Solution Properties of Synthetic Polypeptides. Synthesis and Conformational Properties of Poly(N^{ϵ}-acetoacetyl-L-lysine), Poly(N^{δ}-acetoacetyl-L-ornithine), and Poly(N^{γ}-acetoacetyl-L-diaminobutyric acid) in Aqueous Solution

A. Cosani, M. Terbojevich, M. Palumbo, E. Peggion,* and E. Scoffone

Contribution from the Institute of Organic Chemistry, University of Padova, Padova, Italy.
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ABSTRACT: Poly(N^{ϵ}-acetoacetyl-L-lysine) (PALL), poly(N^{δ}-acetoacetyl-L-ornithine) (PALO), and poly(N^{γ}-acetoacetyl-L-diaminobutyric acid) (PADB) have been synthesized by reaction of poly(L-lysine), poly(L-ornithine), and poly(L-diaminobutyric acid) with diketene in aqueous solution at pH ~9. All polymers are water soluble at pH >3.5, the order of solubility being PALL < PALO < PADB. With time PALL tends to become insoluble in water probably because of the formation of β structures. The conformational properties of three polymers have been investigated by uv absorption and circular dichroism techniques. In acid or neutral aqueous solution the polymers assume the right-handed α-helical conformation, while the random coil form is predominant in alkaline solution. The conformation of the peptide backbone does affect appreciably the optical activity of the side-chain chromophores in all the three polymers. In the case of PADB the side-chain groups have also substantial perturbing effects on the CD pattern of the backbone.

The optical rotatory properties of poly(α-amino acids) containing chromophores in the side chains have been the object of a number of investigations.[1-10] Generally, the presence of optically active side-chain chromophores adds complication to the interpretation of CD and ORD results in terms of conformation, since very often the contributions to the optical activity from the side-chains overlap those from the peptide groups. In spite of these difficulties the conformation in solution of some aromatic poly(α-amino acids) has been quite securely established. In some cases, it has been found that to an ordered helical structure of the peptide backbone there corresponds an ordered structure of the side chains.[11,12] However, by CD or ORD measurements it has not been possible to follow separately changes of the ordered structure of the side chains and conformational changes of the peptide backbone for the reason above discussed.

In the recent literature the use of the acetoacetyl group $-COCH_2COCH_3$ as blocking agent for the amine function of lysine residues in proteins has been described.[13] Acetoacetylation causes inactivation of ribonuclease A, and the biological activity can be partly recovered by removing the blocking groups with hydroxylamine hydrochloride.[14,15]

The behavior of such a group as side-chain chromophore of a homopolypeptide chain is interesting for a number of reasons. First of all, the uv absorption region of the acetoacetyl derivative is well separated from that of the peptide group and it might be therefore possible by uv and CD measurements to observe conformational changes of the side chains independently from those of the peptide backbone.

Furthermore the contribution to the optical activity due to the side chains can be established and this can be useful in order to study the conformational properties of acetoacetylated proteins. Finally, since the β-ketoamide function is a chelating agent for a number of cations, it is possible to observe the effect of chelation on the conformation of the polypeptide chain.

In the present paper we wish to report the synthesis and the conformational properties of the acetoacetyl derivatives of poly(L-lysine), poly(L-ornithine), and poly(L-diaminobutyric acid), namely, poly(N^{ϵ}-acetoacetyl-L-lysine) (PALL), poly(N^{δ}-acetoacetyl-L-ornithine) (PALO), and poly(N^{γ}-acetoacetyldiaminobutyric acid) (PADB).

Experimental Section

Materials. Reagent grade dioxane was refluxed over sodium metal and anthracene until the formation of a persistent blue color. Then it was distilled immediately before use rejecting the first 10% of distillate.

Ethyl acetate (reagent grade) was dried over $CaCl_2$ for 24 hr and then fractionally distilled. Petroleum ether (bp 30–60°, reagent grade) was refluxed over sodium wires and then distilled.

Triethylamine (Carlo Erba RP) was purified by refluxing for several hours over pellets of KOH. Then the KOH was filtered off and the filtrate was refluxed over potassium metal and then fractionally distilled under reduced pressure. Diketene (Fluka purum) was distilled under reduced pressure immediately before use.

N^{ϵ}-Carbobenzoxy-L-lysine, N^{δ}-carbobenzoxyornithine, and N^{γ}-carbobenzoxydiaminobutyric acid were prepared from the corresponding copper complexes according to the literature.[16-18] By reaction with phosgene in anhydrous dioxane the N^{ω}-carbobenzoxylated amino acids were transformed in the corresponding N-carboxyanhydrides according to the literature.[19,20]

***N*-Hexylacetoacetamide.** To a solution of 8 ml of *n*-hexylamine

(1) M. Goodman, A. S. Verdini, N. S. Choi, and Y. Masuda in "Topics in Stereochemistry," Vol. V, E. L. Eliel and N. L. Allinger, Ed., Wiley-Interscience, New York, N. Y., 1970, and references therein quoted; G. D. Fasman in "Poly-α-aminoacids," Marcel Dekker Inc., New York, N. Y., 1969, and references therein quoted.
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(4) J. Engel, *Eur. J. Biochem.*, **21**, 2230 (1971).
(5) G. Conio, V. Trefiletti, and E. Patrone, *Makromol. Chem.*, **154**, 311 (1972).
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(7) M. B. Senior, S. L. H. Garrell, and E. Hamori, *Biopolymers*, **10**, 2387 (1971).
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(10) M. Goodman and E. Peggion, *Biochemistry*, **6**, 1533 (1967).
(11) G. D. Fasman, E. Bodenheimer, and C. Lindblow, *Biochemistry*, **3**, 1665 (1964).
(12) E. Peggion, A. Cosani, A. S. Verdini, A. Del Pra, and M. Mammi, *Biopolymers*, **6**, 1477 (1968).
(13) F. D'Angeli, F. Filira, and E. Scoffone, *Tetrahedron Lett.*, **10**, 605 (1965).
(14) A. Marzotto, P. Pajetta, and E. Scoffone, *Biochem. Biophys. Res. Commun.*, **26**, 517 (1967).
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(18) B. C. Barrass and D. T. Elmore, *J. Chem. Soc.*, 334 (1957).
(19) A. Farthing, *J. Chem. Soc.*, 3213 (1950).
(20) M. Sela and A. Berger, *J. Amer. Chem. Soc.*, **77**, 1813 (1955).

