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The Configurational Changes of Poly-L-proline in Solution

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Two forms of poly-L-proline have been described in the literature. Form I of the polymer exhibits a specific optical rotation $[\alpha]_D$ +50° in acetic acid, whereas Form II shows $[\alpha]_D$ −540° in this solvent. As shown in this paper each of the two forms has a regular and characteristic configurational pattern which is reflected in its hydrodynamic properties as measured by sedimentation and viscosity. The two different homogeneous structures give differing configurational contributions to the specific optical rotation as deduced from a comparison of their rotatory properties with those of low molecular weight prolyl peptides. The unique configuration of each form can be altered in the presence of various solvent systems, whereas low molecular weight prolyl derivatives show only minor variations in rotatory properties in these same solvents. The kinetics of forward mutarotation of Form I in acetic acid and reverse mutarotation of Form II in acetic acid–propanol have been examined at several temperatures between 30 and 45°. From this information an enthalpy of activation of about $\Delta H^* = 20$ kcal./mole prolyl residue was calculated for both reactions. Strong acids such as perchloric acid were found to catalyze both forward and reverse mutarotation processes. These strong acids were shown to combine with the imide peptide bond of proline polymers. The catalytic effect of protons on the mutarotation rate is explained by the assumption that Form I of the polymer has all peptide bonds in the *cis* configuration in solution, whereas in Form II the peptide bonds exist in the *trans* configuration and thus transition from one form to the other in the presence of acids is brought about through the loss of double bond character at the peptide linkage. The kinetics of the transition Form I → Form II have been analyzed and a general formula derived to describe the intramolecular *cis–trans* isomerizations along the macromolecular chain. It has been demonstrated that the transformations in the configuration of Form II in the presence of some neutral salts does not arise from *trans–cis* isomerizations at the peptide bonds but probably involves a *trans′–cis′* isomerization at the α carbon–carbonyl carbon bond. Studies of the viscosity and optical rotatory properties of different samples of poly-L-proline (mol. wt. 12,000–52,000) reveal that while the hydrodynamic properties of poly-L-proline II are determined primarily by the end-to-end distance along the individual molecules, the optical rotatory properties are a reflection of the helical pattern of relatively short segments (10–15 prolyl residues). When taken in conjunction, the hydrodynamic and rotatory properties of poly-L-proline II demonstrate that Form II of the polymer retains the left-handed helical configuration suggested by Cowan and McGavin for the structure in the solid state, but the individual molecules at high molecular weight behave as somewhat flexible rods. Similar studies on the structure of poly-L-proline I suggest that this species possesses a right-handed helical configuration in solution.

During the past few years theories on the structure of collagen have in general agreed that the proline and hydroxyproline residues, which amount to between 15 to 30% of the total weight of the protein, are of crucial importance in the molecular pattern of this substance. The elegant proposal of Ramachandran and Kartha,[2] that collagen might consist of three helical polypeptide chains coiled about each other in a three-stranded rope, has been given strong support by the X-ray studies of Rich and Crick[3,4] and Cowan, McGavin and North.[5] At the present time the most promising structure for collagen appears to be one in which each of the polypeptide chains making up the collagen molecule is coiled into a left-handed helix of the type described for poly-L-proline II by Cowan and McGavin[6] and for polyglycine II by Crick and Rich.[7] The striking similarity in the X-ray diffraction spacings of poly-L-proline II and stretched collagen

(1) Part of Ph.D. thesis to be submitted by I. Z. Steinberg to the Senate of the Hebrew University, Jerusalem, Israel.

(2) G. N. Ramachandran and G. Kartha, *Nature*, **174**, 269 (1954).

(3) A. Rich and F. H. C. Crick, *ibid.*, **176**, 915 (1955).

(4) A. Rich and F. H. C. Crick, "Recent Advances in Gelatin and Glue Research," G. Stainsby, Ed., Pergamon Press, London, 1958, p. 20.

(5) P. M. Cowan, S. McGavin and A. C. T. North, *Nature*, **176**, 1062 (1955).

(6) P. M. Cowan and S. McGavin, *ibid.*, **176**, 501 (1955); V. Sasisekharan, *Acta Cryst.*, **12**, 897 (1959).

(7) F. H. C. Crick and A. Rich, *Nature*, **176**, 780 (1955).

strongly suggest similar chain configurations, and it is therefore reasonable to suppose that a study of the solution properties of synthetic poly-L-proline and its copolymers should help substantially in understanding the chemistry of collagen.

