Biochemistry

Volume 9, Number 5 March 3, 1970

Conformational Properties of Poly-L-proline in Concentrated Salt Solutions*

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ABSTRACT: The intrinsic viscosity, circular dichroism, and optical rotation of poly-L-proline have been measured at 30° in water as functions of molecular weight and calcium chloride concentration. The intrinsic viscosity decreases in the salt solutions, with the relative change increasing with increasing molecular weight. The change in the intrinsic viscosity is most rapid in the more dilute salt solutions and is half-completed at about 1.5 M calcium chloride. The molecular weight dependence of the intrinsic viscosity shows that these effects are caused by the conformational transition from a somewhat flexible rod-like structure in water to a statistical random coil in concentrated calcium chloride. The onset of random coil behavior is at 2.5 to 4.0 M calcium chloride. This conformational transition also affects the optical properties of poly-L-proline. Both the resolved positive and negative bands in the circular dichroism spectra change in the same manner as the intrinsic viscosity with the addition of calcium chloride. The positive Gaussian band disappears completely at very high salt concentrations. However, the manner of change of the observed circular dichroism at a particular wavelength, including the extrema, is not directly related to the change of the intrinsic viscosity or either resolved Gaussian band. This is because the observed circular dichroism at a particular wavelength is sensitive to changes in the intensities and locations of both resolved Gaussian bands.

The specific rotation at wavelengths in the near ultraviolet and visible does not change in the same manner as does the intrinsic viscosity. The transition of poly-L-proline from form II to the random coil is best monitored by the intrinsic viscosity or by resolution of the circular dichroism spectra.

The polyimino acid poly-L-proline has been shown to exist in either one of two ordered helical conformations in the solid state (Cowan and McGavin, 1955; Sasisekharan, 1959; Traub and Shmueli, 1963). In one of these structures, designated form II, the imide group is in a planar *trans* orientation and a left-handed helical conformation results (Cowan and McGavin, 1955; Sasisekharan, 1959). In the other structure, form I, the imide group is *cis* and a right-handed helix is formed (Traub and Shmueli, 1963). When dispersed in dilute solution in appropriate solvents, the isolated molecules retain these ordered conformations as evidenced by optical rotation and circular dichroism studies (Fasman and Blout, 1963; Blout *et al.*, 1963; Carver *et al.*, 1966; Timasheff *et al.*, 1967; Bovey and Hood, 1967; Okabayashi *et al.*, 1968; Tiffany and Krimm, 1968). Since there is no capability for intramolecular hydrogen bonding in this polymer, the ordered structure, in the isolated molecule, must be maintained by severe steric restrictions to rotation about the carbonyl carbon–α-carbon bond, this being the only bond per repeating unit capable of some rotational freedom. This conclusion is supported by the theoretical conformational analysis of Schimmel and Flory (1967). In dilute solution these two ordered forms can be cooperatively interconverted by appropriate alteration of the solvent composition (Gornick *et al.*, 1964; Engel, 1966).

In concentrated aqueous solutions of certain salts, notably calcium chloride and lithium bromide, hydrodynamic properties, such as the intrinsic viscosity (Blout and Fasman, 1957; Harrington and Sela, 1958; Steinberg *et al.*, 1960; Ciferri and Orofino, 1966), as well as optical properties, such as optical rotation (Blout and Fasman, 1957; Harrington and Sela, 1958; Steinberg *et al.*, 1960; Kurtz and Harrington, 1966), have values which are quite different from those exhibited

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† Recipient of Public Health Service Postdoctoral Fellowship 1-F02-GM-41,377-01 from the National Institute of General Medical Sciences.

by either of the helical structures. A collapse or disruption of the ordered structure has been surmized from these results (Harrington and Sela, 1958; Steinberg *et al.*, 1960; Kurtz and Harrington, 1966; Tiffany and Krimm, 1968) since the same salts are known to cooperatively transform the ordered structures of the globular and fibrous proteins to disordered structures (Simpson and Kauzmann, 1953; Bello *et al.*, 1956; Mandelkern *et al.*, 1962; von Hippel and Wong, 1962; von Hippel and Wong, 1965; Mandelkern and Villarico, 1969). Calcium chloride is one of the most effective salts in inducing this structural transition. It has also recently been shown that circular dichroism data down to nearly 200 mμ can be obtained with poly-L-proline in concentrated aqueous solutions of this salt (Tiffany and Krimm, 1968).

In the present work we have examined the hydrodynamic and optical properties of poly-L-proline in dilute solution as a function of molecular weight, calcium chloride concentration, and temperature. Our objective has been to quantitatively define the polymer conformation in the concentrated salt solution, to locate any transition which may exist, and to determine how the different techniques of measurement reflect the structural changes that occur.

Materials and Methods

Materials. The sources of the samples of poly-L-proline are listed in Table I along with the molecular weight data and the abbreviations used in this paper.[1] MIL was converted from form I into form II by heating for 5.5 hr at 100° in glacial acetic acid (Blout and Fasman, 1957). The other samples were supplied as form II. All of the polymers were dialyzed against water and recovered by lyophilization before use. Polymer solutions were prepared in volumetric flasks using poly-L-proline which had been dried in a vacuum using a Dry Ice–acetone trap.

TABLE I: Poly-L-proline Samples Studied.

Polymer	M_n[a]	M_w[b]	M_w/M_n	Supplier
SCC[c]	3,800 ± 300	4,400 ± 300	1.2	Sigma Chemical Co.
L2[c]	7,0 0 ± 500	10,900 ± 700	1.6	M. H. Liberman
MAN	7,4 0 ± 500	9,200 ± 500	1.2	Mann Research Lab.
MIL	15,200 ± 600	16,300 ± 1,000	1.1	Miles Lab.

[a] Number-average molecular weight. [b] Weight-average molecular weight. [c] Molecular weight data taken from Liberman (1968).

Calcium chloride was reagent grade. Solutions were prepared by dilution of a saturated stock solution whose concentration was determined periodically by titration with silver nitrate. Distilled water was used throughout.