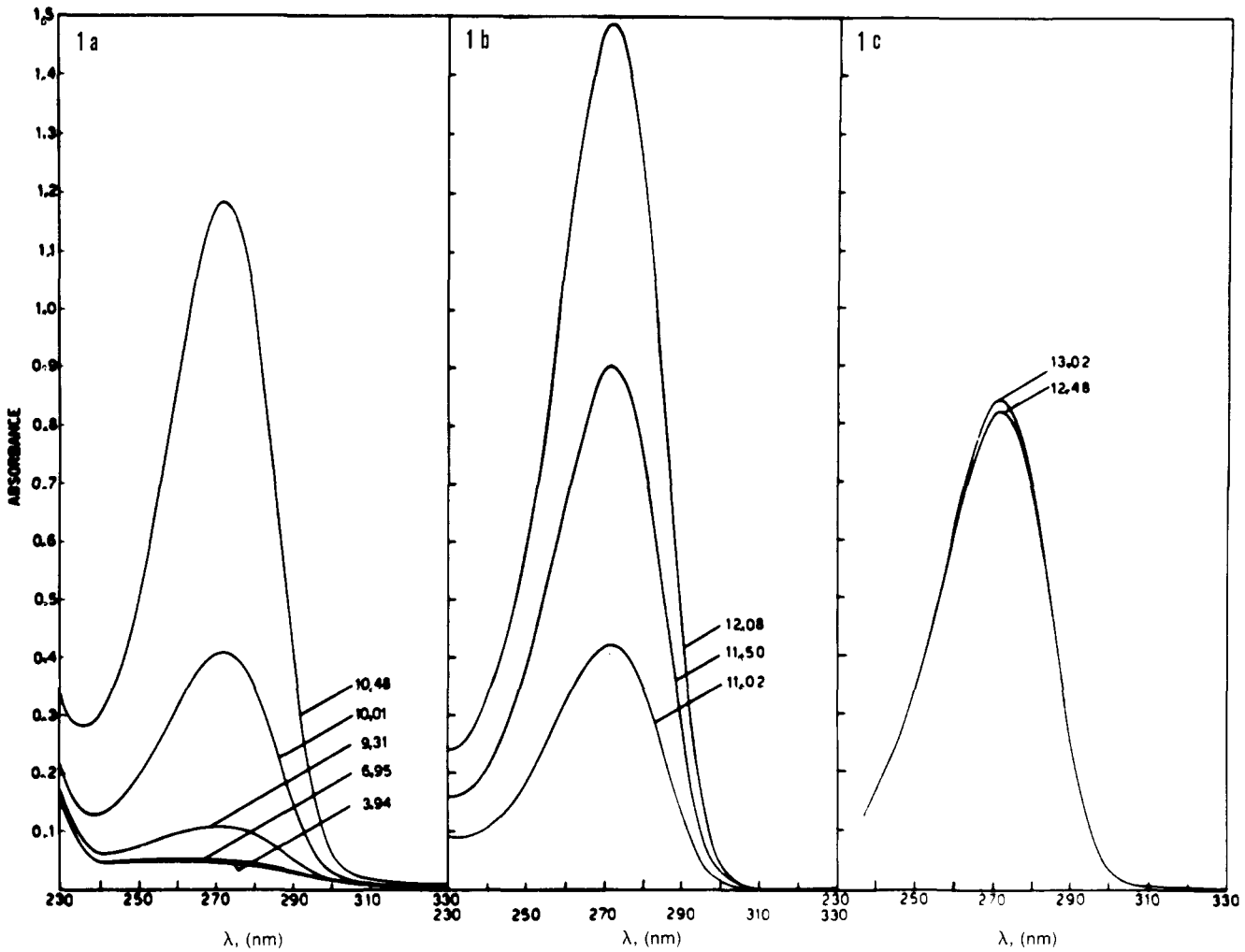


Figure 1. Uv absorption spectra of *n*-hexylacetoacetamide in aqueous solution at different pH values. The concentration was always 1.105×10^{-3} mol/l. The spectra have been recorded using 1-cm (a), 0.1-cm (b), and 0.05-cm (c) path-length cells.

(Fluka puriss.) in 30 ml of ethyl ether, 5 ml of diketene in 15 ml of ethyl ether was added dropwise in ~2 hr. The solution was then refluxed for 2 days. At the end of the reaction the unreacted amine was extracted with aqueous HCl, and the product was recovered by evaporation of the solvent. It was then recrystallized twice from methanol, mp 57°. *Anal.* Calcd for $C_{10}H_{19}NO_2$: C, 64,86; H, 10.29; N, 7.57. Found: C, 65.70; H, 9.21; N, 7.94.

Polymers. Poly(N^{ϵ}-carbobenzoxy-L-lysine) (PCBL), poly(N^{δ}-carbobenzoxy-L-ornithine) (PCBO), and poly(N^{γ}-carbobenzoxydiaminobutyric acid) (PODBA) were prepared by polymerization of the corresponding *N*-carboxyanhydrides in dioxane and using triethylamine as the initiator. In all cases the monomer concentration was 0.1 mole/l. and the monomer to initiator ratio was 50. At the end of the polymerization (checked by ir) the solutions were diluted 1:1 with chloroform. From these mixtures treated with anhydrous HBr poly(L-lysine) (PLL), poly(L-ornithine) (PLO), and poly(L-diaminobutyric acid) (PLDB) were obtained as hydrobromides according to the procedure of Fasman *et al.*[21] The corresponding hydrochlorides were obtained by dialysis against 0.01 *N* HCl. Intrinsic viscosity measurements in 0.2 *M* NaCl on the three deblocked polymers were as follows: PLL·HCl, $[\eta]$ = 0.75 dl/g; PLO·HCl, $[\eta]$ = 0.82 dl/g; PLDB·HCl, $[\eta]$ = 0.67 (dl/g).

