The Protonation of Poly-γ-benzyl-L-glutamate in Mixed Solvents Containing Dichloroacetic Acid*

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ABSTRACT: Spectral studies in the near infrared have been conducted with poly-L-alanine, poly-L-methionine, and poly-γ-benzyl-L-glutamate in mixed solvents containing dichloroacetic acid and ethylene dichloride. The appearance of a band in the neighborhood of 1.51 μ in the spectra indicates that the peptide residues of all three polyamino acids are protonated in this solvent system. Poly-γ-benzyl-L-glutamate exhibits a unique protonation pattern characterized by two transition regions. One occurs at low concentrations of acid and the other between 75 and 80% acid. Only the latter transition can be reversed by an increase in temperature within the range of 11–55°. These transitions parallel those observed in other physical properties of poly-γ-benzyl-L-glutamate in this solvent system. In each

transition, however, the fraction of peptide hydrogen bonded residues calculated from the spectral data differs significantly from that obtained from the optical rotatory dispersion data. The spectral data indicate that roughly 60% of the peptide residues are already protonated at the point where the major transition in the other physical properties begins to occur. The protonation patterns of these polyamino acids can be explained along the lines of the mechanisms previously proposed for poly-L-alanine and poly-L-leucine in mixtures of trifluoroacetic acid and chloroform. Modifications in the present cases can be attributed to the weaker acidity of dichloroacetic acid, as well as the steric and chemical properties of the side chains of poly-γ-benzyl-L-glutamate.

The synthetic polyamino acids have long served as popular models in the study of solvent- and temperature-induced conformational changes in proteins. In particular, the transformations in the physical properties of poly-γ-benzyl-L-glutamate (PBLG)[1] have been extensively examined in solvents containing strong organic acids and an inert diluent. Over the composition range of 70–80% dichloroacetic acid (DCA) in these mixed solvents, marked transitions occur in the viscosity (Doty, 1957), birefringence of flow, optical rotation, the optical rotatory dispersion parameter, b_0 (Doty and Yang, 1956; Moffitt and Yang, 1956; Yang and Doty, 1957), dielectric constant, and electrical birefringence (Watanabe et al., 1964). These transitions, as well as that observed in the partial specific volume of PBLG (Bradbury et al., 1965) can be reversed by heating solutions containing 75–80% DCA to temperatures above 41°.

The changes observed in these studies have normally been assumed to reflect the conversion of a more or less intact peptide hydrogen bonded helix to a random solvated coil. The resulting differences observed in the

magnitude of b_0 in going from one state to the other have been assumed to bear a linear relationship to changes in percent helicity in these solvents, with a value of $-630°$ found for PBLG in noninteracting solvents presumably being representative of a 100% right-handed α-helix.

The use of b_0 as an unqualified measure of helicity, however, has been subject to some question in recent years. Although there are a variety of data which suggest that its use is frequently justified (Urnes and Doty, 1961), there also exist data which indicate that, even for simple model systems, there is not always a linear correlation with the fraction of peptide residues involved in a hydrogen bonded α-helix (Ruttenberg et al., 1965), nor is its magnitude independent of the chemical nature of the solvent (Cassim and Taylor, 1965). Furthermore, recent investigations (Hanlon and Klotz, 1965) of the near-infrared spectra of the simpler polyamino acids, poly-L-alanine (PLA) and poly-L-leucine (PLL), in the mixed solvent containing trifluoroacetic acid and chloroform have revealed that changes in b_0 with increasing acid concentration could not be due to changes in a simple peptide hydrogen bonded system. In their study, reasonably high values of b_0 were correlated with the appearance of a protonated peptide species (I). The transitions in b_0 effected by a change in the solvent composition were found to reflect the conversion of this protonated species (I) to one hydrogen bonded to the acid component of the solvent.

These various peptide species could be clearly identified by the position of the first overtone of the NH stretching vibration in the 1.4–1.6-μ region of the near

[1] The following abbreviations are employed in the text: poly-γ-benzyl-L-glutamate, PBLG; poly-L-alanine, PLA; poly-L-leucine, PLL; poly-L-methionine, PLM; dichloroacetic acid, DCA; ethylene dichloride, EDC; N,N-dimethylformamide, DMF.
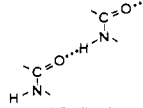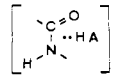
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| CHEMICAL STATE OF -NH IN POLYPEPTIDES	POSITION OF -NH OVERTONES, λ (μ)
Peptide hydrogen bond	1.54 μ, 1.58 μ
Protonated peptide	1.51 μ
Peptide — Acid hydrogen bond	1.49 μ

FIGURE 1: Band assignments for the first overtone of the NH stretching vibration of the peptide residues in polyamino acids.

infrared. Band assignments in this spectral region for the peptide NH were based on an extensive series of investigations conducted with both the low molecular weight amide, N-methylacetamide, as well as with the polyamino acids, PLA and PBLG (Klotz and Franzen, 1962; Hanlon et al., 1963; Klotz et al., 1964; Hanlon and Klotz, 1965). The various band assignments resulting from these studies are shown in Figure 1. Although the reader should consult these earlier papers for details, a summary of the several investigations is given below.

In an unassociated state, solutions of N-methylacetamide in an inert solvent exhibit a spectral absorption band at 1.47 μ. This peak is due to the first overtone of the NH stretching vibration as is evident from its complete absence in spectra of N,N-dimethylacetamide. As the concentration of the amide is increased, linear aggregates form via NH $\cdots$ O=C hydrogen bonds. The spectrum of N-methylacetamide in this state shows a double band with maxima at 1.525 and 1.57 μ. Spectra of the polyamino acid, PBLG, in the helix-forming solvents, CHCl$_3$ and pyridine, reveal bands of similar shape and extinction coefficients, although the maxima are shifted to higher wavelengths, falling at 1.54 and 1.58 μ. These latter positions are presumably characteristic of the peptide NH involved in an intramolecular peptide hydrogen bond.

The spectrum of N-methylacetamide in a relatively weak acid such as acetic shows a peak at 1.485 μ. Undoubtedly this reflects the spectral properties of the NH in the amide hydrogen bonded to the undissociated acid as there is no evidence for extensive protonation of the amide in such solutions. In the stronger acid, trifluoroacetic, however, conductivity and density studies indicate that the amide is protonated by this solvent. The near-infrared spectra of the amide in this solvent exhibit two peaks, one at 1.51 and the other at 1.525 μ. Only the former is predominant in the much

stronger acids, H_2SO_4 and HCl, as well as in a mixed solvent containing perchloric acid and dioxane. Under the conditions of the latter experiment, nuclear magnetic resonance data have shown that the amide is protonated in the form shown in structure I (Berger
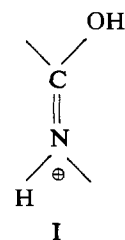
et al., 1959). Spectra of PLA in this same perchloric acid solvent also show a single major peak at 1.51 μ, thus demonstrating that the incorporation of the amide residue into the polypeptide structure results in no pronounced changes in the position of the maximum due to the protonated species. Hence, a band at 1.51 μ has been assigned to the protonated peptide species.

With this background available it was now feasible to extend this study to the more interesting but complex polypeptide PBLG. The spectral properties of this polymer have consequently been examined in mixtures of dichloroacetic acid (DCA) and ethylene dichloride (EDC) under conditions where the transformation in other physical properties have been interpreted in terms of a helix to coil transition. In addition, similar experiments have been conducted with poly-L-alanine and poly-L-methionine (PLM) in the same mixed solvents in order to determine whether variations of the side chain would affect the pattern of the spectral changes observed.