Molecular Weights. Number-average molecular weights for MAN and MIL were determined in water using a Mechrolab 503 high-speed membrane osmometer equipped with a B20 membrane. There was slight permeation of the membrane when MAN was studied. No permeation was observed with MIL. The number-average molecular weights of SCC and L2 were obtained by M. H. Liberman using a Mechrolab vapor pressure osmometer (SCC) and Mechrolab 502 high-speed membrane osmometer (L2) (Liberman, 1968).

Weight-average molecular weights were obtained by low-speed sedimentation equilibrium in water using a Beckman Spinco Model E analytical ultracentrifuge with interference optics. Plates were read with the aid of a Nikon comparator. The partial specific volume was assumed to be 0.758 cm^3/g (Cowan and McGavin, 1955). Apparent molecular weights, *i.e.*, the molecular weights obtained by ignoring the concentration dependence of the activity coefficient and refractive index increment of the polymer and the density of the solution, were obtained from the polymer concentration at the top and bottom of the cell using method I of van Holde and Baldwin (van Holde and Baldwin, 1958). The true molecular weight was obtained by extrapolating a plot of the reciprocal apparent molecular weight *vs.* concentration to infinite dilution (Wales *et al.*, 1951), the data being linear for the system studied here. The true molecular weight of a very high molecular weight synthetic polymer in a good solvent is underestimated by this extrapolation (Mandelkern *et al.*, 1957). The extrapolation can be justified in the present case, however, because of low molecular weights and the narrow molecular weight distributions.

The weight-average molecular weights of SCC and L2 were determined at a single polymer concentration by M. H. Liberman (Liberman, 1968). Based on the more detailed study of the sedimentation equilibrium of MAN and MIL, the failure to extrapolate the results for L2 and SCC to infinite dilution results in a negligible error. The data in Table I show that the molecular weight distribution of SCC, MAN, and MIL are extremely narrow. The molecular weight distribution of L2 is slightly broader than that of the other three samples.

Viscosity. Flow times were measured using Cannon-Ubbelohde semimicro dilution viscometers in baths with the temperature controlled to ±0.03°. Kinetic energy corrections are not required with these viscometers. Flow times for solvents were 122–482 sec.

Circular Dichroism and Optical Rotation. Circular dichroism and optical rotation were measured using a Durrum-Jasco recording spectropolarimeter, Model ORD/CD/UV5. The circular dichroism had been calibrated using a purified and dried sample of 10-camphorsulfonic acid (DeTar, 1969). Temperature control was achieved by circulating water from a constant-temperature bath through jacketed cells or through a hollow metal cell holder when nonjacketed cells were used. The relationship between the temperature in the cell and the temperature in the bath was determined by measuring the former with a thermocouple. Cells with light paths from 0.2 to 5 mm were used. Polymer concentrations were approximately 0.2 mg/ml when measuring circular

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[1] We have found, in some cases, that the molecular weights furnished by the manufacturers are grossly inaccurate and can be in error by as much as a factor of six. Clearly, therefore, if the specification of the molecular weight is important for a particular investigation it is imperative that it be independently determined by an absolute method.

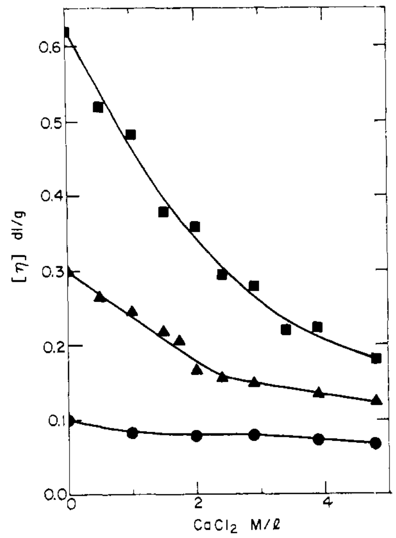

FIGURE 1: Intrinsic viscosity of poly-L-proline MIL (■), MAN (▲), and SCC (●) at 30° as a function of calcium chloride concentration.

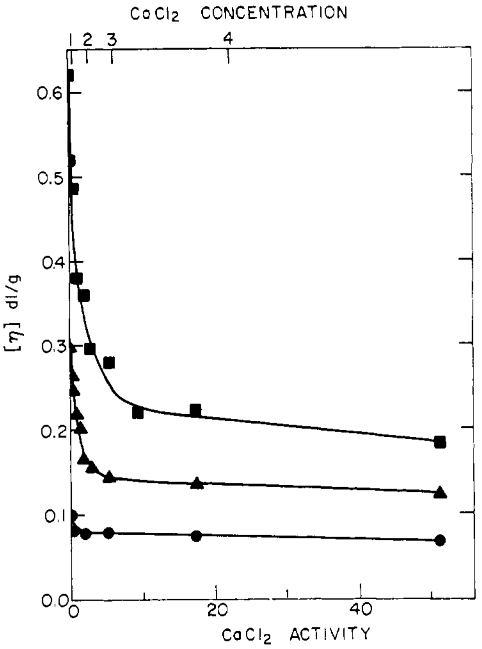

FIGURE 2: Intrinsic viscosity of poly-L-proline MIL (■), MAN (▲), and SCC (●) at 30° as a function of calcium chloride activity. The activity is that of calcium chloride at 25°.

dichroism and 6–11 mg/ml when measuring optical rotation.