All three deblocked polymers at pH 11.2 in 1:1 methanol–water solvent mixture gave CD patterns corresponding *quantitatively to the right-handed α-helical conformation.*

Poly(N^{ϵ}-acetoacetyl-L-lysine) (PALL). To an ice-cooled solution of PLL·HCl (1 g) in water (70 ml) adjusted to pH 9 with 1 *N* NaOH, 0.85 ml of freshly distilled diketene was added with a microsyringe in 1 hr with vigorous stirring. Care was taken to maintain the pH at 9 by addition of NaOH. At the end of the diketene addition the solution of acetoacetylated polymer was left under stirring for 1 hr at 0° and for 1 hr at room temperature. After three extractions with ethyl ether in order to remove the excess diketene, the aqueous polymer solution was dialyzed for 2 days against water, using a 4465-A2 dialyzing tubing (A. Thomas Co., Philadelphia, Pa). About one-half of the dialyzed solution was stored at room temperature. From the remaining portion the polymer was recovered by lyophilization. The solid, white material with time tends to become insoluble in water. All spectroscopic measurements were performed on the polymer solution obtained by dialysis; its concentration was determined either by microkjeldahl nitrogen determination or by the amount of polymer obtained by lyophilization of a known volume of solution. At pH <3 PALL precipitates.

The absence of free amino groups in PALL was checked by reaction with ninhydrin.

Poly(N^{δ}-acetoacetylornithine) (PALO) and poly(N^{γ}-acetoacetyl-L-diaminobutyric acid) (PADB) were prepared following exactly the same procedure as for PALL. Both polymers once isolated by lyophilization were easily soluble in water at pH >3 and gave negative reaction with ninidrine.

Measurements. pH measurements were carried out with a Metrohm Model E 388 precision potentiometer using either Metrohm UX combined glass electrodes or Beckman glass and X calomel electrodes.

Circular dichroism (CD) measurements were performed with a Cary 60 spectropolarimeter equipped with a 6002 CD accessory, uv absorption measurements were carried out with a Cary 15 spectrophotometer. In all spectroscopic measurements fused quartz cylindrical cells with Suprasil windows were used.

Results and Discussion

Uv Absorption Measurements. The uv absorption spectra of the model compound *N*-hexylacetoacetamide at

(21) G. D. Fasman, M. Idelson, and E. R. Blout, *J. Amer. Chem. Soc.*, 83, 709 (1961).

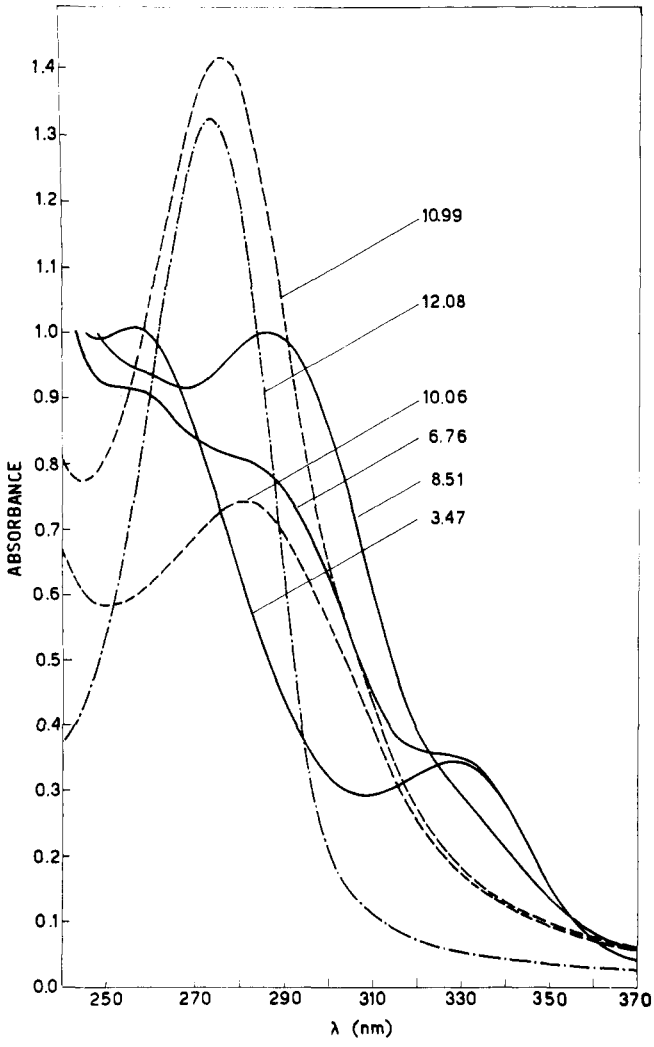


Figure 2. Original uv absorption spectra of poly(N^{ϵ}-acetoacetyl-L-lysine) (PALL) in aqueous solution at various pH values. In all measurements the polymer concentration was 2.09×10^{-3} molar residue. The spectra have been recorded with a 1-cm (——), 0.5-cm (- - - -), and 0.05-cm (—·—·—) path-length cells.