Materials and Methods

The synthetic polyamino acids were obtained from the Pilot Chemical Co. The two benzylglutamate samples employed were standard preparations of mol wt 310,000 and 330,000. The poly-L-methionine was a specially requested product. Its reduced viscosity at 0.5% in CF$_3$COOH was 0.37 dl/g. Making the assumption that in CF$_3$COOH its viscosity is proportional to the first power of the molecular weight, the latter was estimated to be ca. 8000 on the basis of the data given by Perlmann and Katchalski (1962). The value of its Moffitt–Yang parameter, b_0, was somewhat low in both pure ethylene dichloride and in 0.2% DCA–99.8% EDC (the value of b_0 was $-575°$ in both solvents) as well as in 1% DCA–99% EDC ($b_0 = -540°$). Its sedimentation velocity pattern in DCA–EDC (1–99%) revealed a broad but continuous distribution of polymer species. It is thus likely that this PLM sample employed in these studies contained ca. 10% low molecular weight polymer incapable of forming α structures. The molecular weight of the PLA sample was simply specified as high by the manufacturer. The molecular weights of similar samples from Pilot had previously been estimated to be
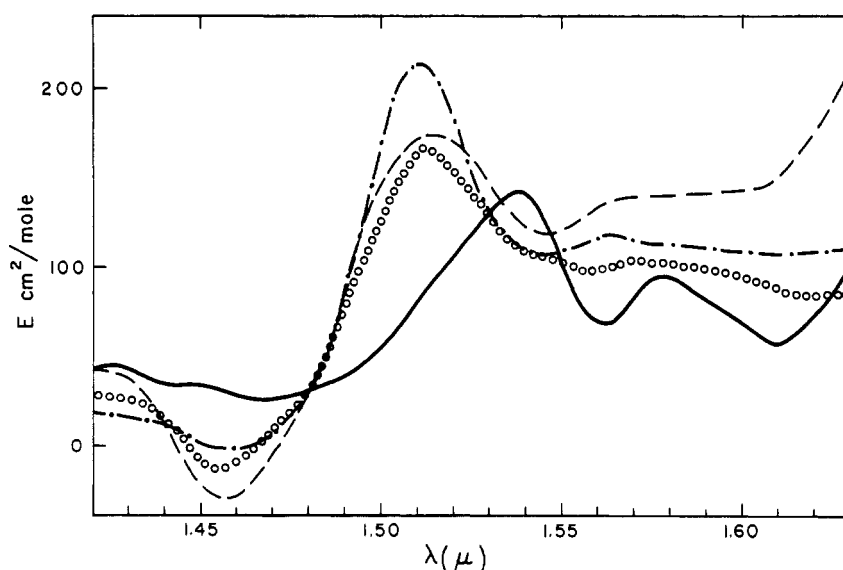
FIGURE 2: Spectra of polyamino acids in the near infrared: — PBLG (1.37%) in EDC; — — — PBLG (6.65%) in DCA; O O O PLM (4.29%) in DCA; ·—·— PLA (2.21%) in DCA.

30,000–45,000 (Hanlon and Klotz, 1965). All polyamino acids were dried at 56° and 1 mm for 24–48 hr and stored in a desiccator over anhydrous $CaSO_4$.

The low molecular weight solutes, benzyl acetate and CCl_4, as well as the solvent, N,N-dimethylformamide (DMF), employed in the centrifugation experiments were reagent grade material and were used without further treatment. The solvents, ethylene dichloride and dichloroacetic acid, were purified by fractional distillation over P_2O_5.

All mixed solvents and solutions were prepared by weight and concentrations were calculated on the basis of the densities of the solutions measured pycnometrically at 25.0 ± 0.05°. With the exception of pure DCA solutions, most spectral measurements were made at solute concentrations of 0.07–0.16 residue molar. For PBLG this corresponds to weight concentrations between 1.5 and 3.4% (w/v). Spectra in pure DCA were obtained at 0.31 residue molar, corresponding to a weight concentration of 6.7% for PBLG. All polymer concentrations are reported as either residue molar or weight/volume (w/v) per cent, whereas all solvent compositions for the mixed solvent, DCA-EDC, are reported in terms of volume (v/v) per cent of DCA.

Spectra were obtained in the near infrared with a Cary spectrophotometer, Model 14 CMR, equipped with a 0–0.1 OD slide wire, thermostatted cell adaptors, and a Yellow Springs Instrument Co. bridge and thermistor assembly. Spectral experiments were normally conducted at 25.0 ± 0.5°; some solutions were examined over the range of 11–55° in order to ascertain the effects of temperature on the spectral properties. Solvent absorption in the near infrared was equalized in the sample and reference solutions by the addition of CCl_4 to the reference solution at a volume fraction equal to that of the solute in the sample solution (Klotz and Franzen, 1962). Path lengths of 5 to 2 cm were employed over most of the range of solvent concentrations. One-centimeter paths were employed for spectral experiments in pure DCA. Extinction coefficients of the various solutes are reported in units of centimeters squared per mole.

When light scattering was appreciable as was the case for many polymer solutions, the spectra were corrected by a logarithmic extrapolation from a spectral region where the solute exhibited no pronounced absorption bands. The spectra of these polyamino acids in the wavelength region between 0.600 and 1.100 μ were normally devoid of structure and showed only a monotonic decline in the value of the absorbance with increasing wavelength. It was assumed that this behavior represented the normal scattering properties of the solute and solution contaminants under the conditions of the experiment. A plot of the log of these absorbances vs. the log of the wavelength was found to be linear. Making the further assumption that the same logarithmic relationship was obeyed in the region of absorption (1.4–1.65 μ), the value of the absorbance due to scattering in the latter region was obtained from these data and subtracted from the observed absorption spectrum. This procedure is a purely empirical one as the wavelength dependence of light scattering in this spectral region for the polymers under these conditions of concentration and cell geometry is not known. The variation in E_s, the increase in the extinction coefficient of the solution due to scattering, with wavelength was not marked in the spectral region of absorption. It was also found to be independent of temperature (within ±1 cm^2/mole) in those cases where the polymer spectrum was examined between 11 and 55°. The values of E_s were almost negligible for the lower molecular weight polyamino acids, but were quite appreciable for PBLG. At 1.52 μ, for instance, E_s for these PBLG solutions ranged from 84.0 cm^2/mole in 100% EDC (the maximum value) to 24.6 cm^2/mole in 80% DCA (the minimum value). Despite this significant contribution to the absorbance of the solution, the correction procedure
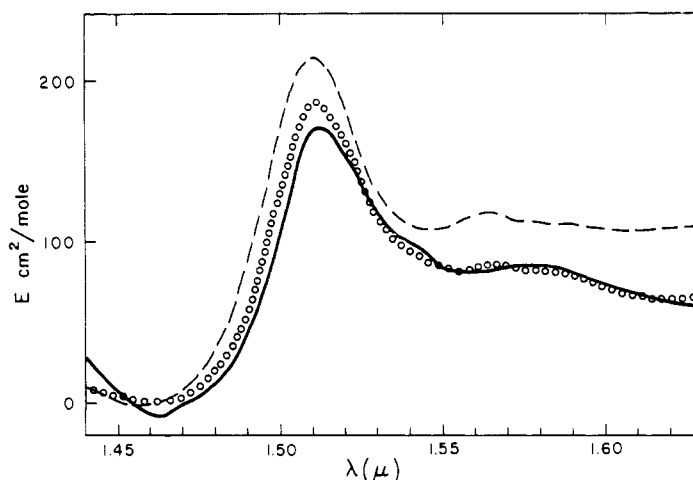
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FIGURE 3: Spectra of poly-L-alanine in DCA–EDC mixtures: —— 0.501% in 6% DCA; O O O 1.19% in 20% DCA; — — — 2.21% in 100% DCA.

outlined above appeared to be reliable. This was evidenced by the fact that in 19.7% DCA, spectra of PBLG at 1.52% and at 3.41% exhibited different values of E. (39.8 and 26.8 cm²/mole, respectively, at 1.52 μ), yet after correcting the observed extinction coefficients for scattering, the two spectra obtained were identical and superimposable within ±3 cm²/mole.

