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Protonation of Polypeptides in "Helix-Breaking" Solvents: Spectral and Optical-Rotatory Properties in Solutions Containing Strong Organic Acids*

Sue Hanlon† and Irving M. Klotz

ABSTRACT: Poly-γ-benzyl-L-glutamate, poly-L-alanine, and poly-L-leucine have been examined spectroscopically in the near infrared in mixed solvents containing strong organic acids. The optical-rotatory-dispersion properties of the alanine and leucine polymers have also been determined in the same solvents. The results indicate that a sizable fraction of the peptide groups of these polyamino acids are protonated by these solvents.

The transition in b_0, commonly interpreted as a helix-coil transition, cannot in these particular cases involve the breakage of intramolecular peptide hydrogen bonds. Rather, the parallel changes observed in the spectral properties support the conclusion that the optical-rotatory transitions reflect the transformation of the protonated polypeptide chain to a form which is partially hydrogen bonded to the organic acid of the solvent.

A knowledge of the conformation of polyamino acids in solution under a variety of conditions is important to the understanding of the forces responsible for the maintenance of protein structure in solution. Yet this knowledge is not easily obtained. Many techniques that might be applied to the problem are limited either by their lack of applicability in a multicomponent system or by the absence of a sound theoretical basis. Thus the interpretation of data from these techniques is a rather hazardous venture, particularly in those cases where a given transformation is effected by the addition of an appreciable quantity of a third strongly interacting component.

Since a direct determination of conformational change in such a system is so difficult, a more fruitful approach to the problem would seem to be a direct assessment of the chemical state of the peptide groups under conditions where other data have been interpreted in terms of a helix-coil transition. Presumably this transition should be reflected in a transformation of the hydrogen-bonded peptide groups to solvent-interacting peptide groups.

On the basis of results for *N*-methylacetamide in a variety of solvents (Klotz and Franzen, 1962; Hanlon *et al.*, 1963; Klotz *et al.*, 1964), it was felt that an identification of the various interactions of the peptide

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N-H groups could be made from their spectra in the overtone region of the N-H stretching vibration. In view of the finding that *N*-methylacetamide is protonated by trifluoroacetic acid (Hanlon *et al.*, 1963; Klotz *et al.*, 1964), we were particularly interested in those systems consisting of the synthetic polyamino acids in mixed solvents containing strong organic acids such as dichloroacetic acid or trifluoroacetic acid.

To this end, the synthetic polyamino acids, poly-γ-benzyl-L-glutamate, poly-L-alanine, and poly-L-leucine, were examined spectroscopically in both "helix-forming" and "helix-breaking" solvents. Poly-γ-benzyl-L-glutamate was employed in the initial studies since this polymer has been most extensively characterized in solution (Doty *et al.*, 1954; Moffitt and Yang, 1956; Doty *et al.*, 1956; Doty and Yang, 1956; Yang and Doty, 1957). Poly-L-alanine and poly-L-leucine were selected for the more detailed studies as these polymers present the least number of complications with respect to either intermolecular or solvent interactions with functional groups on side chains. A preliminary report of this work has appeared elsewhere (Hanlon *et al.*, 1963; Hanlon and Klotz, 1964).

Materials and Methods

The synthetic polyamino acids employed in these studies were obtained from the Pilot Chemical Co. The molecular weights of the poly-γ-benzyl-L-glutamate were given by the manufacturer as 255,000 and 323,000. The molecular weights of the poly-L-alanine and poly-L-leucine samples were roughly estimated by comparing their intrinsic viscosities in trifluoroacetic acid to those of samples of poly-γ-benzyl-L-glutamate of known molecular weight in dichloroacetic acid (Doty *et al.*, 1956). For poly-L-leucine, this procedure yielded values of 100,000 and 70,000 for two samples employed in these experiments. The molecular weights of poly-L-alanine samples were 30,000 and 45,000. All polymer samples were dried to constant weight at 70° and stored in a desiccator over anhydrous $CaSO_4$ until used.

With the exception of pyridine (Fisher Spectranalyzed), all solvents and low molecular weight solutes were purified prior to use. Trifluoroacetic acid (Eastman Kodak) and CCl_4 (Fisher Spectranalyzed Reagent) were distilled over P_2O_5. Chloroform, an Allied Chemicals Reagent Grade product, was rendered free of ethanol and water by extraction with sulfuric acid and water, desiccation over $Mg(ClO_4)_2$, and distillation. Dichloroacetic acid, ethylene dichloride, *N*-methylacetamide, and *N,N*-dimethylacetamide were simply distilled without additional treatment. *N*-Methylacetamide and *N,N*-dimethylacetamide were reagent grade materials obtained from Eastman Kodak Co.

Solutions of poly-γ-benzyl-L-glutamate were prepared by weight. Concentrations at 25° were calculated on the basis of the partial specific volume data of Luzzati *et al.* (1961) and the density of the solvent at 25°. Spectra of the polymer in pyridine and in chloroform were obtained over a concentration range of 0.045 residue M (moles amino acid residue/liter) to 0.23 residue M. This corresponds to a weight concentration of 1–5% (g/dl). The solutions containing dichloroacetic acid were prepared at polymer concentrations of 0.33–0.70 residue M (7.3–15%).

Solutions of poly-L-alanine and poly-L-leucine were also prepared by weight but concentrations were calculated more accurately by means of an experimentally measured density (see below). Most spectra and all optical rotatory dispersions were obtained at a polymer concentration of 0.33 residue M, which corresponds to a weight concentration of 3.7% poly-L-leucine and 2.4% poly-L-alanine. Effects due to concentration were ascertained by running a number of preliminary spectra at concentrations between 0.15 and 0.90 residue M.

Solutions consisting of 0.044 residue M poly-L-alanine (0.3%) in 0.25% CF_3COOH–99.75% $CHCl_3$ and in 0.50% CF_3COOH–99.5% $CHCl_3$ were also prepared for spectral studies.

All operations involving the preparation and transfer of solutions of poly-L-alanine and poly-L-leucine were conducted in a drybox. In most instances these solutions were filtered through a plug of glass wool (to remove lint and a small amount of undissolved polymer) and examined within 30–40 minutes after the first addition of solvent. The effects of time were ascertained by reexamination after 5–32 hours.

The weight concentrations of these solutions were redetermined after use by weighing the residue remaining after evaporation of a known weight of solution to dryness at 110°. The difference between the concentration based on the quantity of polymer and solvent initially weighed out and that determined by evaporating to dryness was never greater than ±3%.