Computer Resolution. The resolution of the circular dichroism into component Gaussian curves of the form

$$\Delta E_{\nu} = \Delta E_{\mathrm{M}} \exp\left[-\left(\frac{\nu - \nu_{\mathrm{M}}}{\omega}\right)^{2}\right] \qquad (1)$$

was accomplished using a Control Data Corp. 6400 computer and computer program CDORD written and made available to us by Professor DeTar. ΔE is the circular dichroism in cm^2/mmole, ν is the frequency in cm^{-1}, and ω is the half-width in cm^{-1} of the Gaussian curve where ΔE has fallen to $\Delta E_{\mathrm{M}}/e$. The subscript M refers to values at the extrema. The calculations were made on the basis of wave numbers. Refractive index corrections were not applied. When two Gaussian curves were required to reproduce the observed spectrum, the following procedure was used to determine the range of uncertainty in the Gaussian parameters. Superscript + and − refer to the positive and negative Gaussian curves, respectively. Trial values of the Gaussian parameters were estimated by inspection of the observed spectrum. ν_{M}^{+} was held constant, and the computer adjusted ω^{+}, $\Delta E_{\mathrm{M}}^{+}$, ν_{M}^{-}, ω^{-}, and $\Delta E_{\mathrm{M}}^{-}$ to fit the observed spectrum. Tolerances of the five adjusted parameters were all 1%. Then ν_{M}^{+} was decreased by 20 to 100 cm^{-1} and the computer readjusted the five variable parameters to tolerances of 1%. The stepwise decrease in ν_{M}^{+} was continued until the quality of the fit had markedly deteriorated. The same procedure was then followed by making stepwise small increases in ν_{M}^{+} above the original trial value.

The program CDORD evaluated the standard deviation in ΔE and the residuals for each computed fit. The residual at each frequency is the observed circular dichroism minus the contributions of the computed Gaussian curves at that frequency. Ideally the absolute value of all residuals would be small and equal to the standard deviation, and the residuals would be randomly distributed on either side of zero. The best fit to the observed spectrum was chosen by requiring the residuals to be as close as possible to the ideal case. The standard deviation in ΔE for the curves designated as best fitting the observed spectra ranged from 0.19 to 0.65% of the maximum observed ΔE.

The ν_{M}^{+} associated with the combination of Gaussian curves giving the best fit to the observed spectrum will now be designated $\nu_{\mathrm{M,FIT}}^{+}$. The series of computed Gaussian curves was then examined in order of decreasing ν_{M}^{+} for values less than $\nu_{\mathrm{M,FIT}}^{+}$. The Gaussian curves associated with each such ν_{M}^{+} were classified as also fitting the observed spectrum until one set was found for which either (1) the distribution of the residuals was not as random as in the spectrum associated with $\nu_{\mathrm{M,FIT}}^{+}$, (2) the computed standard deviation in ΔE became 1.1 times the value associated with $\nu_{\mathrm{M,FIT}}^{+}$, or (3) the calculation diverged. The same procedure was then followed for the set of Gaussian curves with ν_{M}^{+} greater tha $\nu_{\mathrm{M,FIT}}^{+}$. The range of possible values for the area under a Gaussian curve was obtained by inserting the extreme values of ΔE_{M} and ω for that curve into the relationship $0.5 \times (\pi)^{0.5}(\Delta E_{\mathrm{M}})(\omega)$.

Results

Intrinsic Viscosity. The intrinsic viscosity of SCC, MAN, and MIL at 30° is shown as a function of calcium chloride concentration in Figure 1. The addition of calcium chloride brings about a systematic reduction in the intrinsic viscosity which is very substantial for the higher molecular weight sample. These changes in the intrinsic viscosity are reversible. If the calcium chloride is removed by exhaustive dialysis against water and the polymer recovered by lyophilization, the initial intrinsic viscosity is reproduced upon solution in water. The change in the intrinsic viscosity on going from

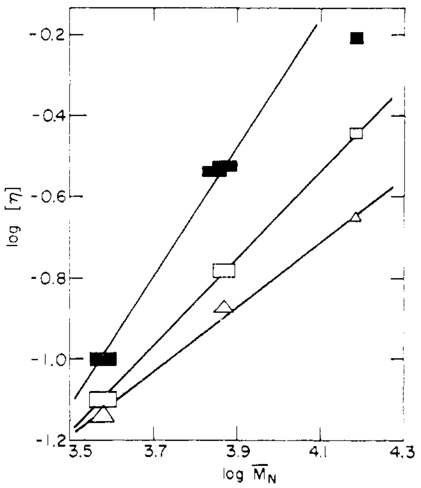

FIGURE 3: Logarithm of the intrinsic viscosity in dl/g plotted against the logarithm of the number-average molecular weight of poly-L-proline in water (■), 2.00 M (□), and 3.90 M (△) calcium chloride.

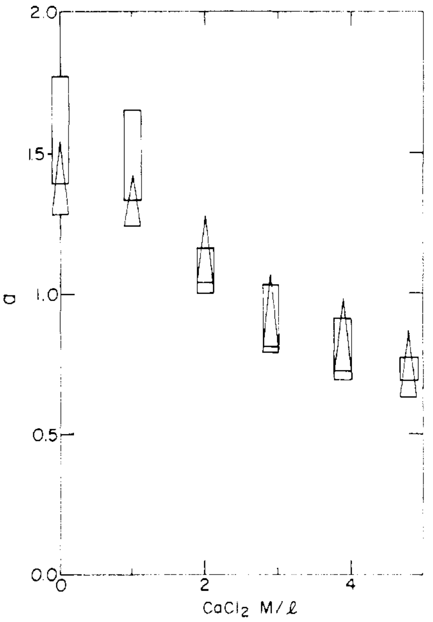

FIGURE 4: Variation of the exponent of the molecular weight dependence of the intrinsic viscosity of poly-L-proline with calcium chloride concentration at 30°. The molecular weights used were the number-average molecular weight (□) and the weight-average molecular weight (△). In water and 1.00 M calcium chloride, *a* derived from the number-average molecular weights was obtained using only SCC, L2, and MAN.

water to 4.80 M calcium chloride becomes much larger in both absolute and relative terms as the molecular weight of the poly-L-proline increases. The value in 4.80 M calcium chloride is 62 and 29% of the value in water for SCC and MIL, respectively. In each case the intrinsic viscosity changes most rapidly at the lower concentrations of calcium chloride and levels off as the concentration of calcium chloride increases. The change in the intrinsic viscosity of MIL and MAN is half-completed near 1.5 M calcium chloride.

The same data are plotted as a function of the activity of calcium chloride in Figure 2. The activity of calcium chloride was calculated using the molal activity coefficient at 25° (Lewis and Randall, 1961) and tabulated densities for aqueous calcium chloride (Washburn, 1928). When plotted in this manner it becomes more apparent that the intrinsic viscosity experiences a rapid initial decrease with added salt and then levels off. The leveling off occurs at a concentration corresponding to about 3 M calcium chloride.