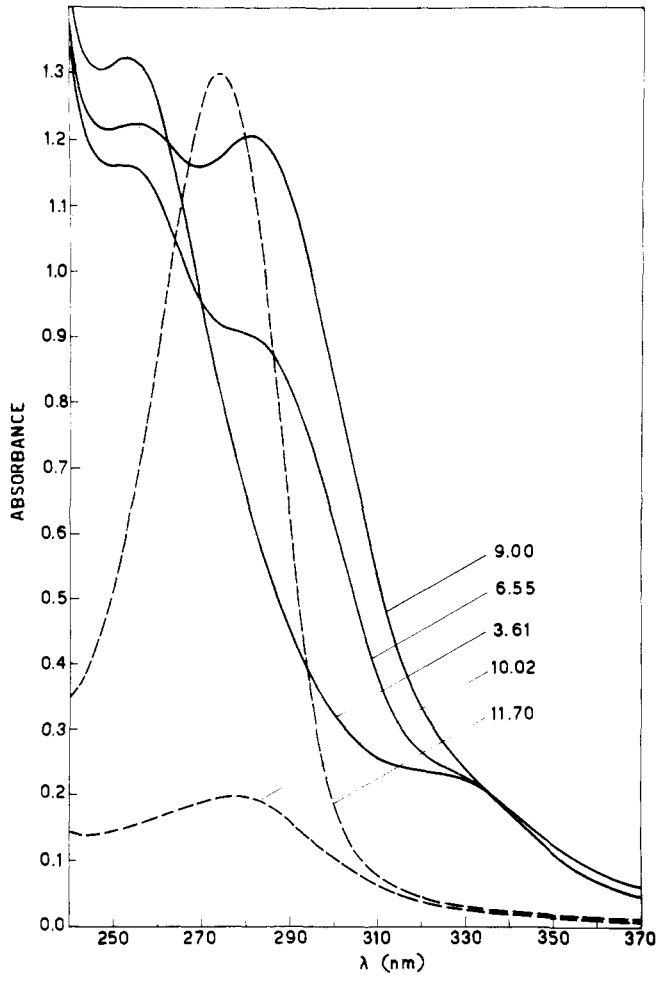
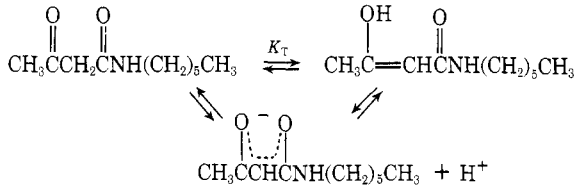


Figure 3. Original uv absorption spectra of poly(N^{δ}-acetoacetyl-L-ornithine) (PALO) in aqueous solution at various pH values. In all measurements the polymer concentration was 4.93×10^{-3} molar residue. The spectra have been recorded using 1-cm (——) and 0.1-cm (- - - -) path-length cells.

various pH values are shown in Figure 1. At acid pH values there is a characteristic absorption band centered at 272 nm, with a molar extinction coefficient ϵ of about 43. From its position and intensity this band can be assigned to the ketonic form of the compound, generally predominant in aqueous solution.[22-23] On increasing the pH the intensity of this band is enhanced, since the ionized form of the mixture in keto-enolic equilibrium is formed. We can in fact assume that the following equilibrium system is present in solution



If K_T is the equilibrium constant for the tautomeric equilibrium, $(K_a)_K$ and $(K_a)_E$ are the dissociation constants for the ketonic and enolic forms, respectively, and K_a is the overall ionization constant, the following equations can be written

$$K_a = \frac{[I][H^+]}{[K] + [E]} \quad (1)$$

$$\frac{1}{K_a} = \frac{1}{(K_a)_K} + \frac{1}{(K_a)_E} \quad (2)$$

$$(K_a)_K = K_a(1 + K_T) \quad (3)$$

$$(K_a)_E = K_a(1 + K_T)/K_T \quad (4)$$

where [I], [K], and [E] are the concentrations of the ionized form, ketonic form and enolic form, respectively. If the tautomeric equilibrium is shifted toward the ketonic form ($K_T \ll 1$), then the measured overall dissociation constant K_a is nearly coincident with $(K_a)_K$. From Figure 1 the molar extinction coefficient of the ionized form at 272 nm is 15,170. From this figure and from the molar extinction coefficient of the nonionized form, the pK_a has been calculated. It was found that $pK_a = 11.45 \pm 0.05$, which is in a fairly good agreement with literature data on similar compounds.[22-23]

The uv absorption spectra of PALL, PALO, and PADB in aqueous solution at various pH values are shown in Figures 2–4. It appears immediately that all spectra exhibit remarkable differences in comparison to the uv spectra of the model compound. At acid or neutral pH the three polymers show a new absorption band at ~330 nm not present in the model compound nor in acetoacetyl deriva-

(22) L. Hevesi, P. van Brandt, and A. Bruylants, *Bull. Soc. Chim. Fr.*, 3971 (1970).
(23) L. Hevesi and A. Bruylants, *Bull. Soc. Chim. Fr.*, 4066 (1971).