Spectra of poly-γ-benzyl-L-glutamate in $CHCl_2COOH$ and in the mixed solvent CH_2ClCH_2Cl-$CHCl_2COOH$ were run in the near infrared in a Beckman DK-2 spectrophotometer at 25°. All other spectra of the polyamino acid solutions were obtained at 25 ± 0.2° by means of a Cary spectrophotometer Model 14 CMR equipped with thermostated cell adapters and a 0–0.1 OD slide wire. Unless otherwise indicated, solvent absorption in this spectral region was compensated by a reference solution with CCl_4 at the same volume fraction as the polymer in the sample solution (Klotz and Franzen, 1962). These reference solutions were handled in a manner identical with that of their matching sample solutions containing the polymers.

For the spectra obtained in the near infrared with the Cary Model 14 CMR, the optical system selected was that in which light from a tungsten source is first sent through a monochromator before passing through the sample and reference compartments. The stray light level of this path coupled with the absorption properties of the organic acids did not permit solution path lengths greater than 2 cm to be used at the higher concentrations of trifluoroacetic or dichloroacetic acid. For the detailed series of spectra of poly-L-alanine and poly-L-leucine, quartz cells with a 1.00-cm light path were employed. For the more transparent solvents, pyridine and chloroform, path lengths up to 10.000 cm were used. In cases where light scattering was

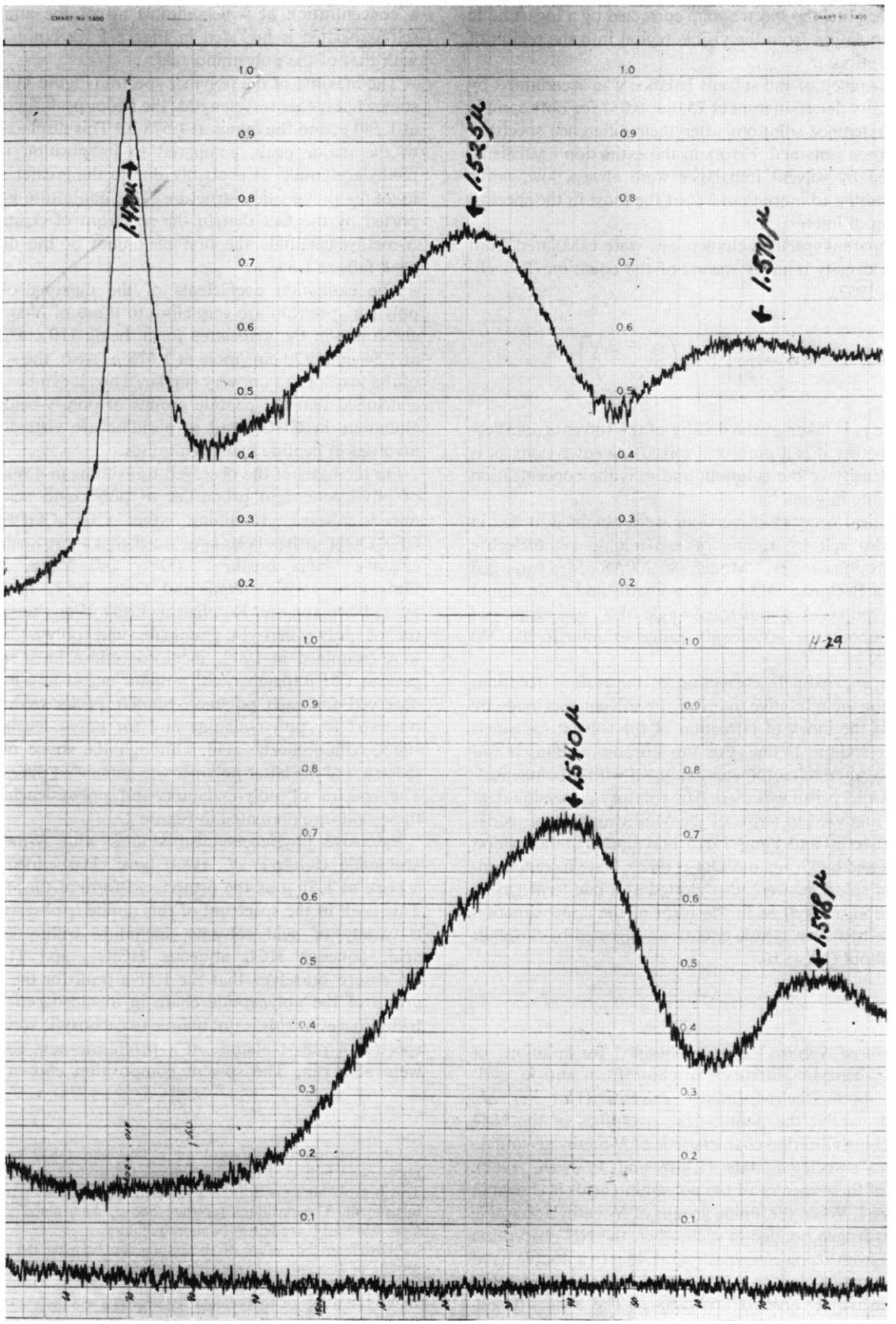


FIGURE 1: Spectra of amides in a self-associated state. Upper, *N*-methyl acetamide (3.0 M) in CCl_4 versus CCl_4; lower, poly-γ-benzyl-L-glutamate (0.053 residue M) in pyridine versus CCl_4 in pyridine. Ordinates represent actual absorbance × 10; abscissa wavelengths are given in microns (μ).

appreciable, the spectra were corrected by a logarithmic extrapolation from the visible region into the region of absorption.

Adequacy of the solvent balance was ascertained by a density determination at 25.0 ± 0.05° for both sample and reference solutions after their difference spectrum had been obtained. Errors in the extinction coefficients caused by solvent imbalance were always low, never amounting to more than 3% at the most in the spectral region of interest.

Apparent specific volumes, φv_2, were calculated from these density data by means of the equation (Schachman, 1957)

$$\varphi v_2 = \frac{1}{\rho_0}\left[1 + \left(\frac{\rho_0 - \rho_1}{c_2}\right)\right]$$

where ρ_0 is taken as the density of the solvent regardless of whether it is a pure or a mixed solvent system, ρ_1 is the density of the solution, and c_2 is the concentration of solute in g/ml.

Optical rotatory dispersions were obtained at 25° in a 1-dm cell by means of a Rudolph photoelectric spectropolarimeter, Model 80/200AS/655, equipped with a Beckman DU monochromator and a zirconium light source. A symmetrical angle of 2° was employed throughout the wavelength range examined, 380–700 mμ.