The variation of the intrinsic viscosity of a polymer with molecular weight can be described by the empirical equation

$$[\eta] = K'M^a \qquad (2)$$

where $[\eta]$ is the intrinsic viscosity, M the molecular weight, and K' and a are constants for a given solvent and temperature (Flory, 1953a). In Figure 3 the logarithm of the intrinsic viscosity is plotted against the logarithm of the number-average molecular weight of poly-L-proline in water, 2.00 M, and 3.90 M calcium chloride. The relative positions of the points for SCC, MAN, and MIL would be essentially the same if the weight-average molecular weights were used because these polymers have very narrow molecular weight distributions. The point corresponding to L2, however, would move further to the right as a consequence of its somewhat broader molecular weight distribution. In Figure 3 there is curvature in the data obtained in water. The slope defined by the three polymers of lowest molecular weight is 1.6. If the weight-average molecular weight were used, the point for L2 would move far enough to the right to allow a straight line with a slope of 1.4 to give a reasonable fit to the data. In 1.00 M calcium chloride (data not shown) there is still curvature when the results are plotted using the number-average molecular weight, and the curvature disappears when the weight-average molecular weight is used. At concentrations of calcium chloride of 2.00 M or higher, the data can be well represented by a straight line for either type of average molecular weight.

The values of the slope *a* are presented as a function of calcium chloride concentration in Figure 4. The size of the symbols reflects the uncertainty in molecular weight and intrinsic viscosity. The exponent *a* is thus seen to vary from 1.6 in pure water to 0.7 at 4.80 M CaCl$_2$. The results presented in Figure 4 are qualitatively similar to the plot of intrinsic viscosity *vs.* calcium chloride concentration in Figure 1. In both cases the major change occurs at the lower concentrations of calcium chloride and there is a leveling off at the higher salt concentrations.

Circular Dichroism. The circular dichroism of MAN in water and in different concentrations of calcium chloride at 30° is shown in Figure 5. The other poly-L-proline samples yielded similar spectra. In water at 30°, MAN exhibits a minimum at 206 mμ with a ΔE of -13, a maximum at 228 mμ with a ΔE of $+0.5$, and a crossover at 224 mμ. No other features are observed down to 190 mμ. Literature values for ΔE at the maximum are generally in the range of 0.4 to 0.6 (Carver *et al.*, 1966; Timasheff *et al.*, 1967; Tiffany and Krimm, 1968), although there is one report of a value nearly an order of magnitude lower (Okabayashi *et al.*, 1968). Literature values for ΔE at the minimum vary from -9 to -16 (Carver *et al.*, 1966; Timasheff *et al.*, 1967;

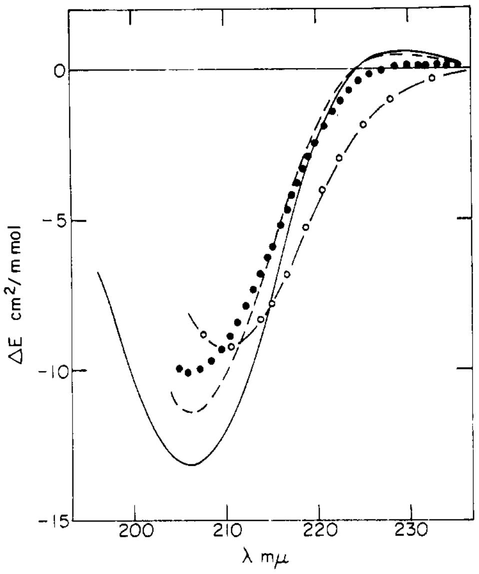

FIGURE 5: Circular dichroism of poly-L-proline MAN at 30° in water (—), 2.00 M (---), 3.90 M (••••), and 5.75 M (-O-O), calcium chloride.

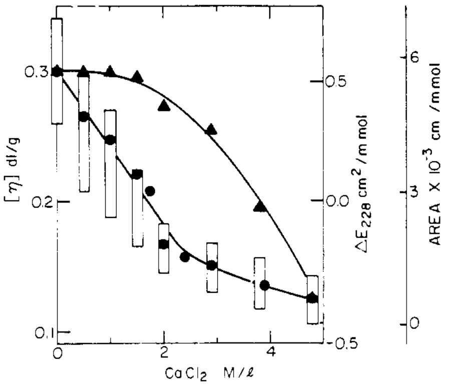

FIGURE 6: Intrinsic viscosity (●), ΔE_{228} (▲), and area under the resolved positive Gaussian circular dichroism band (bars) for poly-L-proline MAN at 30° as a function of calcium chloride concentration. The scales are adjusted so that all points in water and in 4.80 M calcium chloride are coincident.

Tiffany and Krimm, 1968; Okabayashi *et al.*, 1968). In agreement with the work of Tiffany and Krimm (Tiffany and Krimm, 1968), it was found that the magnitude of the minimum is reduced and that the maximum disappears in the most concentrated calcium chloride solution. Figure 5 also reveals that there is no isosbestic point and that the minimum undergoes a red shift of about 4 mμ on going from water to 5.75 M calcium chloride. These latter two features are not apparent in the data reported by Tiffany and Krimm.

The triangles in Figure 6 represent the value of ΔE at 228 mμ, the wavelength at which the maximum is observed in water, as a function of calcium chloride concentration. The circles in Figure 6 represent the intrinsic viscosity of MAN at 30°. The intrinsic viscosity and observed ΔE_{228} clearly do not parallel one another as the salt concentration is increased. The intrinsic viscosity changes rapidly at low concentrations of calcium chloride and levels off at higher concentrations. On the other hand, there is essentially no difference between the value of ΔE_{228} in water and 1.00 M calcium chloride. As the concentration of calcium chloride is increased above 1.00 M, ΔE_{228} becomes more negative. This trend accelerates as the concentration of calcium chloride increases. The intrinsic viscosity has attained half of its total change at about 1.5 M calcium chloride, but ΔE_{228} does not reach half of its change over this concentration range until nearly 4 M calcium chloride.