The spectra of PBLG in solutions containing >10% DCA were found to be independent of the concentration of PBLG in the 1.5–3.4% range. Below 10% DCA similar spectra were obtained for 0.7 and 1.5% PBLG at the same acid concentration. In the heating experiments the spectral changes were found to be perfectly reversible; no significant difference was observed in the spectra at 25° obtained before and after exposure to other temperatures. The spectra of the PBLG solutions at acid concentrations below 70% were invariant with time from 3 to 24 hr. Solubility problems were experienced with PLM in pure EDC but a spectrum of this polymer in this solvent could be obtained if the solution were warmed to 50° and a spectrum were run as soon as the solution had cooled to room temperature. These solutions of PLM were unstable and became turbid with time.

At the concentrations at which the spectral experiments with the polymers were conducted, it was usually necessary to allow the solutions to stand in the spectrophotometer for a period of time to allow bubbles and schliera to clear. If these as well as other elaborate precautions were employed in handling the solutions and obtaining the spectra, reproducibility in the case of the PBLG experiments was generally found to be ±0.003 OD. When these PBLG spectra were compared by matching absorbance in some nonspecific region of the spectrum such as 1.30–1.35 μ, reproducibility was found to be ±0.002 or less. This latter value corresponds to extinction coefficients of ±6 cm²/mole for most of the PBLG spectra.

Due to greater ease of handling and a lower level of scattering, the reproducibility in the spectra of PLA and PLM was much better, corresponding to ±0.0015 or an extinction coefficient of ±3 at the lower acid concentrations. Since most of the extinction coefficient changes were quite small in all these studies, this degree of precision was required.

Optical rotatory dispersion data were obtained with a Zeiss polarimeter equipped with a series of interference filters isolating wavelengths of 365.8, 405.8, 435.6, 545.9, and 577.8 mμ. Concentrations of polymer in the solutions for these experiments covered the range examined spectrally. An approximate correction of the residue rotation for the refractive index of the solvent was made using the value at the sodium D line. The rotatory dispersion data were analyzed in terms of the Moffitt–Yang equation (Moffitt and Yang, 1956) and plotted in the fashion suggested by Urnes and Doty (1961) in order to obtain the helicity parameter, b_0. These data were also analyzed in the manner suggested by Shechter and Blout (1964b).

Sedimentation velocity experiments were conducted at room temperature (ca. 25°) with a Spinco Model E ultracentrifuge equipped with schlieren optics. A single sector (4°) Kel F centerpiece was employed.

Results

The spectra of both PBLG and PLM in ethylene dichloride exhibited the characteristic double band with maxima at 1.54 and 1.58 μ associated with peptide hydrogen bond formation. These results were essentially in agreement with the spectra of PBLG in pyridine and in chloroform obtained in previous investigations (Hanlon and Klotz, 1965). In dichloroacetic acid, the spectra of all three polyamino acids, PLA, PLM, and PBLG, exhibited a peak in the neighborhood of 1.51 μ, presumably due to the protonated peptide residues.
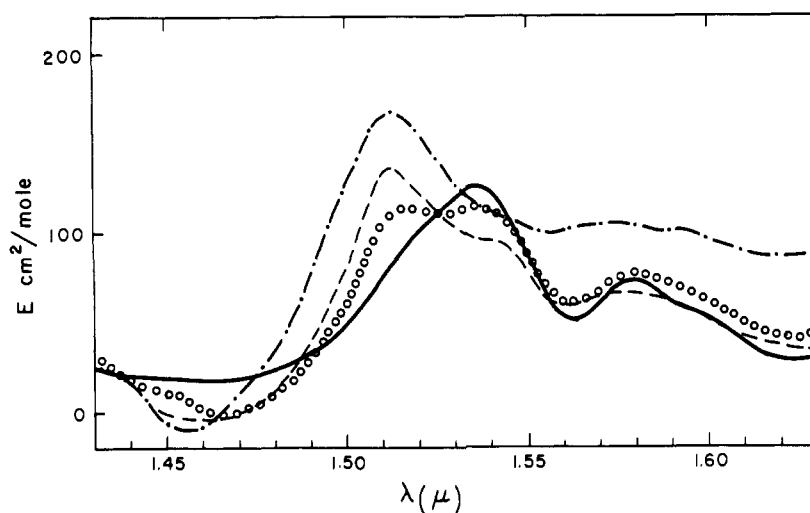
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FIGURE 4: Spectra of poly-L-methionine in DCA–EDC mixtures: —— 1.01% in 100% EDC; o o o 1.02% in 1% DCA; — — — 2.17% in 20% DCA; ·—·—4.29% in 100% DCA.
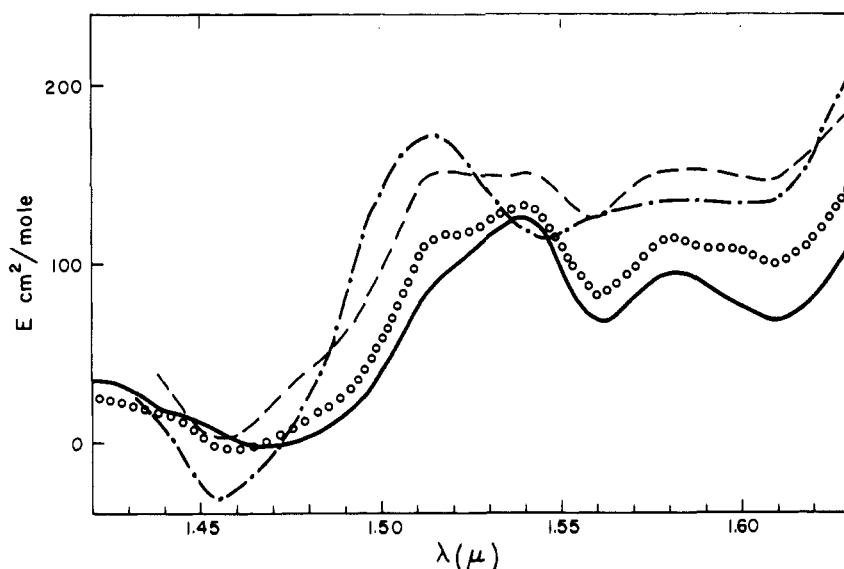

FIGURE 5: Spectra of poly-γ-benzyl-L-glutamate in DCA–EDC mixtures: —— 1.53% (0.0694 residue molar) in 1% DCA; o o o 3.41% (0.156 residue molar) in 10% DCA; — — — 3.43% (0.157 residue molar) in 70% DCA; ·—·— 3.44% (0.157 residue molar) in 80% DCA.