An approximate correction of the residue rotation, [m], for the refractive index of the solvent was made by use of the index of refraction of the solvent measured at the sodium D line. For this purpose, a Bausch and Lomb Abbé refractometer equipped with a compensating Amici prism was used. The rotatory dispersion data were analyzed in terms of the Moffitt and Yang equation (Moffitt and Yang, 1956), and plotted in the fashion suggested by Urnes and Doty (1961). Data from a number of solutions were also plotted as a one-term Drude equation, as well as in the form of the approximation of the two-term Drude equation suggested by Shechter and Blout (1964a,b).

Results

Infrared Spectra. Spectra obtained for solutions of poly-γ-benzyl-L-glutamate in chloroform and in pyridine, both "helix-forming" solvents (Doty *et al.*, 1956), exhibit the double-peak overtones of the N-H stretching vibration characteristic of *N*-methylacetamide in the associated state (Klotz and Franzen, 1962). One of these spectra of the polyamino acids is shown in Figure 1. When the amide groups of *N*-methylacetamide are hydrogen bonded to each other, the NH absorption shifts from a single peak at 1.47–1.48 μ to a double band with a major peak at 1.525 μ and a minor one centered approximately around 1.57–1.58 μ. The extinction coefficients (based on concentration in moles/cc) at these wavelengths are 116 and 84 cm²/mole, respectively, when all of the amide groups are hydrogen bonded. A typical spectrum of *N*-methylacetamide in CCl_4 at a concentration at which almost all of the amide is self-associated is included in Figure 1 for comparison with that of the polyamino acid.

The maxima of the polymer spectra (Figure 1) fall at somewhat higher wavelengths, the major peak occurring at 1.540 μ, and the minor at 1.578 μ.[1] This displacement of the major peak, compared to its position in *N*-methylacetamide, is probably due to the greater rigidity of the polypeptide structure. This conjecture is supported by the fact that in the spectrum of crystalline *N*-methylacetamide the first maximum of the double peak falls at 1.533 μ.

The extinction coefficients of the maxima of the polymer spectrum are very close to those of *N*-methylacetamide in the associated form, being 110 cm²/mole at 1.54 μ and 70 cm²/mole at 1.578 μ. Also, the shapes of the two spectra are very similar. Thus it can be safely concluded that the peptide groups of poly-γ-benzyl-L-glutamate in $CHCl_3$ and in pyridine are virtually all involved in peptide hydrogen bonds.

On the basis of the observed transitions in a number of physicochemical properties of polyamino acids in mixed solvents containing either $CHCl_2COOH$ or CF_3COOH, it has been concluded that these acids are effective "helix breakers" (Doty and Yang, 1956; Doty *et al.*, 1956; Yang and Doty, 1957; Fasman, 1962; Perlmann and Katchalski, 1962). When the spectra of poly-γ-benzyl-L-glutamate and poly-L-alanine were examined in 100% dichloroacetic acid, a major peak at 1.51 μ together with a minor one at 1.56 μ were observed for each polymer. Similar peaks were also observed for poly-L-alanine and for poly-L-leucine in 100% trifluoroacetic acid, although the shape of the spectrum of the latter polymer was somewhat different.[2] The spectra of poly-L-alanine and poly-L-leucine in these acids are presented in Figure 2.

Extensive physicochemical studies with *N*-methylacetamide (Klotz *et al.*, 1964) have clearly identified a peak at 1.51 μ as the protonated form of the amide. This peak in the spectrum of this amide is observed in a variety of acid solvents, including trifluoroacetic acid, aqueous HCl, aqueous $HClO_4$, and H_2SO_4. To assure ourselves that the 1.51-μ peak for the N-H group of the polypeptide chain in dichloroacetic and trifluoroacetic acids is not unique to carboxylic solvents, spectra of poly-L-alanine in a perchloric acid solvent were also run. The solvent composition chosen was

[1] Previous spectra obtained on a Beckman DK-2 spectrophotometer indicated that these peaks fell at 1.53 and 1.57 μ. This is probably caused by faulty wavelength calibration of that instrument. The positions reported above, determined with a Cary Model 14, are felt to be more reliable.

[2] Owing to the nature of the interaction between the amide groups of the polymers and the organic acids, the absorption properties of the solvent are changed appreciably at wavelengths above 1.55 μ. Since CCl_4 cannot compensate for these changes, the additional absorption of the solvent in this region is thus superimposed on the polymer spectrum. For this reason, the actual extinction coefficients of the peaks at 1.56 μ of the polyamino acids in the solvents containing organic acids are somewhat lower than the values reported in Figures 2, 5, and 6.

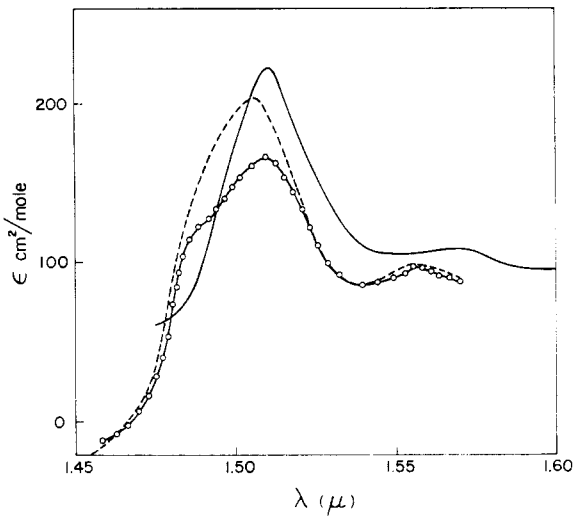


FIGURE 2: Spectra of polyamino acids in strong organic acids. ———, poly-L-alanine (0.155 residue M) in $CHCl_2COOH$ versus CCl_4 in $CHCl_2COOH$; – – – –, poly-L-alanine (0.33 residue M) in CF_3COOH versus CCl_4 in CF_3COOH; O–O–O–, poly-L-leucine (0.33 residue M) in CF_3COOH versus CCl_4 in CF_3COOH.

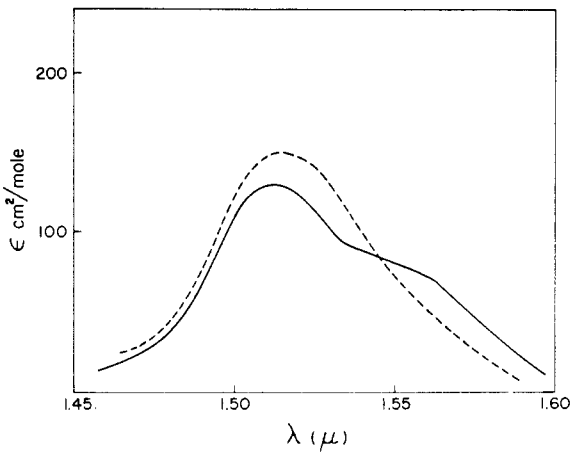
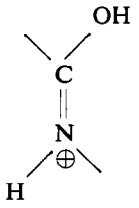


FIGURE 3: Spectra of amides in 8.4 M $HClO_4$, 2.5 M dioxane, 19.2 M H_2O. – – – –, *N*-methylacetamide (1.08 M); ———, poly-L-alanine (0.378 residue M).

the same as that known (from nuclear magnetic resonance studies, Berger *et al.*, 1959) to protonate *N*-methylacetamide.