In Figure 7 the circles again represent the intrinsic viscosity of MAN at 30° and the triangles represent the observed magnitude of ΔE at 206 mμ, the wavelength at which the minimum is observed in water. The latter is nearly a linear function of calcium chloride concentration. Thus ΔE_{206} does not change in the same manner as does the intrinsic viscosity, although this apparent discrepancy is not as dramatic as in the case of ΔE_{228}.

Inspection of Figure 5 suggests that ΔE_{228} may not be a linear function of the intensity of the positive band of the circular dichroism. If the much more intense negative band makes a substantial contribution to the observed ΔE_{228}, a change in ΔE_{228} might be due to a change in the negative band, a change in the positive band, or a change in both bands. Alternatively, a change in the contribution of the positive band to ΔE_{228} might be exactly compensated by a change in the contribution from the negative band. It is immediately obvious from inspection of Figure 5 that the negative band experiences changes in ΔE_M and ν_M, and perhaps also in ω. The causes of the changes in ΔE_{228} may therefore be quite complex. If the negative band experiences changes in ω and ν_M, ΔE_{206} need not be a linear function of the intensity of the negative band.

In order to investigate these possibilities, the spectra were resolved into the minimum number of component Gaussian curves which would reproduce the original spectra. Details of this procedure are given in the Materials and Methods section. The spectrum in 5.75 M calcium chloride could be reproduced by a single negative Gaussian curve. Therefore the positive band does indeed disappear in very concentrated calcium chloride. The uncertainty in the resolution of the spectrum obtained in 4.80 M calcium chloride

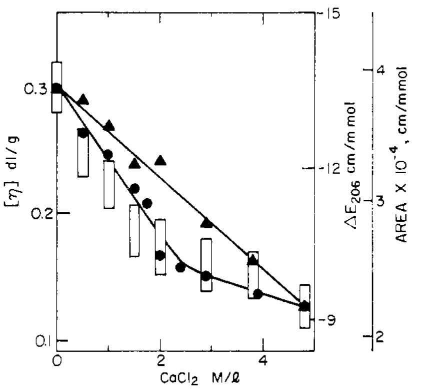

FIGURE 7: Intrinsic viscosity (●), ΔE_{206} (▲), and area under the resolved negative Gaussian circular dichroism band (bars) for poly-L-proline MAN at 30° as a function of calcium chloride concentration. The scales are adjusted so that all points in water and in 4.80 M calcium chloride are coincident.

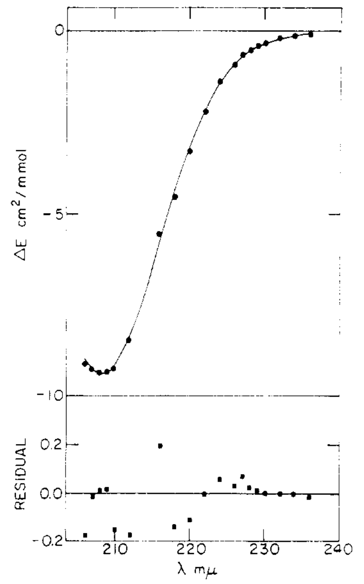

FIGURE 8: Best possible fit to the observed circular dichroism (●) of poly-L-proline MAN at 30° in 4.80 M calcium chloride using a single negative Gaussian curve (—). The residual (■) is the difference between the observed circular dichroism and the Gaussian curve. The computed standard deviation in ΔE is 0.031 cm²/mmole.

TABLE II: Comparison of the Resolved Gaussian Parameters for Poly-L-proline Form II in Water.

Parameter[a]	This Work	Carver *et al.* (1966)[b]
ΔE_M^+, cm²/mmole	3.2 ± 0.6	3.7 ± 0.8
ν_M^+, cm⁻¹	45,220 ± 180	45,250 ± 120
ω^+, cm⁻¹	2,040 ± 90	2,150 ± 70
ΔE_M^-, cm²/mmole	−13.8 ± 0.4	−16.6 ± 0.9
ν_M^-, cm⁻¹	48,500 ± 200	48,330 ± 70
ω^-, cm⁻¹	3,130 ± 90	3,130 ± 80

[a] ΔE_M is the value at the maximum in the Gaussian curve, ν_M is the location of the maximum, and ω is the half-width, where $\Delta E = (\Delta E_M)(e)^{-1}$. Superscripts + and − refer to the positive and negative Gaussian curves, respectively. [b] Carver *et al.* report the Gaussian parameters in different terms. Their results have been converted into the units used here to facilitate comparison. In order to remove the refractive index correction, the term $3(n^2 + 2)^{-1}$ was assumed to be 0.76 and 0.75 for the positive and negative bands, respectively (Yang, 1967); n is the refractive index of water.

is such that it is not possible to decide whether it is reproduced better by a single negative Gaussian curve or by the addition of a small positive Gaussian curve at higher wavelengths. Figure 8 represents the best fit obtained for this salt concentration using a single negative Gaussian curve. Figure 9 shows the result obtained using two Gaussian curves. It is not possible to choose which is the better fit on the basis of the magnitude of the residuals, the appearance of a pattern in the residuals, or the magnitude of the computed standard deviation in ΔE, which is 0.031 cm²/mmole in Figure 8 and 0.030 cm²/mmole in Figure 9. With the spectra obtained at concentrations of calcium chloride of 3.80 M or less, two Gaussian curves of opposite sign were required to fit the observed spectra. Similar results were obtained when the circular dichroism spectra of SCC and L2 in water and at various concentrations of calcium chloride were resolved.