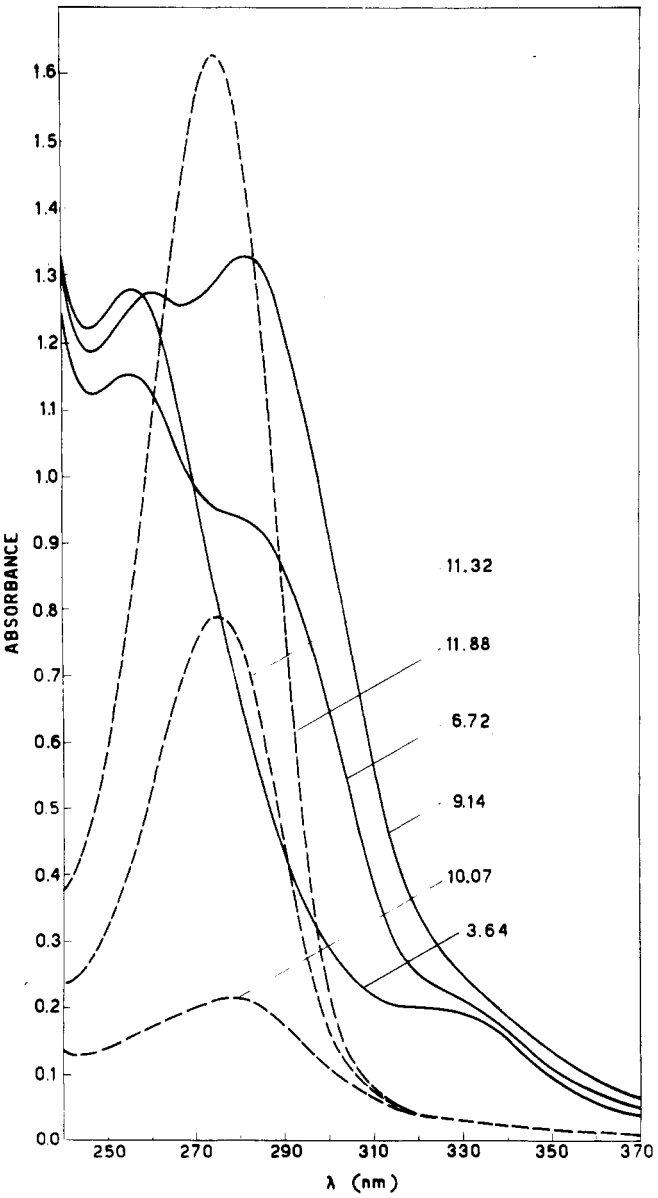


Figure 4. Original uv absorption spectra of poly(N^{γ}-acetoacetyl-L-diaminobutyric acid) (PADB) in aqueous solution at various pH values. The polymer concentration was 4.30×10^{-3} molar residue. The spectra have been recorded using 1-cm (——) and 0.1-cm (- - - -) path-length cells.

tives of amino acids.[24] In acid solutions another absorption maximum centered at 256 nm is present. On increasing the pH, these two bands tend to disappear and a new band is formed at 285 nm, which shifts to 273 nm at pH >12. In no case do the spectra recorded at different pHs and at the same concentration and cell path length show a clear isosbestic point. The molar extinction coefficients of the absorption maxima at various pH, relative to the model compound and to the three polymers, are reported in Table I.

It appears clearly that the relative intensities of the 330- and 256-nm bands are not constant in the three polymers under identical conditions. An interpretation of the uv results in terms of conformation can be made only by assuming that PALL, PALO, and PADB assume the right-handed α-helical conformation at pH <10.

The existence of a uv absorption band at 330 nm in acid or neutral polymer solutions, which is not present in simple acetoacetyl derivatives of amino acids and in the model compound, should be attributed to interactions among the side-chain chromophores when the polymers are in the α-helical conformation. The band at 256 nm should probably be assigned to the ketonic form of the side chains. Since the ratio of the intensities between the 256- and 330-nm bands changes on changing the distance of the acetoacetyl groups from the peptide backbone it appears that the extent of these interactions is not the same in the three polymers.

Finally the band which forms at 285 nm on increasing the pH has to be assigned to the ionized form of the side chains.

The absence of a clear isosbestic point in the uv spectra recorded at various pH clearly means that we are not dealing with a two component equilibrium system. The same result has been found by Longworth and Rahn[25] in the uv spectra of polytyrosine at different pH values. In our case there are two possible explanations for such a behavior. The first is that the keto–enolic equilibrium is not completely shifted toward the ketonic form, so that ketonic form, enolic form and ionized form are simultaneously present. The second explanation could be the following. Even in the hypothesis that the keto–enolic equilibrium is completely shifted toward the ketonic form, at low degrees of ionization there is perturbation of the ordered structure of the side chains. As a consequence, the following species can be simultaneously present in equilibrium: (i) *nonionized* acetoacetyl side chains either in ordered or in disordered arrangement, (ii) ionized side chains in ordered and disordered arrangement. This sec-

Table I

Sample	pH	λ_{max} (nm)	ϵ at λ_{max}
PALL	3.47	330	148
		256	485
	10.06	280	712
	13.00	272.5	13,820
PALO	3.61	330	45
		254	270
	12.12	273	2,650
PADB	3.64	330	45
		256	300
	11.88	274	3,800
Model compound	3.94	272	43
	13.02	272	15,170

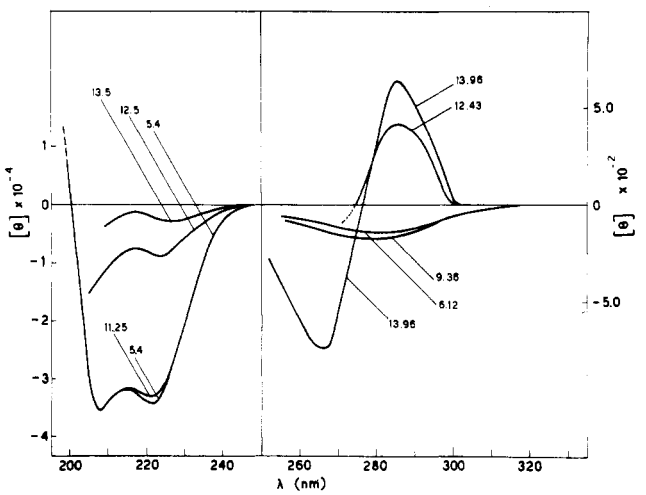


Figure 5. CD spectra of PALL in aqueous solution at various pH values.

(24) C. Toniolo, F. Filira, and C. DiBello, *Biopolymers*, **10**, 2275 (1971).