Some of these results are shown in Figure 2. There is no evidence in the spectra in DCA of a shoulder at 1.49 μ as had been previously observed for PLA and PLL in CF_3COOH. The broadening of the peaks at 1.51 μ for PLM and PBLG in contrast to PLA may be due to the interaction of the functional groups of the side chains with the protonated residues.

The effects of varying the solvent composition on the spectra of these three polymers are demonstrated in Figures 3–5. In these figures are shown the spectra of PLA, PLM, and PBLG, in solvent mixtures consisting of varying proportions of DCA and EDC. At the

lowest concentrations of acid required for polymer solubility (6%), PLA is predominantly in the protonated form as is evident from the appearance in Figure 3 of the peak at 1.51 μ with only a small shoulder at 1.54 μ. The disappearance of the shoulder is accompanied by a gradual increase in the extinction coefficient of the 1.51-μ peak as the acid concentration increases.

The spectra of PLM, which could be obtained over the entire range of acid concentrations, demonstrated more pronounced evidence of a conversion of peptide hydrogen bonded species (absorbing at 1.54 and 1.58 μ) to the protonated form (1.512 μ). The spectra shown
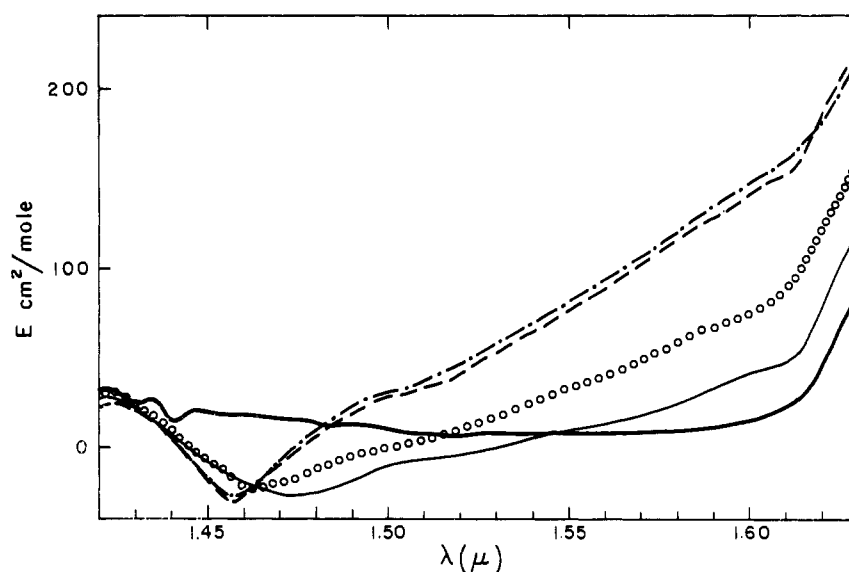
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FIGURE 6: Spectra of benzyl acetate in DCA–EDC mixtures: —— 0.572 M in 100% EDC; ——— 0.0718 M in 1% DCA; O O O 0.174 M in 10% DCA; — — — 0.178 M in 70% DCA; ·–·– 0.166 M in 80% DCA.

in Figure 4 exhibit a common isosbestic point at 1.525 μ up to 20% DCA. Above this concentration of acid, the spectra are increasingly distorted by an increase in all extinction coefficients. The extinction coefficients at 1.525 μ for these spectra are presented in the second column of Table I.

TABLE I: Extinction Coefficients of Poly-L-methionine and Poly-γ-benzyl-L-glutamate at Their Isosbestic Point, 1.525 μ.

Compn of Solvent (% DCA)	PLM, E (cm²/mole)	PBLG, E_c (cm²/mole)
0 (100% EDC)	108	110
1	111	116
6		113
10	111	112
20	111	105
70		112
80 (25°)	141	117
80 (41°)		111
100	143	124

In the spectra of PBLG shown in Figure 5, this distortion is much more serious and is evident over the entire acid concentration range. It is obvious from these data, nevertheless, that a sizeable fraction of peptide NH is absorbing in the 1.51-μ region even at fairly low concentrations of acid, and that this fraction increases with increasing concentration of DCA. Its relationship

to the disappearance of peptide hydrogen bonded NH absorbing at 1.540 and 1.580 μ, however, is unclear from these results alone.

It could be demonstrated by studies with benzyl acetate in the same solvent system that the distortions of the PBLG spectra could be attributed mainly to the interaction of the benzyl ester side chains with the acid component of the solvent. Benzyl acetate in ethylene dichloride has virtually no absorption in the spectral region between 1.4 and 1.6 μ. In all of the mixed solvents as well as in pure DCA, however, the apparent extinction coefficients of the solute increase in the manner illustrated in Figure 6. In this experiment, solutions of benzyl acetate in EDC as well as in the mixed solvents consisting of varying proportions of EDC and DCA were run vs. reference solutions containing CCl₄ at the same volume fraction in these solvents. The changes observed in the shape of these spectra are undoubtedly due to changes in the absorption properties of DCA when it interacts with the benzyl ester.

On the basis of the assumption that the low molecular weight model, benzyl acetate, closely approximates the effect of the benzyl ester side chains on the solvent absorption properties, these benzyl acetate spectra were employed to correct the PBLG spectra. For each acid concentration, the extinction coefficients of the benzyl acetate spectrum were subtracted from the extinction coefficients of the PBLG spectrum at the same wavelength after the two spectra had been superimposed at 1.35 and 1.36 μ. This difference, designated as E_c, was then plotted vs. wavelength. The results for several acid concentrations are presented in Figure 7.

The negative values of E_c for some of the spectra above 1.60 μ suggest that benzyl acetate has a somewhat greater effect on the solvent absorption properties
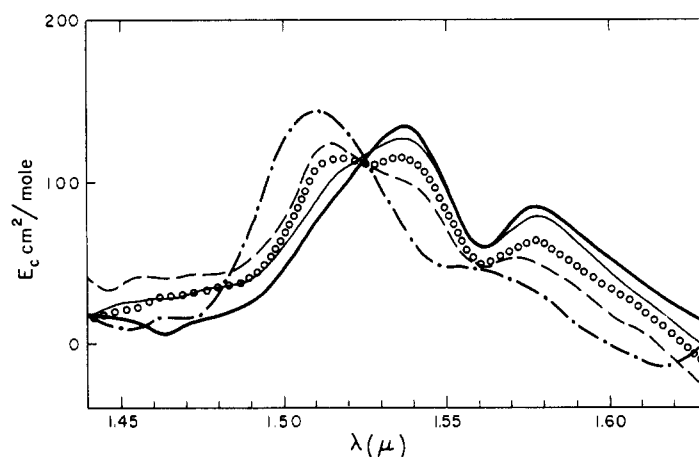
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FIGURE 7: Spectra of poly-γ-benzyl-L-glutamate corrected for benzyl ester side chain effects in DCA–EDC mixtures: —— 100% EDC; ——— 1% DCA; ○ ○ ○ 10% DCA; — — — 70% DCA; ·—·· 80 to 100% DCA.

than has the benzyl ester side chain of the polymer. Nevertheless, the corrected spectra appear to cross at an isosbestic point around 1.525 μ, the point also found for PLM in this same solvent system. As the data in the third column of Table I demonstrate, these values of E_c at 1.525 μ are also very close to the extinction coefficients of the spectra of PLM solutions at the same wavelength, at the lower acid concentrations. In fact, as Figure 8 shows, a corrected spectrum of PBLG and a spectrum of PLM at presumably the same fractions of protonated and peptide hydrogen bonded species are very similar and superimposable between 1.50 and 1.55 μ. The shapes of the corrected spectrum of PBLG and the observed spectrum of PLM in 100% DCA are, however, somewhat different. Evidently there is some distortion of the PLM spectra at the higher acid concentrations due to the interaction of the methionyl side chains with DCA. In addition, it is likely that the nature of the side chain influences, to a certain extent, the structure of the complex formed between the solvent components and the protonated peptide groups. Thus, one might expect the absorption band due to the protonated peptide NH to exhibit different shapes for different polyamino acids. This is, in fact, what is observed.