The values for the parameters characterizing the Gaussian curves which reproduce the circular dichroism of MAN in water at 30° are given in Table II. They are compared with values obtained by Carver *et al.* (Carver *et al.*, 1966). The agreement in the band locations, widths, and the relative values at the extrema is excellent. There is a discrepancy of about 20% in the absolute values at the extremes. This might be due to differences in the calibration of the circular dichroism. In any case, the disagreement is small compared with the variation in the values in the literature for the observed minimum and maximum in the circular dichroism of poly-L-proline form II in water (Carver *et al.*, 1966; Timasheff *et al.*, 1967; Okabayashi *et al.*, 1968; Tiffany and Krimm, 1968). Even when allowance is made for the uncertainty in the resolution of the spectra, the salient features of the analysis emerge clearly. It is obvious that the change in the areas under *each* of the Gaussian curves follows the same path as do the changes in the intrinsic viscosity (see Figures 6 and 7). Therefore the transition brought about by the addition of calcium chloride is accompanied by simultaneous changes in the hydrodynamic and optical properties when the circular dichroism is analyzed in the manner described.

The change in ΔE at a particular wavelength may follow a different course because the Gaussian bands experience changes in their locations and widths as well as in their maxima. In the case of ΔE_{228}, the contribution from the positive Gaussian band at this wavelength decreases throughout the range of calcium chloride concentration. The decrease is most rapid on going from water to 2 M calcium chloride. The contribution of the negative Gaussian band to ΔE_{228} becomes substantially less negative due to decreases in ΔE_M and ω^- on going from water to 2 M calcium chloride. This almost exactly compensates for the changes in the contribution from the positive band, and the result is little change in the observed ΔE_{228}. At the other end of the concentration range, from 3.80 to 5.75 M calcium chloride, the contribution of the negative Gaussian band to ΔE_{228} becomes increasingly more negative with increasing calcium chloride concentration due to the red shift of the negative Gaussian band. As a result the observed ΔE_{228} rapidly becomes more negative as the concentration of calcium chloride increases.

Optical Rotation. The optical rotatory dispersion of MAN was obtained in the visible and near ultraviolet at 30° as a function of calcium chloride concentration. The polymer concentrations were within the range used to obtain the intrinsic viscosity of MAN. At a constant wavelength the specific rotation is nearly a linear function of calcium chloride concentration with only a slight tendency to level off at the higher concentrations. In a recent study of poly-L-proline in aqueous solution (Schleich and von Hippel, 1969), it was reported that, when corrected for the change in refractive index of the solvent, the specific rotation at 546 mμ is a linear function of salt concentration for a large number of salts.

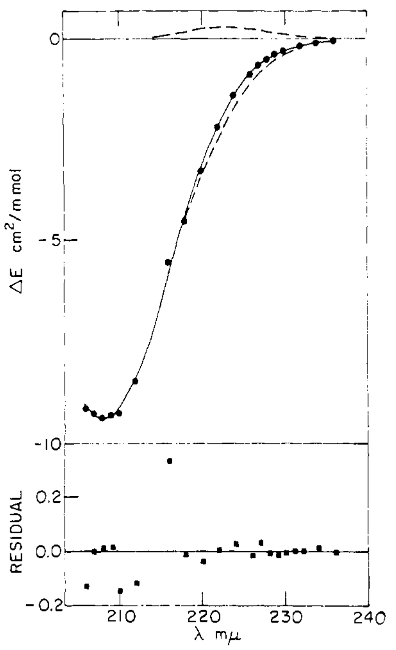

FIGURE 9: A fit to the observed circular dichroism (●) of poly-L-proline MAN at 30° in 4.80 M calcium chloride using two Gaussian curves (---) of opposite sign. The summation of the Gaussian curves is shown by the solid line. The computed standard deviation in ΔE is 0.030 cm²/mmole.

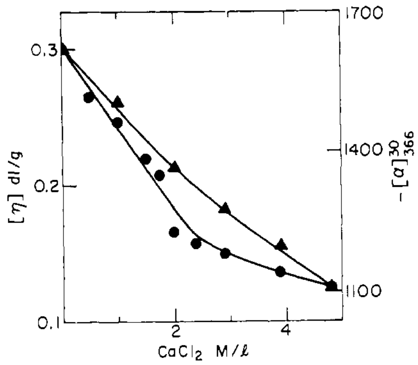

FIGURE 10: Specific rotation at 366 mμ (▲) and the intrinsic viscosity (●) of poly-L-proline MAN at 30° as a function of calcium chloride concentration. The specific rotation was measured at polymer concentrations similar to those used to determine the intrinsic viscosity.

The relationship between the change in the specific rotation at 366 mμ and the change in the intrinsic viscosity is shown in Figure 10. The relative change in the intrinsic viscosity from 2 to 4.8 M calcium chloride is much less than the relative change in the specific rotation.

Even though the circular dichroism of poly-L-proline form II in water exhibits two Cotton effects above 200 mμ, the optical rotatory dispersion at high wavelengths obeys a one-term Drude equation (Blout and Fasman, 1957; Harrington and Sela, 1958; Gratzer *et al.*, 1963) of the form

$$[\alpha]_\lambda = \frac{A}{\lambda^2 - \lambda_0^2} \tag{3}$$

where $[\alpha]_\lambda$ is the specific rotation at wavelength λ, and λ_0 and A are constants for a given temperature and solvent. Linear plots of $[\alpha]_\lambda\lambda^2$ *vs.* $[\alpha]_\lambda$ were obtained using data from the wavelength range 290 to 600 mμ. The constant A became less negative as the concentration of calcium chloride increased. From 0 to 2.90 M calcium chloride, λ_0 was constant at 204 ± 1 mμ. At higher calcium chloride concentrations λ_0 experienced a red shift, reaching 213 mμ in 5.75 M calcium chloride.

Discussion

This detailed study of the behavior of the intrinsic viscosity of poly-L-proline as a function of molecular weight and calcium chloride concentration establishes that poly-L-proline undergoes a conformational change in concentrated aqueous calcium chloride. Previous studies of the change produced in the intrinsic viscosity of poly-L-proline in concentrated aqueous solutions of neutral salts which disrupt the ordered structures of fibrous and globular proteins have been limited to obtaining results at a single high concentration of lithium bromide (Harrington and Sela, 1958; Steinberg *et al.*, 1960), at two concentrations of potassium thiocyanate (Blout and Fasman, 1957), and at four concentrations of potassium thiocyanate (Ciferri and Orofino, 1966). In each case the intrinsic viscosity was reduced in the concentrated salt solutions. The change in the intrinsic viscosity implies that a conformational change has occurred, but does not define the conformational states involved. In none of these studies was the intrinsic viscosity in the aqueous salt solutions examined as a function of the molecular weight of poly-L-proline.