The results of this experiment, shown in Figure 3, demonstrate that the essential feature of a protonated amide spectrum (the 1.51-μ absorption peak) is present in the polypeptide in different types of acidic solvents, just as it is in the simple model amide. It seems clear, therefore, that a protonated form



characterizes the state of the amide group of polypeptides dissolved in $CHCl_2COOH$ and CF_3COOH solutions.

There are, nevertheless, minor differences between the two spectra. The shoulder observed near 1.525 μ in the spectrum of *N*-methylacetamide in strong oxyacids (Hanlon *et al.*, 1963) is not found in the poly-L-alanine spectrum. The absorption in this region in the *N*-methylacetamide spectrum may be attributed (Klotz *et al.*, 1964) to additional hydrogen bonding of the NH group in the triplet and higher ion aggregates of this amide in CF_3COOH solutions. Hence its absence in the poly-L-alanine spectrum is not surprising. The polymer spectrum, on the other hand, exhibits an additional small absorption, evident as a break in the curve at 1.56 μ. The nature of the group giving rise to this peak is not known. It may represent a small amount of additional or alternate protonation on the peptide nitrogen. A basis of such an assignment is the fact that the N-H stretching overtone of ammonium groups falls in this region, as has been found by Mr. H. DePhillips in this laboratory.

Under conditions which presumably bring about a helix-coil transition, the spectra of all three polyamino acids gave no evidence of a transition involving a peptide hydrogen-bonded structure. Instead, the 1.51-μ peak was a persistent feature under all conditions. For instance, poly-γ-benzyl-L-glutamate in the mixed solvent, 80% (v/v) dichloroacetic acid–20% ethylene dichloride, has been reported to undergo an inverted transition in its optical-rotatory properties (Doty and Yang, 1956) over the temperature range 10–40°. A spectrum of a solution of the polymer in this solvent, however, revealed no marked changes in the 1.51-μ absorption band at 11°, 21°, and 40°.

Similarly, transitions in the b_0 value for both poly-L-alanine and poly-L-leucine in the mixed solvent, $CHCl_3$-CF_3COOH, have been reported by Fasman (1962). The spectra of our samples of these polymers in the same solvent system exhibited a 1.51-μ peak at all concentrations of CF_3COOH. In fact, this peak could even be demonstrated for solutions of poly-L-alanine at acid concentrations as low as 0.25–0.5%. A spectrum of one of these latter solutions is shown in Figure 4. At this low acid concentration, however, there was also an appreciable amount of peptide hydrogen-bond formation, as evidenced by a pronounced 1.53- to 1.54-μ shoulder and a peak at 1.58 μ.

As Figures 5 and 6 show, there were changes in the spectra of poly-L-alanine and poly-L-leucine over the range where optical-rotatory-dispersion data previously reported (Fasman, 1962) showed transitions, but these changes did not involve the appearance or disappearance of the characteristic double absorption peak at 1.54–1.58 μ associated with peptide hydrogen bonds.

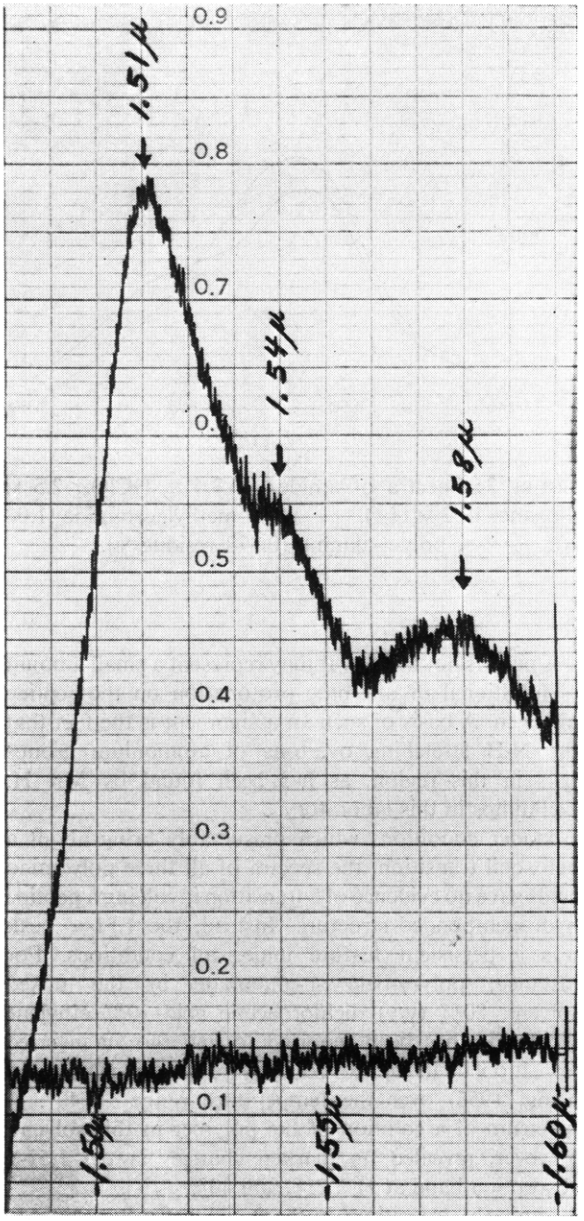


FIGURE 4: Spectrum of poly-L-alanine (0.0442 residue M) in 0.50% of CF_3COOH, 99.5% $CHCl_3$ versus 0.50% of CF_3COOH, 99.5% $CHCl_3$. The ordinate values represent the actual absorbance × 10.