From these several lines of evidence, it can be safely concluded that the appearance of the protonated peptide species in PBLG is accompanied by a concomitant disappearance of the peptide hydrogen bonded form. Since there is an isosbestic point, it is likely that there are only two interconvertible peptide species, protonated, and peptide hydrogen bonded, whose extinction coefficients are invariant with solvent composition within the limits of experimental error. If the further assumption is made that the extinction coefficients at the two extremes of solvent composition represent the values for the 100% peptide hydrogen
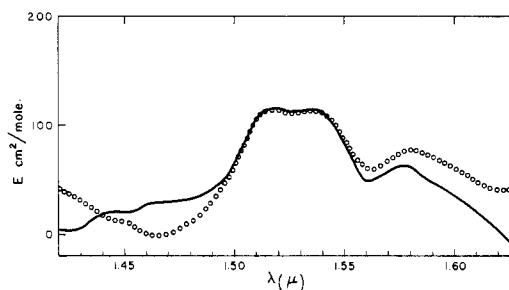

FIGURE 8: Spectra of poly-γ-benzyl-L-glutamate and poly-L-methionine at comparable fractions of protonated peptide residues: —— spectrum of PBLG in 10% DCA, corrected for side chain effects; ○ ○ ○ spectrum of PLM in 1% DCA.

bonded form and the 100% protonated form, respectively, the fraction, α_P, of protonated residues can be calculated by means of the formula

$$\alpha_P = \frac{E_c - E_{c_{100\% \text{ EDC}}}}{E_{c_{100\% \text{ DCA}}} - E_{c_{100\% \text{ EDC}}}} \tag{1}$$

The subscripts for E_c denote the values in the solvent extremes. A graphical representation of the values of α_P, calculated from the data at several wavelengths, is shown in Figure 9A. The several values of α_P calculated for the same DCA concentration are in rather poor agreement with one another. Nevertheless, it is obvious from these data that the protonation reaction at 25° occurs in two steps, one at very low concentrations of acid and the other between 70 and 80% DCA; roughly 50% of the total peptide residues are involved in each transition.
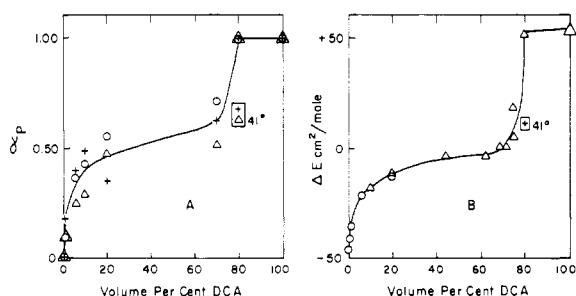
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FIGURE 9: Protonation of the peptide residues of poly-γ-benzyl-L-glutamate in DCA–EDC mixtures. A: fraction of protonated residues, α_P, at 25° as a function of solvent composition. Values were calculated from E_c of the corrected spectra at 1.510 (+), 1.540 (Δ), and 1.580 μ (O). Points enclosed in boxes represent data obtained from the spectrum in 80% DCA at 41°. B: Spectral transformations in mixed solvents at 25°, shown as $\Delta E = E_{1.515\ \mu} - E_{1.540\ \mu}$: 1.5% PBLG (O), 3.4% PBLG (⌄), and 6.7% PBLG (Δ). Point enclosed in box represents ΔE for the spectrum in 80% DCA at 41°.
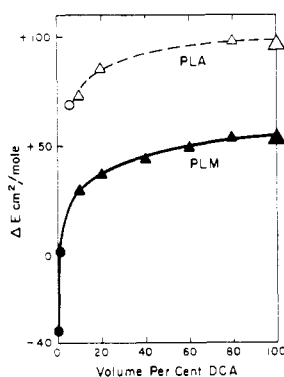

FIGURE 10: Spectral transformations of poly-L-alanine and poly-L-methionine in mixed solvents at 25° shown as $\Delta E = E_{1.515\ \mu} - E_{1.540\ \mu}$: — — — — PLA at 0.5% (O), 1.2% (⌄), and 2.2% (Δ); ——— PLM at 1.0% (●), 2.2% (▲), and 4.3% (▲).

Essentially the same conclusion was derived from a more direct treatment of the experimental data. If the same assumptions employed in calculating α_P are again made, the observed extinction coefficient, E_λ, of the uncorrected PBLG spectra shown in Figure 5 can be expressed as

$$E_\lambda = \alpha_P E_{P_\lambda} + (1 - \alpha_P)E_{H_\lambda} + E_{S_\lambda} \qquad (2)$$

where E_{P_λ} and E_{H_λ} are the extinction coefficients of the fully protonated and fully peptide hydrogen bonded polyamino acids at wavelength λ. E_{S_λ} is the perturbed absorption of the solvent due to its interaction with the polymer. Although this term is partially dependent
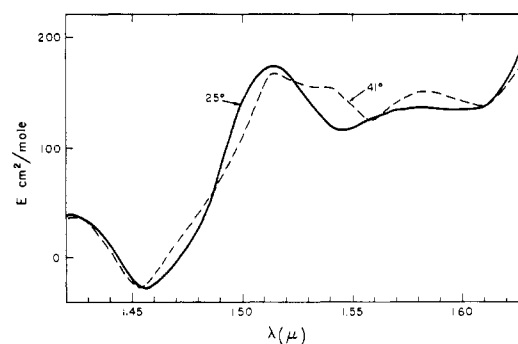

FIGURE 11: Effect of temperature on the spectral properties of poly-γ-benzyl-L-glutamate in 80% DCA; spectra obtained at 25° (———) and 41° (— — —).

on α_P, model studies indicate that the predominant contribution comes from the interaction of the side chains with the solvent.

If the observed extinction coefficient, E_λ, at one wavelength, λ_2, is subtracted from that at another wavelength, λ_1, of the same spectrum (the subscripts 1 and 2 denote the order of increasing wavelength) the difference, ΔE, is given by

$$\Delta E = \alpha_P(\Delta E_P - \Delta E_H) + \Delta E_H + \Delta E_S \qquad (3)$$

where the ΔE values are $(E_{\lambda_1} - E_{\lambda_2})$. With rearrangement, an expression for α_P can be obtained in terms of a difference, ΔE, of the observed extinction coefficients of the solution

$$\alpha_P = \frac{\Delta E}{\Delta E_P - \Delta E_H} - \frac{\Delta E_H}{\Delta E_P - \Delta E_H} - \frac{\Delta E_S}{\Delta E_P - \Delta E_H}$$

$$(4)$$

For the order of the wavelengths given, the values of ΔE_S above 1.46 μ become increasingly more negative. Thus, if the ΔE_S term is neglected the value of ΔE will be proportional to minimum values of α_P and transitions in ΔE as a function of solvent concentration will reflect minimum changes in the magnitude of α_P. The behavior of ΔE may consequently be employed to describe the protonation pattern with the reservation that any changes in the magnitude of ΔE will underestimate the number of residues converted to the protonated form.