The theory of solutions of linear polymers predicts that the exponent a in eq 2 should be 1.5 to 1.8 for a rigid rod-like polymer and 0.5 to 0.8 for a random coil (Benoit *et al.*, 1967; Flory, 1953b). The application of these values to the results presented in Figure 4 defines the conformational change produced in poly-L-proline by calcium chloride. The high value of a in water reflects a rod-like behavior over a substantial number of residues. The polymer is not completely rigid, as evidenced by a decreasing with increasing number-average molecular weight and being at the lower limit of the range associated with rod-like behavior when evaluated from the weight-average molecular weight. These results would be anticipated for a polymer possessing a small but finite degree of rotational freedom at each residue. Experimental studies of hydrodynamic properties from other laboratories also indicates the restricted conformational freedom of poly-L-proline (form II) in water (Harrington and Sela, 1958; Steinberg *et al.*, 1960).

This interpretation is consistent with the theoretical conformational analysis of Schimmel and Flory (Schimmel and Flory, 1967). These authors presented their results as the characteristic ratio, $\langle r^2\rangle_0/xl^2$, as a function of the degree of polymerization. In the above x is one less than the degree of polymerization, l is the distance between adjacent α carbons, and r is the end-to-end distance. If the molecule had precisely that conformation deduced for poly-L-proline form II in the solid state (Cowan and McGavin, 1955; Sasisekharan, 1959), a plot of the linear end-to-end distance against degree of polymerization would exhibit a straight line with slope

3.12 Å per residue. The linear end-to-end distance obtained from the results of Schimmel and Flory is less than that predicted from the crystallographic work for a polymer of the size of MIL, but approaches the crystallographic prediction as x goes to zero.

Theoretically, the value of the exponent a could assume a value as large as 0.8 for the random coil conformation in a good solvent (Flory, 1953a) The exact utilization of this limit would indicate that poly-L-proline attains this conformation in the range 3–4 M $CaCl_2$. However, there are several reasons why this concentration and value of a may not precisely represent the onset of random coil behavior. One of these derives from the restricted molecular weight range presently available for study which limits the experimental accuracy to which the quantity a can be delineated.

Another reason for caution is that for samples of the relatively low degree of polymerization studied here the theoretical possibility exists that the constant in Figure 3 could change in the low molecular weight range (Kirkwood and Riseman, 1948; Flory, 1953a). For synthetic polymers, such as polystyrene and polyisobutylene when studied under Θ conditions, the constants are maintained down to very low degrees of polymerization (Krigbaum *et al.*, 1952).

However, a review of the results obtained with low molecular weight samples of ten different polymers possessing a random coil conformation suggest that deviations from eq 2 could be encountered in good solvents (Bianchi and Peterlin, 1968). A plot of the logarithm of the intrinsic viscosity *vs.* the logarithm of the molecular weight exhibits either upward or downward curvature at low molecular weights. Since the manner in which a changes at very low molecular weights is dependent upon the type of polymer and solvent, it would be most helpful to know how a behaves with low molecular weight polypeptides in a good solvent. Pertinent data are available for three kinds of disordered polypeptides. In only one case is deviation form eq 2 observed in the degree of polymerization range of interest. Poly-γ-benzyl-L-glutamate in dichloroacetic acid obeys eq 2 with a equal to 0.87 to 0.89 for degrees of polymerization from 100 to 2000 (Doty *et al.*, 1956; Bradbury and Fenn, 1968). There are deviations from this behavior at lower degrees of polymerization (Mitchell *et al.*, 1957; Bradbury and Fenn, 1968). The most representative a for the degrees of polymerization corresponding to the poly-L-proline samples is 0.53 to 0.60. Polysarcosine of degree of polymerization 94 to 228 was found to obey eq 2 in water (Fessler and Ogston, 1951). The best a was 1.0, but the standard deviation was large enough so that the authors suggested the true value might be as low as 0.90. It has been demonstrated (Tanford *et al.*, 1967a; Nozaki and Tanford, 1967; Tanford *et al.*, 1967b; Lapanje and Tanford, 1967) that a variety of proteins behave as random coils in 6 M guanidine hydrochloride containing 0.1 M mercaptoethanol at 25°. A linear relationship was observed between the logarithm of the intrinsic viscosity and the logarithm of the viscosity average number of amino acid residues per chain with a equal to 0.66. This relationship was valid for chains containing an average of from 26 to 627 amino acid residues. It can be concluded that the values of a associated with random coil behavior for polypeptides of the degree of polymerization studied here could be as low as 0.5 and as high as 0.9, or perhaps even 1.0.

With this background, the results shown in Figures 3 and 4 can be interpreted as follows: In accord with theoretical calculations, in water poly-L-proline exists as a rod with limited flexibility, based on a being near 1.5. In 4.8 M calcium chloride poly-L-proline exists as a random coil, based on a being less than 0.8. By this criteria random coil behavior is well established at 4 M calcium chloride, and could have been attained at concentrations as low as 2.5 M calcium chloride. It should be reemphasized at this point that when the intrinsic viscosity, for a given molecular weight, is plotted against calcium chloride activity, as in Figure 2, the transition is completed by about 3 M calcium chloride. This observation is consistent with the behavior of the exponent a. The extent of the degree of cooperativity of this structural change is difficult to define. However, it is clear that it does not exhibit the high degree of cooperativity observed in the transformation from form I to form II (Gornick *et al.*, 1964; Engel, 1966), the disruption of the α helix (Zimm *et al.*, 1959), or in the denaturation of fibrous (von Hippel and Wong, 1963; Mandelkern and Villarico, 1969) and globular (Mandelkern and Roberts, 1961; von Hippel and Wong, 1965) proteins.