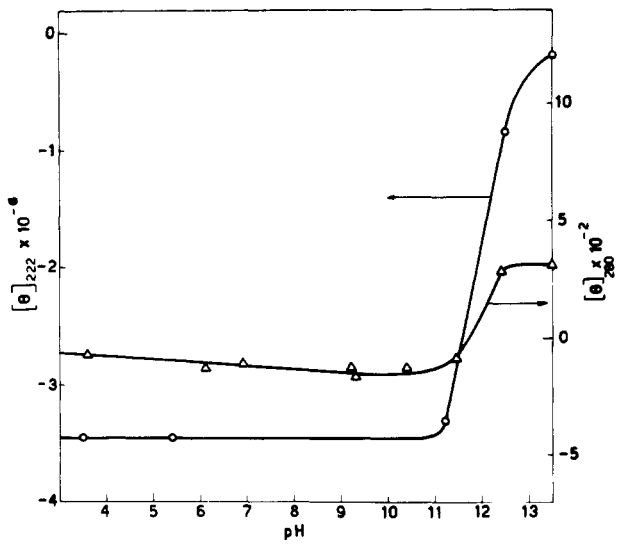


Figure 6. Molar ellipticity values of PALL at 280 nm and 222 nm as a function of pH.

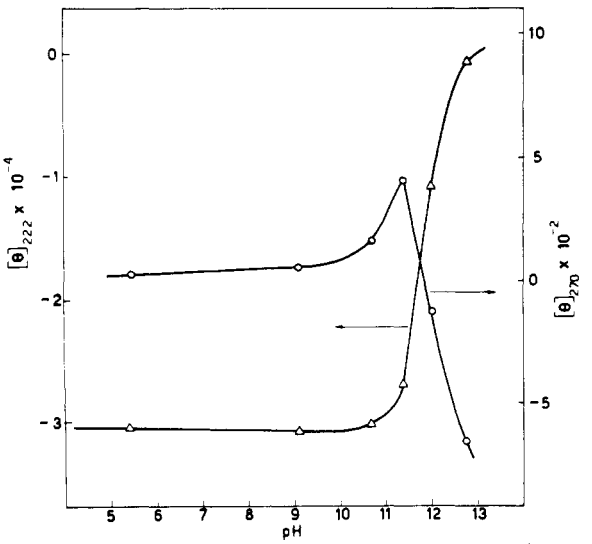


Figure 8. Molar ellipticity values of PALO at 280 nm and 222 nm, as a function of pH.

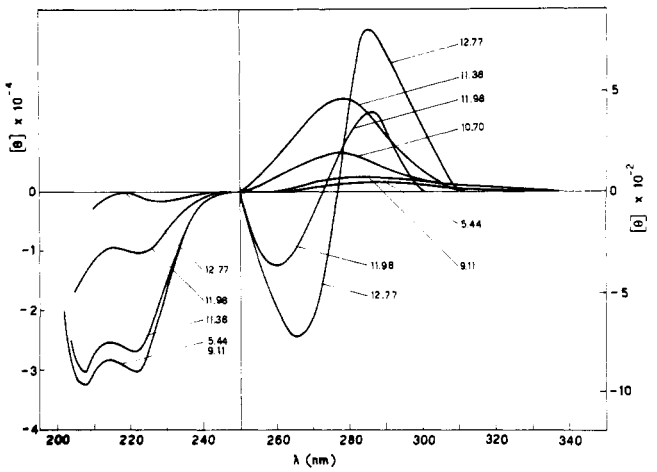


Figure 7. CD spectra of PALO in aqueous solution at various pH values.

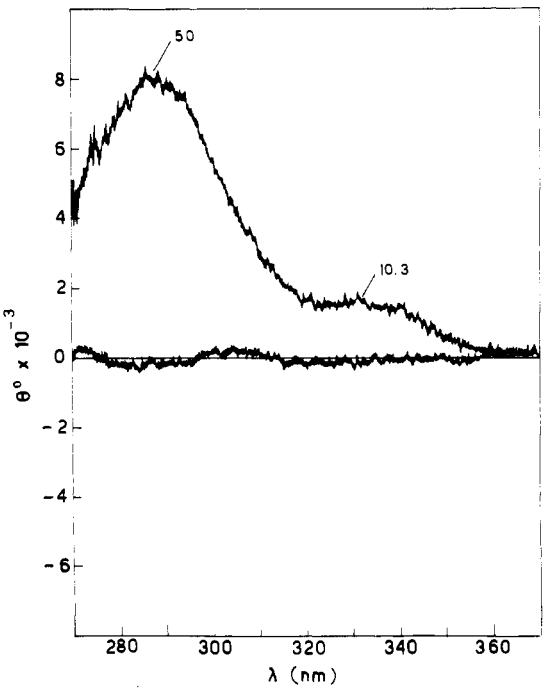


Figure 9. Original CD pattern of PALO in aqueous solution at pH 5.5 in the absorption region of the side-chain chromophore. The polymer concentration was 1.644×10^{-2} molar residue in a 1-cm path-length cell. The ordinate scale corresponds to the 0.02 sensitivity of the Cary 60 instrument. The molar ellipticity values at the wavelengths of maximum dichroic absorption are indicated in the figure.

ond explanation was also suggested by Longworth[25] in the case of poly(L-tyrosine).

Finally we have to comment on the blue shift of the 285-nm band at strongly alkaline pH. Under these conditions the α-helical form of the three polymers collapses because of the ionization of the side chain (see the next section). The uv absorption pattern of the coiled polymers becomes very similar to that of the model compound at the same pH conditions.

CD Measurements. The results of CD measurements on PALL, PALO, and PADB at different pH values are shown in Figures 5-13. Since pH changes affect the CD patterns of the three polymers in different ways, for sake of clarity it is worthwhile to discuss separately the CD results relative to each polymer.