The work of Kurtz, Berger and Katchalski[8] has demonstrated that the synthetic polymer, poly-L-proline, can exist in two forms which exhibit markedly different optical rotations. If the polymer is precipitated from the polymerization medium (pyridine) with ether, and redissolved in water[9] or acetic acid, its initial specific rotation, $[\alpha]^{25}D$, is + 50°. This material has been denoted Form I.[10,11] On standing at room temperature, however, the aqueous or the acetic acid solution slowly changes in rotation over a period of several days, reaching a final value of $[\alpha]^{25}D$ − 540°.[8,12] The polyproline with this specific rotation has been denoted Form II. The mutarotation may be greatly accelerated by heating, but once the state characterized by an $[\alpha]D$ − 540° has been reached, no further optical rotatory changes are observed. A number of other solvents including the aliphatic acids, benzyl alcohol and chloroethanol, also favor the mutarotation of Form I into Form II. Thus formic acid brings about the mutarotation at 30° in less than five minutes.

More recent studies demonstrate that these optical rotatory changes may be reversed by means of certain solvent systems.[13] Thus, dissolution of Form II in a solvent consisting of 10% acetic acid and 90% 1-propanol leads to a gradual decrease in levorotation yielding a final value of $[\alpha]D$ approaching that of Form I. Since this cyclic process of mutarotation in water (or in formic or acetic acid) followed by reverse mutarotation in the acetic (or formic) acid–1-propanol (or 1-butanol) system may be repeated indefinitely and since both Form I and Form II yield L-proline on hydrolysis,[8] it may be concluded that the variations in specific optical rotation observed are not induced by chemical changes but are rather a reflection of configurational transitions in the individual polymer molecules. This view is supported by the infrared absorption studies of Blout and Fasman[10] and of Steinberg, Berger and Katchalski[13] showing that Form I and Form II exhibit distinctly different infrared spectra in the solid state. Moreover, the X-ray diffraction powder diagrams of Form I[14] and Form II[6] suggest substantial differences between the molecular structure of the two forms.

A number of neutral salts in aqueous solution also bring about dramatic changes in the optical rotatory properties. Thus aqueous solutions of LiBr, $LiClO_4$[11] and NaSCN[10] lower the value of the specific levorotation of the sodium D line of poly-L-proline II by nearly 300°. This phenomenon is accompanied by a marked decrease in the viscosity of the polymer solution (at least in the case of the two salts which have been investigated, LiBr[11] and NaSCN[10]) suggesting, as in the reverse mutarotation in organic solvents, that the configurational pattern along the polypeptide chain has been altered.

The configurational changes in the polymers of proline in various solvents take on special significance when viewed against the background of the alterations in viscosity and optical rotation of collagen and gelatin as a function of temperature and solvent. Neutral salts such as LiBr, NaSCN and $CaCl_2$ effect drastic changes in the specific optical rotations of various collagens,[15–17] lowering their specific rotations by 250–300°. These same salts also have strong effects on the thermal shrinkage temperature of collagens. For example, NaSCN at a concentration of 6–8 *M* is capable of lowering the thermal shrinkage temperature of certain collagens to as low a value as 3°.[15] Since these salts strongly affect the optical rotatory and viscosity properties of polyproline, which is devoid of peptide hydrogen bonds, it seems possible that their mechanism of action on collagen may be of similar origin and may not necessarily be related to interchain hydrogen bond rupture.

It has been suggested[8] that the polyproline I–polyproline II interconversion results from a series of *cis–trans* isomerizations at the peptide bonds of the polymer. This possibility has been explored in an earlier paper[11] and shown to be consistent with observed viscosity, sedimentation and optical rotatory properties of the two forms. It was there suggested that Form I in solution is a right-handed helix with all peptide bonds in the *cis* configuration, whereas Form II in solution exhibits the configuration of the left-handed helix described by Cowan and McGavin[6] with all peptide bonds in the *trans* configuration.

In this paper we present a detailed study of the hydrodynamic and optical rotatory properties of poly-L-proline in various solvent systems, as well as of the kinetics of the interconversion of the two forms of polyproline. Conclusions as to the configurations of the polypeptide chain under these conditions, as well as to the pathway of the mutarotation, will be drawn in the light of the limited number of homogeneous structural patterns possible for this molecule.

