed with ether, and dried *in vacuo* to yield an off-white polymer, 70% yield, $[\eta]^{0.2\ N\ NaBr}_{pH\ 1.0}$ 0.37.

Acknowledgment

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