In the pH range 3.50-11.25, PALL is completely in the right-handed α-helical form (Figure 5). In fact, the intensities of the negative CD bands at 222 and 208 nm are in quantitative agreement with the values reported for the helical conformation.[1b]

Above pH 11.25, PALL undergoes a cooperative helix-coil transition (Figure 6) induced by ionization of the side-chain groups. In the absorption region of the acetoacetyl chromophores there is a very small optical activity which is also pH dependent. At pH 3.5 the polymer exhibits a broad, weak *negative* CD band at *ca.* 280 nm (Figure 5). The intensity of this band increases continuously with the pH until pH 9.36, this behavior being parallel to that of the corresponding uv absorption band. The band can be therefore assigned to the ionized form of the acetoacetyl groups whose concentration increases with the pH. At pH >11.25, where the conformational transition occurs, the band splits sharply into two bands opposite in sign (Figure 5). Even if we cannot offer a detailed explanation for this splitting, the variation of the CD properties at 280 nm as a function of pH *indicates that the conformational*

(25) J. W. Longworth and R. O. Rahn, *Biochim. Biophys. Acta*, **147**, 526 (1967).

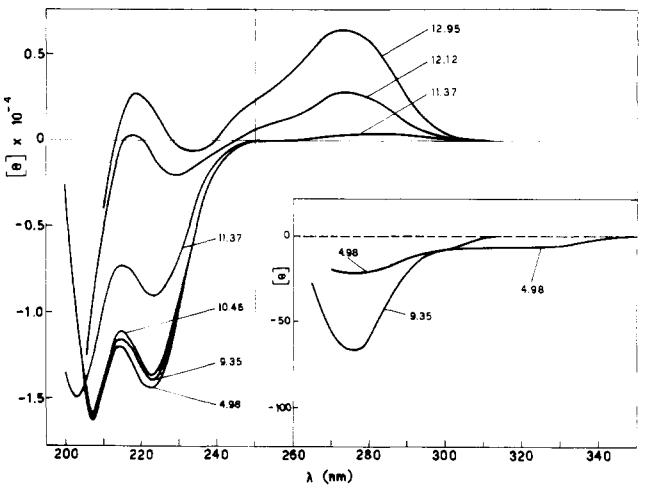


Figure 10. CD spectra of PADB in aqueous solution at various pH values.

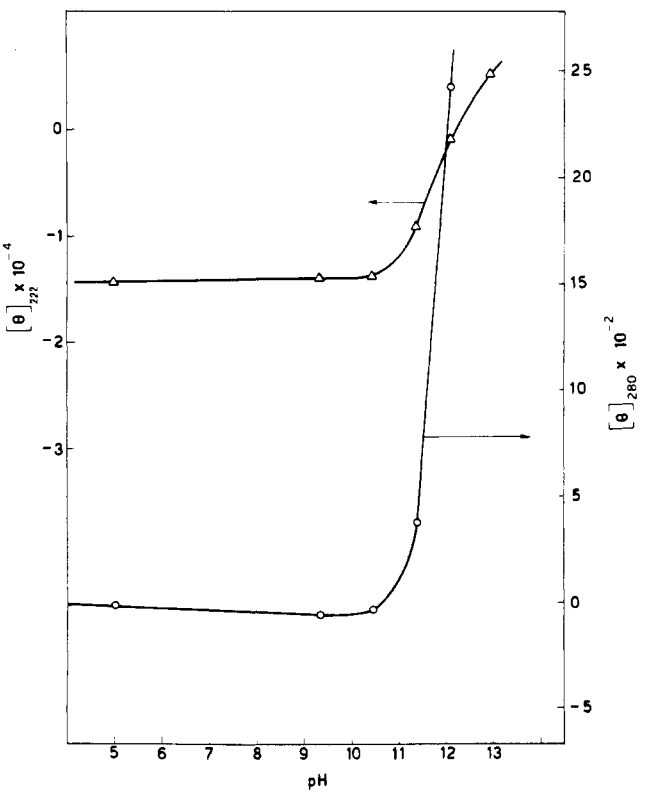


Figure 11. pH dependence of the molar ellipticity values of PADB at 280 and 222 nm.

transition of the peptide bond does have an effect on the optical activity of the side chains. This is clearly evident from Figure 6, where the pH profile of the 280-nm band is compared to that of the 222-nm CD band.

In the 330-nm region no optical activity has been detected, at least in the limits of the instrument sensitivity. The uv band attributed to interactions among side chains is therefore not optically active in the polymer.

From all these data it appears that in PALL, contributions to the optical activity in the peptide absorption region are absent and that the presence of the helical conformation does affect in some way the optical activity of the side chains.

Let us now consider the CD results on PALO, which are shown in Figures 7–9. Again, the polymer is completely in the α-helical conformation until pH 11.38 (Figure 7). On further increasing the pH, a cooperative helix–coil transition takes place (Figure 8). Also in this case it appears that the side-chain chromophores do not give an appreciable contribution to the optical activity in the peptide absorption region, since the CD pattern is almost quantitatively that of an α-helix below pH 11.38. It is interesting to observe the effect of pH changes on the CD pattern in the absorption region of the side-chain groups. At pH 4.98 PALO exhibits two clear positive CD bands at 330 and 286 nm, whose molar ellipticity values are 10.3 and 50, respectively (Figure 9). Within the pH range in which the α-helical conformation is stable, the pH dependence of these bands is closely parallel to that of the corresponding uv absorption bands; in fact, the 286-nm band increases with pH and shifts toward the blue, while the 330-nm band disappears. At pH higher than 11.38, when the α-helix collapses, the same behavior as that of PALL is observed: the band around 280 nm splits into two bands opposite in sign (Figure 7). In this case too, the conformational transition of the peptide backbone does have an effect on the optical activity of the side chains.