Materials and Methods

Materials.—Gelatin, U.S.P. granular, was obtained from Fisher Scientific Company. Ichthyocol collagen was a gift from Dr. Paul N. Gallop. Lithium bromide, sodium thiocyanate, sodium acetate, sodium formate and perchloric acid were reagent grade and were used without further purification. Formic acid and glacial acetic acid were of analytical purity. Propionic acid, Eastman Kodak, was distilled before use. 1-Propanol and 1-butanol were dried for a week over calcium oxide and distilled. Acetic anhydride was kept for a week over metallic sodium and distilled.

(8) J. Kurtz, A. Berger and E. Katchalski, *Nature*, **178**, 1066 (1956).

(9) Only low molecular weight (under 5000) poly-L-proline I samples are water-soluble.

(10) E. R. Blout and G. D. Fasman, "Recent Advances in Gelatin and Glue Research," G. Stainsby, Ed., Pergamon Press, London, 1958, p. 122.

(11) W. F. Harrington and M. Sela, *Biochim. Biophys. Acta*, **27**, 24 (1958).

(12) J. Kurtz, A. Berger and E. Katchalski, "Recent Advances in Gelatin and Glue Research," G. Stainsby, Ed., Pergamon Press, London, 1958, p. 131.

(13) I. Z. Steinberg, A. Berger and E. Katchalski, *Biochim. Biophys. Acta*, **28**, 647 (1958).

(14) A. Rich, private communication.

(15) K. H. Gustavson, "The Chemistry and Reactivity of Collagen," Academic Press, Inc., New York, N. Y., 1956.

(16) D. C. Carpenter and F. E. Lovelace, THIS JOURNAL, **57**, 2337 (1935).

(17) W. F. Harrington, *Nature*, **181**, 997 (1958).

Dichloroethane was refluxed for 2 hr. over phosphorus pentoxide and distilled.

The synthesis of poly-L-proline, polyhydroxy-L-proline and poly-O-acetyl-hydroxy-L-proline has been described in earlier papers.[18,19] The various samples were stored in a desiccator over phosphorus pentoxide and all solutions of the polymers were made up by weight, assuming them to have a negligible water content.

Poly-DL-proline was prepared from N-carboxy-DL-proline anhydride in analogy to the synthesis of poly-L-proline from N-carboxy-L-proline anhydride.[18] The N-carboxy-DL-proline anhydride was prepared from DL-proline and phosgene, according to the improved procedure for the synthesis of N-carboxy-L-proline anhydride.[19] The N-carboxy-DL-proline anhydride was obtained as an oil.

p-Toluenesulfonyl-L-prolyl-L-proline was synthesized as reported earlier.[20]

L-Proline anhydride (L-proline diketopiperazine) was prepared from L-proline ethyl ester, at room temperature, by keeping it for several days in the desiccator over sulfuric acid.[21]

Methods.—Optical rotations and optical rotatory dispersions were measured with a Rudolph precision ultraviolet polarimeter, model 80, equipped with a Rudolph photoelectric polarimeter attachment and an oscillating polarizer prism. Some optical rotations at the sodium D line were also measured with a Rudolph Model 70 polarimeter. Dispersion studies included the spectral range from 350 to 750 mμ and were obtained through the use of a xenon compact arc lamp provided with the instrument. Water-jacketed polarimeter tubes were used in the kinetic studies; water from a thermostated water-bath served to control the temperature within ± 0.1°.

In some cases the specific rotations have been corrected for the index of refraction of the solvent according to the equation

$$[\alpha]^{20}_{W}\,(\text{corr.}) = [\alpha]^{20}_{S} \times \frac{n^2_{D,w} + 2}{n^2_{D,s} + 2}$$

where $[\alpha]^{20}_{S}$ is the specific optical rotation of the solution, $[\alpha]^{20}_{W}$ (corr.) is the expected specific optical rotation in water $n_{D,W}$ is the index of refraction of water at 20° (1.333), and $n_{D,s}$ is the index of refraction of solvent at 20°. Indices of refraction for the various solvents were obtained from Landolt–Börnstein Tabellen.[22]

Viscosity measurements were made either with 2 ml. or 10 ml. volume Ostwald–Fenske viscometers. Temperature was controlled within ± 0.03°.