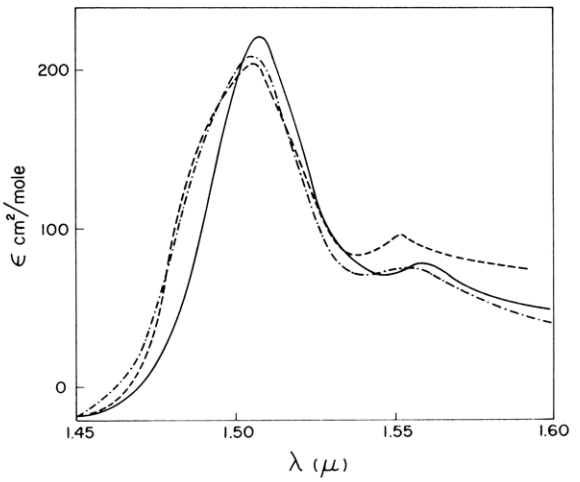
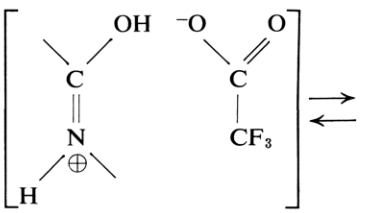
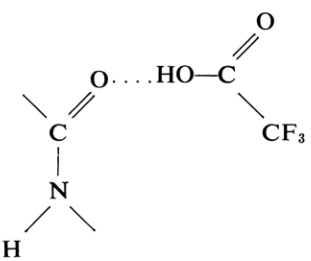


FIGURE 5: Spectra of poly-L-alanine (0.33 residue M) in $CHCl_3$-CF_3COOH. ———, 34–70% CF_3COOH; –·–·–·–, 90% CF_3COOH; – – – –, 100% CF_3COOH.

The most important of these changes in the spectra of the polyamino acids in the CF_3COOH-$CHCl_3$ solvent is the change in the absorption in the 1.48- to 1.49-μ region. In the case of poly-L-alanine, there is a growth of a slight shoulder in this region coupled with a decrease in the absorption at 1.51 μ as the acid concentration increases from 70 to 100% trifluoroacetic acid. For poly-L-leucine these changes are quite pronounced. As Figure 7 shows, they can be correlated with changes in the peak at 1.51 μ. As the concentration of CF_3COOH increases from 30% upward, there is an increase in the absorption at 1.49 μ paralleled by a corresponding decline at 1.51 μ. This trend continues until a concentration of 60% CF_3COOH is reached, at which point the process is reversed. The spectral changes in the extinction coefficients at 1.51 μ are most marked in going from 50 to 60% CF_3COOH.

From the spectrum of *N*-methylacetamide in acetic acid (Klotz *et al.*, 1964), an absorption peak at 1.485 μ has been assigned to the amide that is hydrogen bonded to the acid. Hence the spectral changes observed for the polymer solutions may be interpreted as an interconversion of the solvent-protonated species to the solvent-hydrogen-bonded species, i.e.:





An additional small peak in the 1.56-μ region is present in the spectra of both poly-L-alanine and poly-L-leucine. This is similar to the small peak observed in the spectrum of poly-L-alanine in perchloric acid and is also probably due to additional or alternate protonation on the nitrogen.

TABLE I: Apparent Specific Volumes of Poly-L-alanine and Poly-L-leucine in $CHCl_3$-CF_3COOH.

Poly-L-alanine (0.33 mole amino acid residue/liter)						
CF_3COOH (%, v/v)	33.9	49.7	70.1	90.0	100	
φv (ml/g)	0.668	0.667	0.649	0.635	0.659	
Poly-L-leucine (0.33 mole amino acid residue/liter)						
CF_3COOH (%, v/v)	30.4	50.2	61.9	69.8	90.2	100
φv_2 (ml/g)	0.918	0.910	0.890	0.909	0.899	0.929

Polymer Residue	φv_2 Amino Acid Residue (ml/g) in H_2O
Alanine	0.74[a]
Leucine	0.90[a]

[a] Taken from Cohn and Edsall (1943), p. 372.

These spectral changes were found to be independent of time, and of concentration over a range of 0.15–0.33 residue M. There were no appreciable differences between the corrected spectra of different lots of the same polyamino acid, despite the estimated differences in molecular weight.

Unfortunately, parallel spectral studies on the model *N*-methylacetamide in the mixed solvents could not be conducted. Although *N*-methylacetamide is miscible in all proportions with both $CHCl_3$ and with CF_3COOH separately, *N*-methylacetamide in the presence of both solvents forms a two-phase system over the acid concentration range of interest. Presumably this phenomenon is brought about by the decreased activity of the ion pairs formed by the protonation reaction in the medium of low dielectric constant (Friedman, 1962).

Apparent Specific Volumes. The apparent specific volumes of the polyamino acids in the various mixed solvents are given in Table I. The precision is estimated to be about ±0.015 ml/g, and hence the observed variations with concentration of CF_3COOH are within experimental error. The apparent specific volumes of poly-L-alanine are considerably lower than those of the neutral amino acid residue in water (Cohn and Edsall, 1943). Since the apparent volume of a solute depends not only on solvent interaction but also on how well a given solvent molecule can fit into the available space about a solute or polymer chain, this value reported for water is not strictly a valid standard of comparison. Nevertheless, on steric grounds alone, one would expect the apparent volume of a solute to be larger in a solvent whose molecules are larger than those of water. The fact that the apparent volume of poly-L-alanine is actually smaller in CF_3COOH and in the CF_3COOH-$CHCl_3$ mixtures supports the contention that the peptide groups are protonated in these solvents. The apparent specific volumes are thus reduced by electrostriction.

The apparent specific volumes of poly-L-leucine in the mixed solvents containing CF_3COOH are actually slightly higher than that of the leucine amino acid residue in water, and hence the density data alone

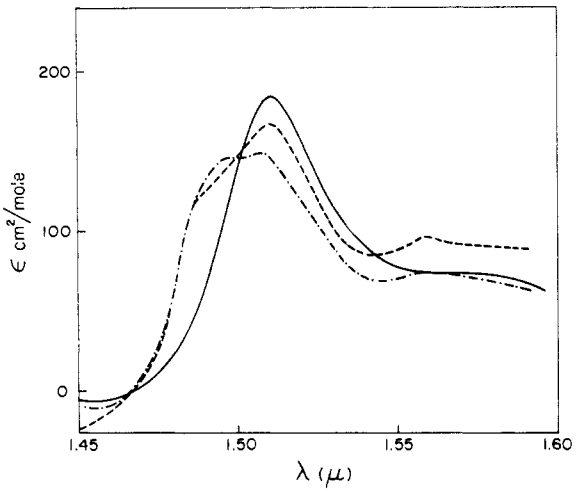


FIGURE 6: Spectra of poly-L-leucine (0.33 residue M) in $CHCl_3$-CF_3COOH. ———, 30% CF_3COOH; –·–·–·–, 62% CF_3COOH; – – – –, 100% CF_3COOH.