The results of CD measurements on PADB are shown in Figures 10–12. In the range of pH between 4.98 and 10.46, the CD pattern is practically constant and its shape is typical of the right-handed α helix. However, the intensities of the two characteristic negative CD bands are about one-half of the expected values. This could be due either to a lower helix content of the polymer or to a perturbing effect of the side chains in a fully helical polymer. The first hypothesis implies that the helical structure of PADB is substantially less stable than that of PALL and PALO under identical conditions. In this case one should expect that the conformational transition of PADB should occur at pH values much lower than those of PALL and PALO. This does not seem to be case here (Figure 13), the half-transition pH of PADB being only slightly lower than that of PALO and PALL. Furthermore, the transition appears to be quite sharp, in contrast to that of poly(L-diaminobutyric acid), which in aqueous solution and in the uncharged state has a very little helical content.[1,26]

Mainly on these bases we lean toward the hypothesis that PADB is completely in the α-helical form in the pH range between 4.92 and 10.46, the distortion of the CD pattern being due to a perturbing effect of the side chains. At pH higher than 10.46 the helix content drastically decreases and becomes practically zero at pH >12.5 (Figure 11). Again the CD properties of PADB in the absorption region of the acetoacetyl groups before and after the conformational transition deserve comment. At pH 4.98, there are two negative CD bands around 320 and 280 nm (Figures 10 and 12). On increasing the pH from 4.92, the 280-nm band is enhanced while the 320-nm band disappears well before the transition; this behavior is consistent with that of the corresponding uv absorption bands. At pH >10.46, where the conformational transition occurs, the 280 nm band becomes positive and then increases with the pH. In this case, too, we can therefore conclude that the optical activity of the side chains is substantially affected by the conformational transition of the side chains.

Conclusions

The results presented in this paper give evidence for effects of the peptide conformation on the optical activity of the side chains. In fact, in the pH range where the conformational transition occurs, all the polymers examined ex-

(26) M. J. Grourke and J. H. Gibbs, *Biopolymers*, **10**, 795 (1971).

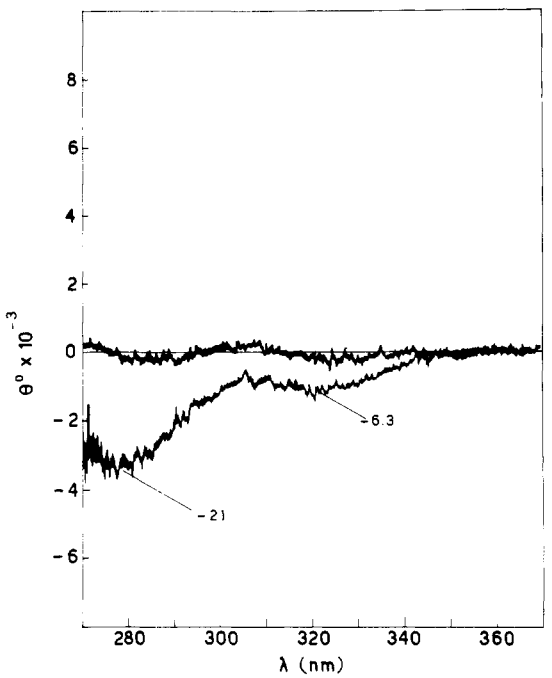


Figure 12. Original CD pattern of PADB in aqueous solution at pH 5.5 in the absorption region of the side-chain chromophore. The polymer concentration was 1.435 × 10^{-2} in a 1-cm path-length cell. The ordinate scale corresponds to the 0.02 sensitivity of the Cary 60 instrument. The molar ellipticity values at the wavelengths of maximum dichroic absorption are indicated in the figure.

hibit a discontinuous variation of the CD properties at 280 nm. As shown by uv absorption spectra, interactions among side-chain chromophores in helical polymers cause the appearance of a new band centered at about 330 nm. Optical activity in this spectral region has been found only for PALO and PADB; this optical activity disappears at a pH much lower than that of the conformational transition. This result supports the hypothesis, based on the absence of an isosbestic point in the uv absorption spectra at various pH values, that the ordered arrangement of the side chains is perturbed before the conformational transition, when the degree of ionization is still very small.

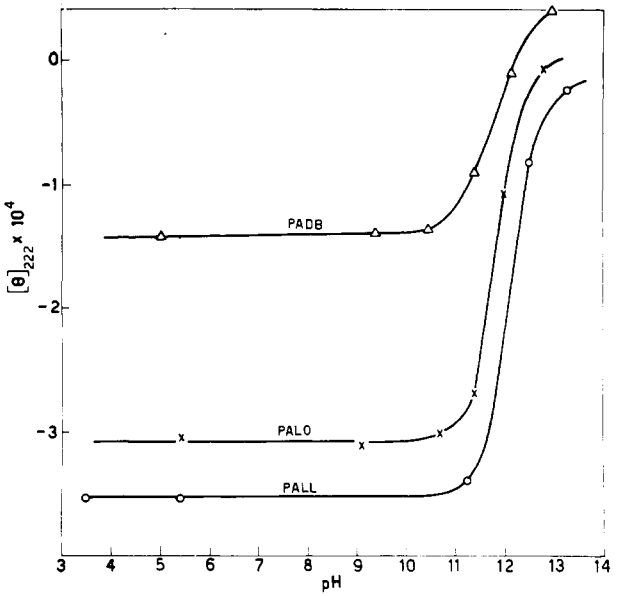


Figure 13. Comparison of the pH dependence of the 222-nm CD band for PALL, PALO, and PADB.

Concerning the perturbation of the CD pattern of the α helix by the acetoacetyl groups, substantial effects seem to be present only in the case of PADB, and this is probably due to the proximity of the side chain to the peptide backbone. The half-transition pH values (Figure 13) indicate that there are small differences in the helix stability of the three polymers toward pH changes.

The order of conformational stability is the same as that of the corresponding deblocked polymers,[1,26] namely, PALL > PALO > > PADB. The higher conformational stability toward ionization of PALL can be explained by assuming that the destabilizing repulsion among negatively charged side chains is partly balanced by favorable hydrophobic forces among the hydrocarbon portions of the side chains. This explanation obviously implies that the acidity constants of the side chains in the three polymers are nearly the same so that the degrees of ionization of the side chains are approximately identical under identical conditions of pH and concentration.