Measurements of sedimentation velocity were obtained through the use of a Spinco Model E ultracentrifuge, operating at 59,780 r.p.m. at 20°. Temperature was controlled within ± 0.5° by means of the RTIC unit of this instrument. The synthetic boundary cell[23] was used in all velocity sedimentation experiments in view of the low sedimentation coefficients observed. The "approach to sedimentation equilibrium" technique of Archibald[24] was utilized to obtain molecular weight data on one sample of poly-L-proline II in water. In this study the operating speed of the centrifuge was 11,000 r.p.m. Ultracentrifuge patterns were analyzed by means of a two-way Hauser Profil P219, Switzerland, microcomparator. The concentration of the aqueous poly-L-proline II solution was determined from a synthetic boundary cell run, and the molecular weight calculated from the meniscus position according to the procedure described by Schachman.[25]

Osmotic pressure measurements were carried out in a modified Zimm–Myerson osmometer.[26]

The nuclear magnetic resonance spectrometer used in this investigation has been described in detail by Grunwald, Loewenstein and Meiboom.[27]

Anhydrous potentiometric titrations were carried out in acetic anhydride[28] purified as described above. The electrode pair used consisted of a Beckman glass electrode which had been immersed in acetic anhydride for at least 12 hr. and a calomel electrode, with a bridge of 0.1 *N* anhydrous lithium perchlorate in acetic anhydride. The titrant, 0.2 *N* perchloric acid in dichloroethane, was prepared by mixing 72% aqueous perchloric acid with the solvent and adding an amount of acetic anhydride equivalent to the amount of the water present. A clear solution was obtained. A direct reading *p*H-meter (Radiometer, Type TTT1, Copenhagen) was used for millivolt readings.

For titration, a weighed amount of polymer (poly-O-acetylhydroxy-L-proline) was dissolved in 5 ml. of acetic anhydride. Perchloric acid reagent was added from a micrometer syringe buret. A blank titration with 5 ml. of pure acetic anhydride was also run. The corrected titration curve was obtained by subtracting, at each millivolt reading, the volume required by the blank from that required by the polymer solution.

Results

Hydrodynamic Properties.—It seems clear that any proposal concerning the spatial distribution of the L-proline residues of Form I and Form II of poly-L-proline in solution should be consistent with the hydrodynamic properties of the two forms.

In earlier work,[11] sedimentation and viscosity studies on Form I were complicated by the fact that this substance is unstable in the solvents investigated, mutarotating rapidly in water, formic and acetic acids to Form II. Moreover, sedimentation patterns of low molecular weight Form I ($DP = 50$) in water revealed the presence of a significant amount (about 25%) of a high molecular weight aggregate ($S^{w}_{20} = 14$) which prevented an appraisal of hydrodynamic parameters before and after mutarotation. We have now found that mutarotation of Form I in propionic acid is a relatively slow process at room temperature, amounting to a rotatory change of only 17° in $[\alpha]^{25}D$ in 24 hr. Sedimentation studies on Form I in propionic acid reveal that in this solvent the polymer sediments with a single, nearly symmetrical, boundary at all concentrations examined. Fig. 1 presents a plot of sedimentation coefficient *versus* concentration of

TABLE I

VISCOSITY OF POLYPROLINE, FORM II

Polymer no.	Molecular weight	Intrinsic viscosity (dl./gr.) In water	In acetic or propionic acid[a]	Axial ratio Found[b]	Calcd.[c]
F8	12000[d]	0.35	0.66 (0.67)	33	49
P6	13200[e]		.86	39	54
P2	14300[d,f]	.55	.75 (0.75)	36	59
B4	19000[f,g]	.54	.90 (0.90)[h]	40	78
D33[i]	52000	.67	1.43	52	214

[a] Intrinsic viscosities in propionic acid are given in parentheses. [b] Calculated according to eq. 1. [c] Calculated from the average number of residues per polymer molecule, the unit translational distance per residue of the Cowan–McGavin helix[8] (3.12Å.) and the estimated hydrodynamic diameter (7.8Å.). [d] Determined from osmotic pressure measurements. [e] Determined from sedimentation and diffusion measurements. [f] Number average molecular weights, determined by dinitrophenylation[20] were 15000 for P2 and 19000 for B4. [g] For molecular weight determinations of this sample, see text. [h] $[\eta]$ in trifluoroacetic acid: 1.02 dl./g. [i] From Blout and Fasman.[10]

(18) A. Berger, J. Kurtz and E. Katchalski, THIS JOURNAL, **76**, 5552 (1954).

(19) J. Kurtz, G. D. Fasman, A. Berger and E. Katchalski, *ibid.*, **80**, 393 (1958).