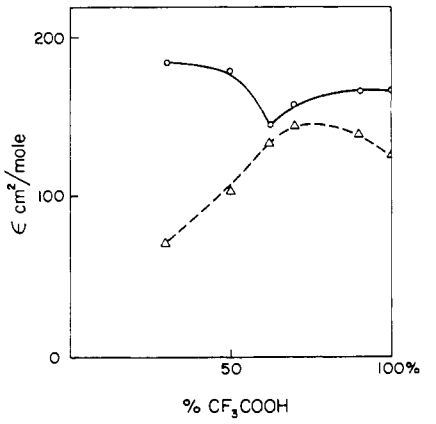


FIGURE 7: Variation in extinction coefficients, ε, of poly-L-leucine (0.33 residue M) in $CHCl_3$-CF_3COOH. ———, ε (cm²/residue mole) at 1.51 μ; – – – –, ε (cm²/residue mole) at 1.49 μ.

TABLE II: Optical Rotatory Dispersion Parameters of Poly-L-alanine in $CHCl_3$-CF_3COOH.

(A) Moffitt-Yang Analysis

Solvent CF_3COOH (%, v/v)	λ_0 = 212 mμ		λ_0 = 230 mμ
	b_0	a_0	b_0
34.0	−331	124	−219
70.0	−295	29	−181
90.0	−181	−377	− 71
100.0	−118	−616	0

(B) Shechter-Blout Analysis

Solvent CF_3COOH (%, v/v)	$A_{(\alpha,\rho)_{225}}$	$A_{(\alpha,\rho)_{193}}$	Per Cent Helix				
			Aqueous[a] Solutions		Organic[b] Solutions		Average[c]
			H_{225}	H_{193}	H_{225}	H_{193}	H
34.0	−961	1459	45	61	51	57	55
70.0	−871	1219	41	54	46	50	49
90.0	−774	599	36	37	41	33	33
100.0	−648	137	30	24	34	20	26

[a] Calculated from equations (10) and (11), Shechter and Blout (1964a). [b] Calculated from equations (4) and (5), Shechter and Blout (1964b). [c] Calculated from equation (8), Shechter and Blout (1964b).

neither support nor contradict the protonation hypothesis. In the light of the spectral results, nevertheless, it is felt that although there is electrostriction of the solvent about the peptide groups in these systems, owing to protonation, the contraction is counterbalanced by the poor packing of solvent molecules about the bulky leucyl side chains.

Optical Rotatory Dispersions. The results of the optical-rotatory-dispersion experiments with the same samples of poly-L-alanine and poly-L-leucine in the mixed solvent, $CHCl_3$-CF_3COOH, are presented in Figure 8 as a plot of $-b_0$ versus % CF_3COOH. These results are similar to, although not identical with, those of Fasman (1962). The numerical values for the two dispersion parameters, b_0 and a_0 (calcd for λ_0 = 212 mμ), are presented in Tables II and III.

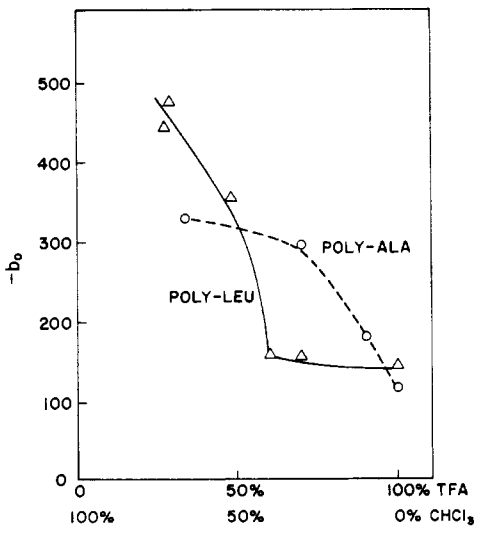


FIGURE 8: Variation in the optical-rotatory-dispersion parameter, b_0, of the polyamino acids in $CHCl_3$-CF_3COOH. – – – –, poly-L-alanine (0.33 residue M); ———, poly-L-leucine (0.33 residue M).

The scatter in the data points in the dispersion plots was too great to determine accurately whether or not 212 mμ was the value of λ_0, which alone would yield a linear plot of the data. In fact, if the data for poly-L-alanine and poly-L-leucine in 100% CF_3COOH are plotted as a one-term Drude equation, the plot appears to be perfectly linear and a value of λ_c = 230 mμ is obtained. If this value of λ_0 is then employed in the calculations of b_0 for both polymers, the transitions are still apparent, although the magnitude of b_0 is reduced. At the lower concentrations of CF_3COOH (30–60% in the case of poly-L-leucine and 34–70% for poly-L-alanine) two terms were required, however, for a linear plot of the rotatory dispersion data; curvature could be observed when the data for both poly-L-alanine and poly-L-leucine were plotted in the form of a one-term Drude equation.

These points are illustrated in Figure 9, which presents the optical-rotation data for poly-L-leucine in 29.5% CF_3COOH–70.5% $CHCl_3$ plotted as a Moffitt equation for λ_0 = 212 mμ and λ_0 = 230 mμ as well as a one-term Drude equation. In Tables II and III, the numerical values of the b_0 values calculated for λ_0 = 230 mμ are also given.

Time seemed to have no significant effect on the values of b_0 and a_0 up to 14–24 hours at the lower CF_3COOH concentrations. In 100% CF_3COOH,

TABLE III: Optical Rotatory Dispersion Parameters of Poly-L-leucine in $CHCl_3$-CF_3COOH.

(A) Moffitt-Yang Analysis

Solvent CF_3COOH (%, v/v)	λ_0 = 212 mμ		λ_0 = 230 mμ
	b_0	a_0	b_0
27.5[a]	−445	− 45.7	−266
29.5	−476	− 56.6	−274
48.5	−355	−270	−190
60.0	−156	−585	− 38.6
69.9	−156	−635	− 20.4
100.0	−145	−700	0

(B) Shechter-Blout Analysis

Solvent CF_3COOH (%, v/v)	$A_{(\alpha,\rho)_{225}}$	$A_{(\alpha,\rho)_{193}}$	Per Cent Helix				
			Aqueous[b] Solutions		Organic[c] Solutions		Average[d]
			H_{225}	H_{193}	H_{225}	H_{193}	H
27.50[a]	−1418	1876	68	72	75	68	71
29.53	−1495	1966	72	74	79	71	74
48.49	−1220	1335	58	57	64	54	57
60.00	− 831	432	39	32	44	29	34
69.90	− 796	323	37	29	42	26	32
100.0	− 802	248	37	27	42	23	31

[a] Solution containing 1.1% ethanol; dispersion measured at 22°. [b] Calculated from equations (10) and (11), Shechter and Blout (1964a). [c] Calculated from equations (4) and (5), Shechter and Blout (1964b). [d] Calculated from equation (8), Shechter and Blout (1964b).

however, the a_0 changed significantly over a 5-hour period. Whether this change was due to a degradation or a disaggregation of the polyamino acid chains is not known at present.