When the spectral data for PBLG are calculated in this fashion, results of the type shown in Figure 9B are obtained. In this figure, values of ΔE for the wavelength pair 1.515 and 1.540 μ ($\Delta E = E_{1.515\ \mu} - E_{1.540\ \mu}$) are plotted vs. the composition of solvent. Similar patterns were also observed for other wavelength pairs above 1.46 μ. In agreement with the conclusions based on the data from the corrected spectra (Figure 9A), two transitions in the protonation are apparent, with a sizeable fraction of peptide residues involved in each.

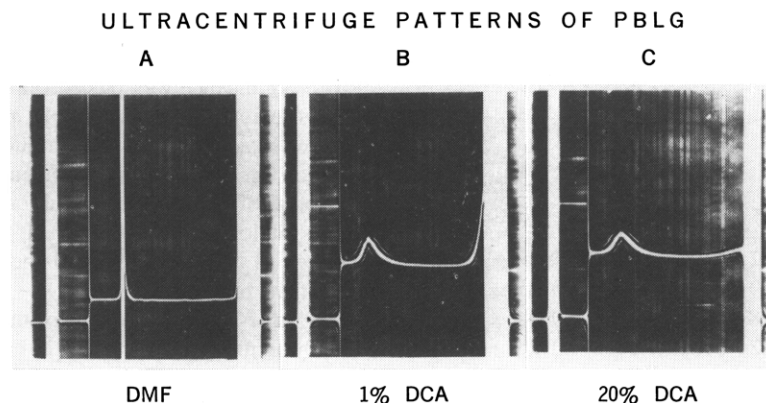A similar treatment of the spectral data for PLA and

ULTRACENTRIFUGE PATTERNS OF PBLG

FIGURE 12: Sedimentation velocity experiments with poly-γ-benzyl-L-glutamate. A: PBLG (1%) in *N,N*-dimethylformamide. This picture was taken 96 min after attaining a speed of 59,780 rpm. The bar angle was 80°. B: PBLG (0.5%) in 1% DCA, 99% EDC. This picture was taken 645 min after attaining a speed of 52,640 rpm. The bar angle was 70°. C: PBLG (0.5%) in 20% DCA, 80% EDC. This picture was taken 1219 min after attaining a speed of 52,640 rpm. The bar angle was 70°.

PLM yielded the results presented in Figure 10, which shows a plot of ΔE for the wavelength pair 1.515 and 1.540 μ *vs.* solvent composition. Neither polymer appears to undergo the two-stage protonation seen in the case of PBLG.

Since the transition in other physical properties of PBLG in 80% DCA can be reversed by heating to higher temperatures, it was of interest to ascertain whether the spectral transition would be similarly affected. To this end, spectra of PBLG in 10, 75, and 80% DCA were obtained over the range of 11–55°. In addition, similar experiments were conducted with PLA in 6, 20, and 80% DCA. Only the spectra of PBLG at the higher acid concentrations, 75 and 80% DCA, were affected in this temperature range. The results of one of these heating experiments at the higher acid concentration are presented in Figure 11 which shows spectra of PBLG in 80% DCA at 25 and 41°. Cooling below 25° or heating above 41° had no further effect. The spectra corrected for the benzyl ester side chain effects showed the same results. As is evident from the decrease in E at 1.51 μ and the increase at 1.54 and 1.58 μ, an increase in temperature results in a partial recovery of the peptide hydrogen bonded structure.[2] The spectra at 41° are similar in appearance to

the ones obtained at 25° in 70–75% DCA, and in fact, the values of α_P as well as ΔE are similar to the average for these values at 75% DCA. This is demonstrated in Figure 9A and B, by the position of the boxed points at 80% DCA. Similar results were obtained for the solution in 75% DCA.

The optical rotatory dispersion properties of PBLG in a few of the mixed solvents were determined in order to ensure the fact that these samples of PBLG at these concentrations behaved in the fashion described for the more dilute solutions. In agreement with the report of others (Karasz *et al.*, 1964), this was indeed found to be the case. The values of the Moffitt–Yang parameter, b_0, calculated for λ_0 of 212 mμ are presented in Table II for several acid concentrations. Employing the standard value of $-630°$ for the b_0 of the hydrogen bonded α-helix, the fraction of peptide residues in the peptide hydrogen bonded form, α_H, were calculated for these acid concentrations. Similar results were obtained if the data were analyzed in the manner suggested by Shechter and Blout (1964b). Equivalent values of $(1 - \alpha_P)$ were also taken from the graphical data presented in Figure 9A. These separate sets of α_H are both presented together in Table II. As is apparent, the values for the two sets of experiments do not agree at all. The high values of b_0 at the lower acid concentrations rule out the possibility that the double transition in the protonation pattern of PBLG may be due to the presence of low molecular weight β structures which protonate more readily than the high molecular weight α-helices.

Additional evidence from ultracentrifuge experiments also ruled out the possibility that there existed a significant fraction of lower molecular weight material

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[2] This is contrary to previous reports of experiments of a similar nature conducted with a 7% PBLG solution in 80% DCA and a Beckman DK-2 (Hanlon and Klotz, 1965). The discrepancy can be attributed mainly to the poor resolution and high stray light level of the latter instrument compared to the present Cary Model 14CMR. The actual measured optical density differences in these present experiments were quite small (on the order of 0.010) and well within the instrumental error of the DK-2.

2057

TABLE II: Calculation of the Fraction of Peptide Hydrogen Bonded Residues, α_H, Poly-γ-benzyl-L-glutamate.

Compn of Solvent ($\%$ DCA)	b_0 (deg)	α_H	
		from b_0 Data	from Spectral Data
0 (100$\%$ EDC)	-667	1.06 ± 0.05	1.00 ± 0.10
1	-629	1.00 ± 0.05	0.85 ± 0.10
20	-588	0.93 ± 0.05	0.54 ± 0.10
70	-552	0.88 ± 0.05	0.38 ± 0.10
80	$+67$	0 ± 0.05	0 ± 0.10
100	$+36$	0 ± 0.05	0 ± 0.10

in the PBLG samples. As the sedimentation velocity patterns in Figure 12 illustrate there was no low molecular weight material remaining at the menisci in solutions of PBLG in DMF, or in the mixed solvents, 1$\%$ DCA or 20$\%$ DCA. In fact, the schlieren patterns of these latter experiments show no bimodal distribution of polymer species of any kind. This rules out an all or none protonation phenomenon. These results also indicate that there is a marked preferential interaction of the polyamino acids with the acid component of the solvent. The calculated value of $(1 - \bar{v}\rho)$ for PBLG in 20$\%$ DCA is -0.013 at $25°$, yet the polymer sedimented instead of floating in this experiment and did so with no apparent convective disturbance.

The characteristics of the self-association of the acid component of the solvent were also examined in this spectral region in an effort to ascertain whether either of the observed protonation transitions of PBLG as well as the effect of temperature could be attributed to changes in the structure of the solvent. Several dilutions of DCA in ethylene dichloride were run against CCl$_4$ in the same solvent over the wavelength range of 1.40–1.65 μ. The first overtone of the OH stretching vibration of the DCA monomer fell at 1.475 μ. There was little change in the spectra above 20$\%$ DCA (2.37 M). Heating these solutions to $51°$ resulted in an increase in the extinction coefficient at 1.475 μ, but there were no dramatic effects evident at 80$\%$ DCA. In short, the transition shown by PBLG in the higher acid concentration cannot be attributed to a change in solvent association properties at that concentration of DCA.