The disruption of the poly-L-proline form II structure also causes changes in the circular dichroism. The most obvious change is the loss of the positive band in the extremely concentrated calcium chloride solutions. However, as has been previously indicated, the magnitude of the circular dichroism at the wavelength at which the maximum is observed, or at any other wavelength, does not change in the same manner as does the intrinsic viscosity. This apparent contradiction between the intrinsic viscosity and the circular dichroism results can be eliminated by resolution of the circular dichroism spectra into component Gaussian bands. The area under *both* Gaussian bands changes in concert throughout the calcium chloride concentration range. These changes occur in unison with the changes in the intrinsic viscosity. The transformation of the poly-L-proline form II helix to the random coil is accompanied by simultaneous changes in the circular dichroism and the intrinsic viscosity, and circular dichroism can be used to measure the transition if the spectra are resolved into component Gaussian curves.

Since poly-L-proline in aqueous calcium chloride at 30° has the hydrodynamic properties of a random coil at 4 M calcium chloride, and may have attained this state at 2.5 M calcium chloride, it is correct to conclude that when the observed positive peak in the circular dichroism disappears, poly-L-proline has a random conformation (Tiffany and Krimm, 1968). However, it may be quite erroneous to identify this point with the onset of random coil behavior. A small positive Gaussian band is present in the circular dichroism at 3.80 M, and perhaps also at 4.80 M, calcium chloride. It is not contradictory for a small Gaussian band to be present in the circular dichroism at the same time that random coil hydrodynamics is observed. Order over only a very few residues is sufficient to develop this band (Okabayashi *et al.*, 1968). Therefore, the polymer could have a few short sequences of ordered or partially ordered residues and still exhibit the statistical properties for random coil hydrodynamics.

The ability to fit the circular dichroism spectrum in water by using two Gaussian curves is in agreement with Pysh's theoretical treatment of the circular dichroism of poly-L-proline form II (Pysh, 1967). However, there has been a recent report (Rosenheck *et al.*, 1969) on the linear dichroism of oriented poly-L-proline films which is in disagreement with this theoretical treatment. In the oriented film a band with parallel polarization is found at 209 mμ and a band with perpendicular polarization is found at 191 mμ. Presumably the band at 221 mμ is too weak to be observed. The theoretical treatment of Pysh predicts perpendicular polarization for the band at about 209 mμ and does not predict the existence of the band at 191 mμ.

Our ability to fit the observed spectra by using no more than two Gaussian curves does not necessarily contradict the results of Rosenheck *et al.* (1969). We have used measurements down to only 196 mμ in water and to only 204 to 206 mμ in the calcium chloride solutions. Rosenheck *et al.* indicate that the bands they observe in the film would probably undergo a blue shift upon solution. If the band appearing at 191 mμ in the film experiences a blue shift upon solution, and if it possesses only weak circular dichroism, it is possible that this band may make such a minor contribution in the spectral region we have examined that it does not interfere with the ability to resolve the spectra using only two Gaussian curves. This conclusion is supported by the method used by Timasheff *et al.* (1967) to resolve the circular dichroism spectrum of poly-L-proline form II from about 188 to 237 mμ in water into two Gaussian bands. They assumed that the circular dichroism due to the negative Gaussian band was symmetrical about 207 mμ and that the circular dichroism at wavelengths lower than 207 mμ was due entirely to this negative Gaussian band. Subtraction of the negative Gaussian band from the observed spectrum yielded a positive Gaussian band at 221 mμ. It is doubtful that such a result could have been obtained if a third circular dichroism band were making a significant contribution to the observed circular dichroism spectrum. The specific rotation in the region far to the red of the optical transitions in poly-L-proline becomes less negative as the calcium chloride concentration increases. The manner of the change in specific rotation differs from the change in the intrinsic viscosity in that there is only a hint at the leveling effect at the highest concentrations of calcium chloride studied. This may be caused by the specific rotation in this region being due not only to those bands which have been observed in the circular dichroism spectra but also to any other optically active electronic transitions located at shorter wavelengths. The solvent effects upon these latter electronic transitions is apparently sufficient to obscure the leveling effect in the bands located above 200 mμ.

The molecular mechanism by which calcium chloride and similar salts produce this conformational change is not reflected in these results. Various mechanisms for the effects of salts upon the conformations assumed by polypeptides and proteins have been suggested in the literature. Among these are an increase in the rotational freedom about the carbonyl carbon–α-carbon bond in poly-L-proline (Steinberg *et al.*, 1960; Kurtz and Harrington, 1966; Tiffany and Krimm, 1968; Schleich and von Hippel, 1969), an increase in rotational freedom about the peptide bond (Mandelkern *et al.*, 1962; Kurtz and Harrington, 1966), reducing the energy difference between the *cis* and *trans* isomers of a planar peptide bond (Harrington and Sela, 1958; Mandelkern *et al.*, 1962), and changes in the properties of the solvent (Harrington and von Hippel, 1961; von Hippel and Wong, 1965). It has been suggested that there may be direct binding of the salt to the peptide group (Mandelkern *et al.*, 1962; Mandelkern and Stewart, 1964; Kurtz and Harrington, 1966; Schleich and von Hippel, 1969).

It has not been possible to reproduce the circular dichroism spectra of poly-L-proline in concentrated calcium chloride using a linear combination of the spectra for poly-L-proline forms I and II. Accordingly it is not possible that the random state is made up of reasonably long alternating sequences of residues in one form and then the other. However, if the length of the sequence of residues with a particular conformation of the peptide bond was always very short, the circular dichroism would not be expected to be that obtained by the linear combination of the spectra for long sequences. The barrier to *cis–trans* isomerization in poly-L-proline is due in part to electrostatic interactions (Holzwarth and Backman, 1969). These interactions would certainly be altered if salt were to bind to the peptide group. If binding of salt were to cause a significant reduction in this electrostatic barrier, *cis–trans* isomerization could be possible. It is clear that other types of analysis and experiment need to be employed in order to establish the detailed changes in bond structure which result in the conformational change.

Acknowledgments

We wish to thank Professor DeTar for providing us with the program CDORD and for discussions concerning its use. We would also like to thank Professor Krimm for making available preprints prior to publication.

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