The results of the analysis of the dispersion data in the manner suggested by Shechter and Blout (1964a,b) are also presented in Tables II and III. Within experimental error, the plots of $[m'][(\lambda^2 - \lambda^2_{193})/\lambda^2_{193}]$ against $[\lambda^2_{225}/(\lambda^2 - \lambda^2_{225})]$ were linear and the scatter was no greater than that exhibited by the data for the same solution plotted in the form of the Moffitt-Yang equation. Furthermore, a plot of the values of $A_{(\alpha,\rho)_{225}}$ against $A_{(\alpha,\rho)_{193}}$ yielded a straight line for each polymer. In the case of poly-L-alanine, this linear relationship is given by

$$A_{(\alpha,\rho)_{225}} = -0.22A_{(\alpha,\rho)_{193}} - 624$$

For poly-L-leucine, the equation is

$$A_{(\alpha,\rho)_{225}} = -0.41A_{(\alpha,\rho)_{193}} - 674$$

The slopes and intercepts of these relationships differ from the results of Shechter and Blout (1964a,b) for polyamino acids in water and in organic solvents. These lines, however, do intersect those obtained by Shechter and Blout (1964a,b) in such a fashion that, if their appropriate empirical equation is applied, the estimates of per cent helicity from the value of $A_{(\alpha,\rho)_{225}}$ and from $A_{(\alpha,\rho)_{193}}$ are very close for a number of solutions. This is demonstrated by the data given in columns 4, 5, 6, and 7 of Table IIB, and in columns 4 and 5 of Table IIIB. Such agreement would normally be interpreted to mean that these particular solutions consisted of mixtures of α helices and random coils.

A "% helix" (H) was also calculated from the equation

$$H = \frac{A_{(\alpha,\rho)_{193}} - A_{(\alpha,\rho)_{225}} + 650}{55.8}$$

(equation 8, Shechter and Blout, 1964b). When these values (given in the last columns of Tables IIB and IIIB) were plotted against solvent concentration, the curves obtained were found to be identical in shape to those derived from the Moffitt-Yang (1956) analysis (Figure 8).

Discussion

On the basis of the infrared data, it seems reasonable to conclude that a substantial proportion of the peptide groups of both poly-L-alanine and poly-L-leucine are protonated at all concentrations of trifluoroacetic acid. Even in the presence of only 0.25–0.50% CF_3COOH in $CHCl_3$, protonated peptide groups can be seen. This conclusion might perhaps seem surprising in view of the fact that the apparent pK of the peptide in a poly-

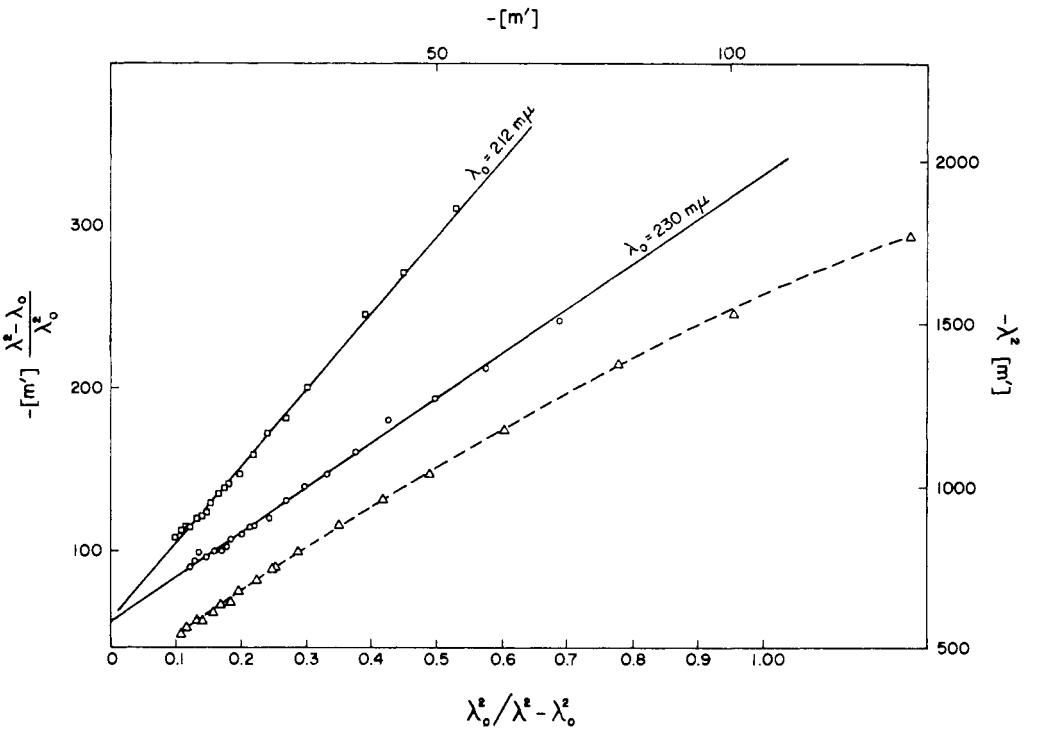
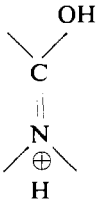


FIGURE 9: Optical-rotatory-dispersion data for poly-L-leucine (0.333 residue M) in 29.5% CF_3COOH, 70.5% $CHCl_3$. Plotted in the form: □—□, a Moffitt-Yang equation with λ_0 = 212 mμ, ○—○, a Moffitt-Yang equation with λ_0 = 230 mμ (left and lower coordinates); △---△, a one-term Drude equation (right and upper coordinates).

peptide chain has been estimated to be −3 to −4 (Goldfarb *et al.*, 1958). This value is appreciably lower than that of zero for the low molecular weight amides (Goldfarb *et al.*, 1955). It must be remembered, however, that these estimates were made for polypeptides and amides in water and presumably reflect the greater basicity of water as well as solvation and charge effects. When water is either present at a very low concentration or excluded altogether, it has been shown repeatedly that proteins, polypeptides, and polyamides are protonated in a variety of acid solvents (Greenberg and Larson, 1935; Schaefgen and Trivisonno, 1951, 1952; Steinberg *et al.*, 1960; Johansen and Reyerson, 1961).