Conclusion

These results clearly show that these three polyamino acids can be protonated by fairly low concentrations of DCA. Poly-γ-benzyl-L-glutamate, in contrast to PLM and PLA, exhibits a complex pattern with two transition regions, one occurring at fairly low concentrations of acid and the other between 75 and 80$\%$ DCA. Both stages appear to involve roughly equal fractions of peptide residues. Early experiments of Doty and Yang (1956) had indicated that PBLG underwent two transi-

tions in this solvent, but the effects at the lower concentrations of acid were, in general, found to be small. Little significance was placed on the changes observed in this region until Watanabe et al. (1964) conducted a detailed examination of the influence of solvent composition on the dielectric constant and electrical birefringence of PBLG. These authors found pronounced effects at low concentrations of DCA which they attributed to the protonation of the three residues at the end of the helix.

The near-infrared spectral results, however, indicate that the extent of the protonation at low concentrations of DCA is far greater than can be accounted for on the basis of the protonation of three residues at the end of a PBLG molecule of an average DP of 1500. Even with a generous allowance for error, an estimate of α_P at the foot of the second transition (about 70$\%$ DCA) reveals that roughly half of the peptide residues are protonated. Yet it is at this second transition that the major changes in the hydrodynamic properties, optical rotation, and optical rotatory dispersion parameters occur.

Although it is difficult to assess quantitatively the meaning of changes in the hydrodynamic properties of the polymer in these mixed solvents, the evidence seems fairly convincing that a major conformational change occurs at 75$\%$ DCA. We are thus faced with the problem of rationalizing these several apparently contradictory conclusions. More specifically, we must explain (1) why PBLG, in contrast to the other two polypeptides, exhibits a complex two-step protonation pattern, (2) how this polyamino acid maintains a fairly rigid, rodlike structure even though about half of its peptide residues are protonated, and (3) why b_0 remains high for all three of these polyamino acids under conditions where the spectral data indicate a considerable fraction of the peptide residues are protonated.

As far as the first question is concerned, we may again invoke essentially the same explanation offered for the differences in the PLA and PLL protonation patterns in the mixed solvent, trifluoroacetic acid and chloroform (Hanlon and Klotz, 1965). These present results, however, permit a more general scheme, shown in Scheme I, to be outlined, and are consistent with the following explanation. The weaker acid strength of DCA apparently permits the survival of unprotonated peptide hydrogen bonded species at a higher concentration of acid than was observed in the solvents containing trifluoroacetic acid. Even so, however, when there is little steric hindrance to the approach of the acid as is the case for PLM and for PLA, to an even greater degree, the peptide residues are almost completely titrated by ca. 20$\%$ DCA. The peptide residues of PBLG, on the other hand, are protected to a certain extent not only by the steric properties of the benzyl ester side chain, but by its chemical properties as well, since the side chain also competes for the acid component of the solvent. The net effect is a lower value of α_P for PBLG at the same concentration of acid.

At these low concentrations of DCA, the equilibrium seems to favor the production of the ion pairs formed
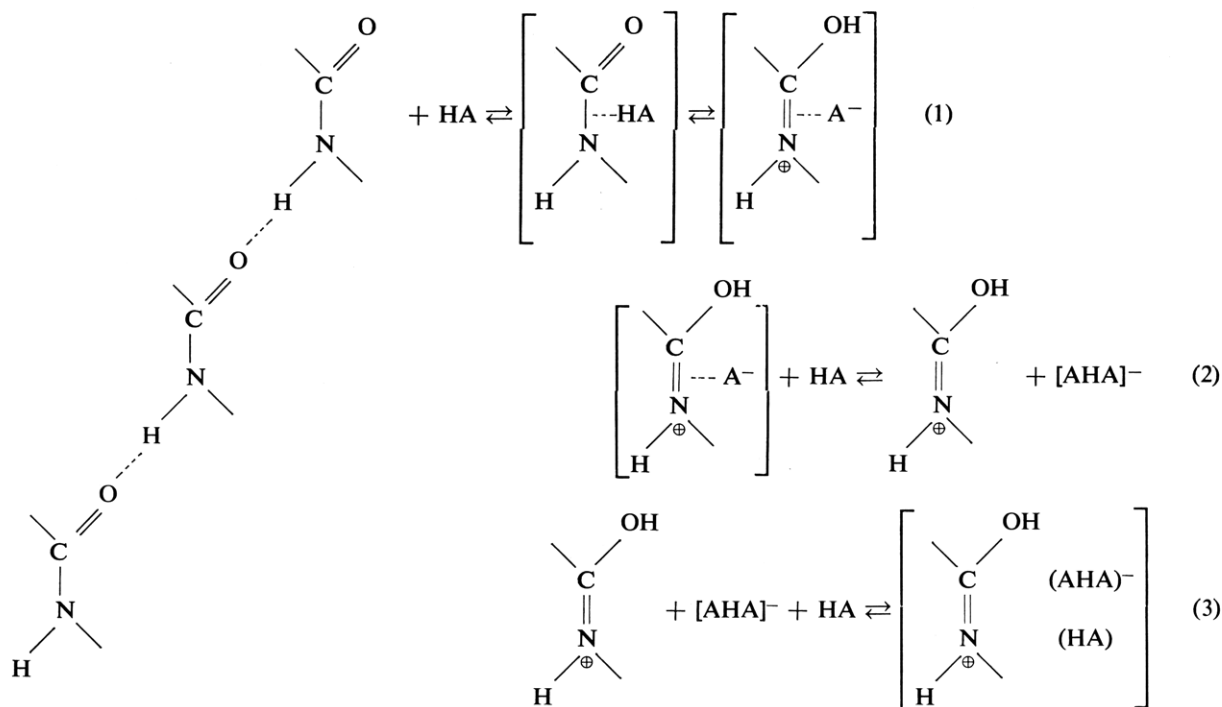
Scheme I: The Protonation of the Peptide Residues of Polyamino Acids in Solvents Containing Strong Organic Acids.

between the protonated residue and the acid anion as opposed to the acid peptide hydrogen bonded form. As the acid concentration increases, however, this anion is removed from the intimate ion pair by the reaction shown in eq 2 of Scheme I. The acid anion, together with the undissociated acid, form the hydrogen bonded homoconjugate (AHA⁻) (Kolthoff and Chantooni, 1963). This competition for the anion leaves a charged peptide residue which, in the absence of any steric restrictions, can be partially stabilized by the associations shown in eq 3 of Scheme I. The efficiency of this stabilization mechanism depends on the size of the side chain, the concentration of solvent, and how well these various solvent species fit into the available space about the peptide residue. In no case is this mechanism as effective as the intimate ion pair formation. The net result is that the further protonation of the polypeptide chain is partially inhibited and proceeds more slowly as the acid concentration continues to increase.

For PBLG, this region of inhibition occurs at a point where a large fraction of the peptide hydrogen bonded structure is intact. This, together with the lack of free rotation about the C_1–N bond due to protonation, the bulky side chains, and the hydrogen bonding possibilities available to the protonated residues, presumably serves to preserve the rodlike characteristics of the macromolecule.

Ultimately, however, a point is reached where the number of protonated species as well as the charge which is building up at the higher DCA concentrations greatly weakens the partially hydrogen bonded struc-
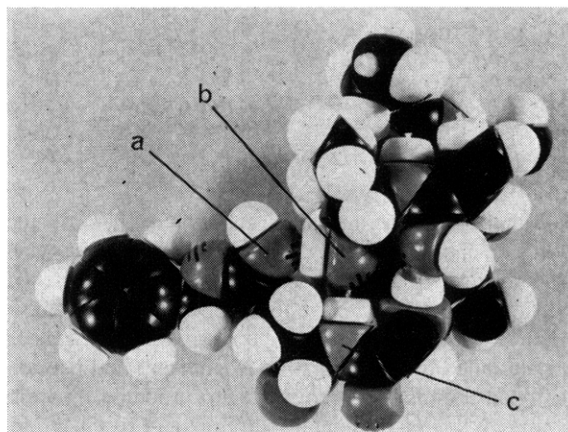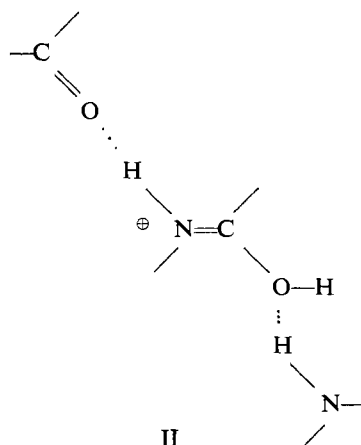

FIGURE 13: Model of the partially protonated polypeptide chain of poly-γ-benzyl-L-glutamate showing possible side chain protonated peptide hydrogen bond: a: carbonyl oxygen of the benzyl ester of the side chain in the protonated form (or hydrogen bonded to the acid component of the solvent); b: carbonyl oxygen of the protonated peptide group; c: peptide nitrogen.

ture. The conformation consequently collapses with the resulting protonation of the remaining peptide residues.

As has been suggested before (Doty and Yang, 1956), the effect of an increase in temperature is presumably to dissociate the solvated complex formed by the reac-

2059

tion in eq 3 of Scheme I. The resulting charged species are unstable in this medium and some revert to the peptide hydrogen bonded form. This is similar to the mechanism proposed for the effects of CF_3COOH on PLL, with the exception that in the latter case the greater acidity and hydrogen bonding properties of CF_3COOH competed far more successfully for the peptide species to form the acid peptide hydrogen bonded complex rather than the peptide hydrogen bonded structure.

This is the simplest interpretation of these results which invokes no specific property of the side chains of PBLG other than their steric and chemical exclusion effects. There may, however, be additional complementary effects. Models constructed of the polypeptide chain reveal that some of the protonated residues can be partially accommodated in a helix *via* bifurcated but highly distorted hydrogen bonds of the type in structure II. Additional interactions between the

II

protonated residue and the carbonyl oxygen of the ester side chain are also possible. One such interaction is demonstrated in Figure 13, which shows a model of the partially protonated polypeptide chain of PBLG wound in two turns of an α helix. The α-helical register is maintained by the formation of conventional peptide hydrogen bonds between the unprotonated peptide groups of the first and the fifth amino acid residues and the fifth and the ninth amino acid residues. This conformation can be stabilized by additional types of hydrogen bonds, as well. All but one of the benzyl ester groups in this model have been removed so as to expose the two protonated peptide groups belonging to amino acid residues 6 and 7. These OH groups are able to form hydrogen bonds with the carbonyl oxygen of the side chains of the residues directly below. In the photograph this is demonstrated by the interaction of the benzyl ester group of amino acid residue 2 with the protonated peptide OH of residue 6. The benzyl ester is shown as a protonated group, although this is not crucial to the structure. Presumably the same orbital type would be present if the carbonyl oxygen were involved in a set of bifurcated hydrogen bonds with one hydrogen provided by DCA and the other by the protonated peptide group.

Although these side chain protonated peptide hydrogen bonds can be formed at all residues, the resulting distortions in such a structure do not permit the weak hydrogen bonds of the type shown in structure II to form. It is tempting to propose, therefore, that an ordered structure of the type shown in Figure 13 is formed at low concentrations of DCA containing some combination of the two amide species, protonated and peptide hydrogen bonded, in interaction with the functional groups of the ester side chains. This structure resists further disruption until the acid concentration reaches such overwhelming proportions that the remaining peptide species as well as the remaining benzyl ester side chains are protonated and solvated. This hypothesis is currently being investigated.

The third question regarding the discrepancy between the fraction of peptide hydrogen bonded residues calculated from the values of b_0 and the near-infrared spectral data was encountered in the earlier studies with PLA and PLL (Hanlon and Klotz, 1965). As in that case, no definitive answer can be given. It would seem, however, that the analysis of Cassim and Taylor (1965) is particularly appropriate here. As was pointed out by these authors, as well by Shechter and Blout (1964a), the b_0 parameter reflects the complexities of a dispersion due to two or more Cotton effects in the ultraviolet. Any factors which influence the position or the rotatory strength of the electronic transitions giving rise to these Cotton effects will also influence the magnitude of b_0.

It is conceivable that the electronic transitions of the protonated polypeptide chain under conditions where ion pair formation is extensive and/or some asymmetric conformation can exist would have characteristics similar to those of the peptide hydrogen bonded α-helix. The b_0 for this protonated chain would thus be quite high irrespective of the fraction of peptide residues existing in the form of a peptide hydrogen bonded helix.

In any event, it is clear that the transition of PBLG at 75% DCA, as well as the thermal reversal of this transition, involves the conversion of a structure which already has a significant fraction of its peptide residues protonated. Thus, this transition appears to be much more complicated than has hitherto been envisaged. Evaluations of various theoretical parameters of this system, based on the assumption that a completely intact peptide hydrogen bonded conformation exists below 70% DCA or above the transition temperature, should be revised.

Acknowledgments

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The Preparation and Tryptic Hydrolysis of S-Aminoethylated Tobacco Mosaic Virus Protein*

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ABSTRACT: A method of preparative aminoethylation of tobacco mosaic virus (TMV) protein with ethylenimine under reducing and denaturing conditions has been described. The reaction proved to be essentially quantitative and specific for the sulfhydryl group. The aminoethylcysteinyl bond has been shown to be completely digested with trypsin though more slowly than lysyl and arginyl bonds.

A single phenylalanine residue (position 10) in TMV protein is also attacked upon prolonged digestion with

trypsin, and this action is inhibited by the chymotrypsin inhibitor, 1-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone (TPCK). The combined introduction of one additional tryptic site by aminoethylation of the cysteine residue and liberation of aspartic acid by dilute acid have permitted the facile allocation of an amino acid exchange in a nitrous acid mutant (No. 273) of TMV. A serine residue was thus found to replace one of four aspartic acid residues (33) in the core peptide containing 41 residues.

Trypsin continues to be the most specific reagent available for the cleavage of polypeptide chains. Alkylation of cysteine residues with $CH_2CH_2NH_2$ groups has, therefore, been proposed as a means of transforming

these residues into trypsin-susceptible sites (Lindley, 1956). Raftery and Cole (1963) have demonstrated that ethylenimine may be used advantageously in place of haloethylamines. This reaction would be of particular advantage for the tobacco mosaic virus (TMV) protein where the single cysteine residue is located near the middle of the large N-terminal tryptic peptide consisting of 41 residues. Although the difficult task of determining the sequence of this peptide has now been completed (Funatsu *et al.*, 1964), the location of the frequent exchanges of amino acid residues in various virus strains in this part of the molecule makes methods for its clear-cut fragmentation most valuable. This appears to be the case if ethylenimine is used to alkylate the cysteine residues of the protein or peptide. In addi-

2061