The fact that a polyamino acid can be protonated at such extremely low concentrations of CF_3COOH can be explained on the basis of the structure of polymer solutions. As Flory (1945) has pointed out, polymer solutions even at fairly low concentrations are never really dilute in the classical chemical sense, but must necessarily consist of high local concentrations of monomer separated by larger solvent volumes. Thus the acid CF_3COOH always finds itself in the presence of a high amide concentration. As was shown in previous studies on *N*-methylacetamide and *N,N*-dimethylacetamide (Klotz *et al.*, 1964) this situation will result in an acid dissociation via protonation of the amide, even when the dielectric constant of the medium is low.[3]

These protonated peptide groups can hardly be involved in N—H ··· O=C< bonds since the carbonyl group is converted to



(Berger *et al.*, 1959) on protonation, as has been already mentioned. Furthermore, the 1.51-μ peak is the predominant absorption even when the concentration of CF_3COOH is only as high as 30% and hence a major fraction of peptide groups must be protonated. Electrostatic repulsions along the polypeptide chain would surely disrupt a compact hydrogen-bonded helix long before the concentrations are reached at which marked changes appear in optical-rotatory properties. In any event the changes in the near-infrared spectra of the peptide N-H groups in the range of concentrations

[3] Recently Watanabe *et al.* (1964) have found transitions in the dielectric constant and the Kerr effect at concentrations of $CHCl_2COOH$ (about 70%) in CH_2ClCH_2Cl which correspond to those producing sharp changes in b_0. It is pertinent to note that very much larger changes in the dielectric constant and the Kerr effect were observed when only very small amounts (0.1%) of $CHCl_2COOH$ were added to ethylene dichloride, which these authors agree must indicate protonation.

of CF_3COOH-$CHCl_3$ over which $-b_0$ drops do *not* correspond to a transformation involving breaking of N—H···O=C< bonds. Rather, the spectra indicate that structural changes in the polypeptide chain occur parallel with conversions of some protonated residues to a deprotonated form, hydrogen bonded to the solvent. A possible mechanism for such a conversion is illustrated schematically in Figure 10.

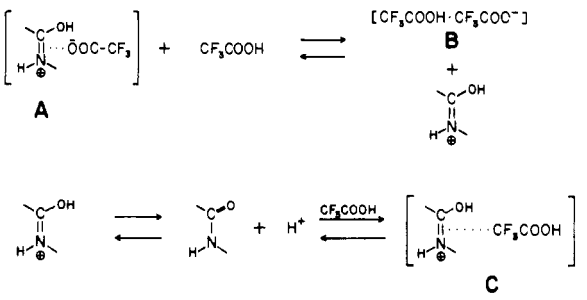


FIGURE 10: Changes in state of peptide residue with changes in the solvent, CF_3COOH-$CHCl_3$.

At the lower concentrations of CF_3COOH, both polymers are protonated but exist in the form of specific ion pairs, **A**. As the concentration of acid increases in the solvent, these specific ion pairs are partially dissociated by the competition of the acid for the acid anion (Kolthoff and Chantooni, 1963; Klotz *et al.*, 1964). Complex **B** is formed by this latter association. The removal of the counterion results in an uncompensated charge on the polymer. In poly-L-alanine, the charge can be almost entirely stabilized by a loose association with the complex **B** as well as undissociated acid molecules which can group themselves about the peptide groups. This stabilization mechanism, however, is not as effective as the specific ion-pair formation, with the result that some deprotonation of the polymer does occur. This is evidenced by the appearance of a slight shoulder in the 1.48- to 1.49-μ region, owing to amide N-H hydrogen bonded to the acid solvent.

In the case of poly-L-leucine, the branched side chains prevent effective association and stabilization of the charged peptide groups by the complex **B**. As the counterion is removed, therefore, the polyamino acid undergoes a significant deprotonation with the consequent appearance of a sizable hydrogen-bonded NH peak. As the acid concentration in the solvent is further increased, however, the concentration of CF_3COOH builds up sufficiently to stabilize the charged species to a certain extent. The deprotonation is thus partially reversed, and the extinction coefficients at 1.49 and 1.51 μ change in the expected fashion.

In keeping with this picture, it is felt that the transition in the optical rotatory dispersion is caused, either directly or indirectly, by the transition from a specific ion pair, complex **A**, to the loose and relatively non-specific associations (**B** or **C**) at higher acid concentrations. This transformation could result in a change in the optical-rotatory-dispersion parameters themselves such that values of λ_0 or b_0 applicable to an uncharged, fully hydrogen-bonded polymer in a noninteracting medium do not apply. In fact, it is hardly to be expected that the Moffitt-Yang analysis (Moffitt and Yang, 1956) or the Shechter-Blout analysis (Shechter and Blout, 1964a,b) would apply at all. The fact that the dispersions require two terms at the lower CF_3COOH concentrations is no guarantee of this since even the amino acids themselves (Katzin and Gulyas, 1964) exhibit dispersions which can be closely approximated by the Moffitt-Yang equation.

Indirectly, the change in charge distribution might result in a conformational change which would, in turn, affect the optical-rotatory properties. It seems likely, particularly in the case of poly-L-leucine, that there would be a considerable lack of freedom of rotation not only about the C_1-N bond but also about the C_1-C_α and the N-C_α bonds. It has been predicted theoretically that polymers with bulky side chains will, in good solvents, tend to assume a loose helical conformation (Krigbaum, 1958). (This conformation would be similar to the polyproline II conformation rather than a compactly folded α helix.) Such a conformation has been suggested to account for the chain extension observed in isotactic polystyrene (Krigbaum *et al.*, 1958). Hence, one might suppose that the steric hindrance imposed by the side chains of the polypeptide chains as well as the restriction on the rotation about the C_1-N bond of poly-L-alanine and poly-L-leucine, owing to protonation at the lower concentrations of CF_3COOH, would result in a loose helical array of chromophoric groups giving rise to a complex dispersion. As the concentration of CF_3COOH is increased and the CF_3COO^- anion consequently is removed, the increased charge repulsion, as well as the increase in rotational freedom about many C_1-N bonds arising from partial deprotonation, leads to a disruption of the regular conformation with a consequent loss of the complex dispersion properties.

In any event, these changes in optical-rotatory properties with solvent composition cannot reflect helix-to-coil transitions involving disruption of amide NH ··· O=C< hydrogen bonds in these systems of poly-L-alanine and poly-L-leucine in CF_3COOH-$CHCl_3$. Even in the absence of corresponding specific infrared data, it seems reasonable to believe that the same conclusion applies to other cases involving polyamino acids in solvents containing strong organic acids.

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