(20) S. Sarid, A. Berger and E. Katchalski, *J. Biol. Chem.*, **284**, 1740 (1959).

(21) J. Kapfhammer and A. Matthes, *Z. physiol. Chem.*, **228**, 47 (1934).

(22) Landolt-Börnstein Tabellen E. I., 5th Ed., Berlin, 1931.

(23) E. G. Pickels, W. F. Harrington and H. K. Schachman, *Proc. Natl. Acad. Sci. U. S.*, **38**, 943 (1952).

(24) W. J. Archibald, *J. Phys. Chem.*, **51**, 1204 (1947).

(25) H. K. Schachman, "Methods in Enzymology," Vol. IV, Academic Press, Inc., New York, N. Y., 1957, p. 32.

(26) E. Riesel and A. Berger, *J. Polymer Sci.*, **37**, 337 (1959).

(27) E. Grunwald, A. Loewenstein and S. Meiboom, *J. Chem. Phys.*, **27**, 630 (1957).

(28) D. C. Wimer, *Anal. Chem.*, **30**, 77 (1958).

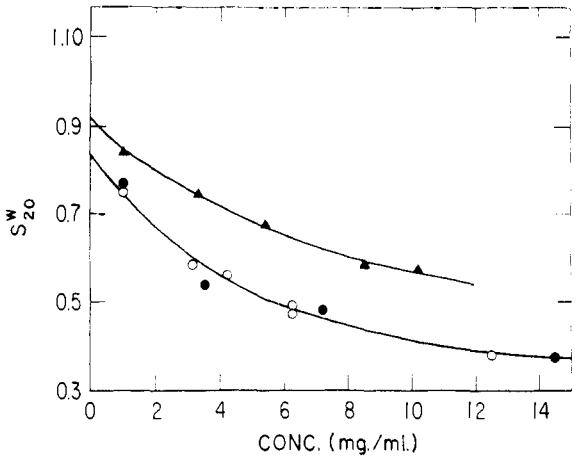


Fig. 1.—Sedimentation coefficients of poly-L-proline (sample B4) as a function of concentration: ▲-▲, form I in propionic acid; ●-●, form II in propionic acid; O-O, form II in acetic acid.

Form I in propionic acid ($[\alpha]^{25}D$ +50°) and Form II in propionic and acetic acids ($[\alpha]^{25}D$ −540°). All sedimentation studies were performed on sample B4 (see Table I).

The curves given in Fig. 1 for sample B4 show that, within the whole range of concentrations measured, the sedimentation coefficients of Form II are lower than those of Form I. Similarly, significant differences were observed between the reduced viscosity, η_{sp}/c, of Form I in propionic acid and Form II in either propionic or acetic acid (Fig. 2).

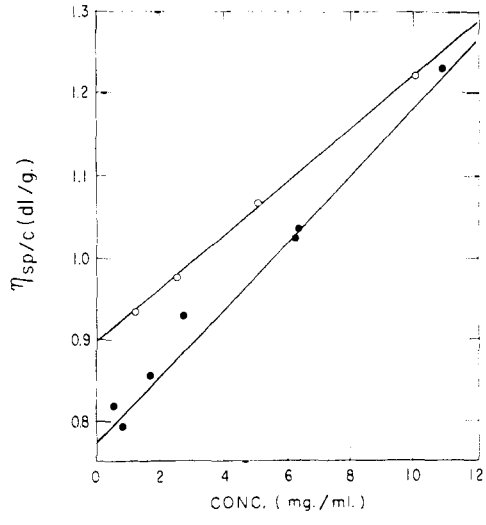


Fig. 2.—The reduced viscosity of poly-L-proline (sample B4) in propionic acid at 30° as a function of concentration: O-O, form II; ●-●, form I.

The weight average molecular weight of Form II, sample B4, in acetic acid was determined from an Archibald "approach to sedimentation equilibrium" study[24] at a concentration of 0.5%, giving 18,500 and 17,900 at times of 1 and 3 hr., respectively. Number average molecular weights of 19,000 and 15,000 were determined for the same sample by end group analysis and osmotic pressure (Fig. 3). Assuming the molecular weight of this sample to be between 18,000 and 19,000 a "β" factor for the effective hydrodynamic ellipsoid of Form II can be calculated according to the treatment of Scheraga and Mandelkern[29] (see equation 1) utilizing the sedimentation and viscosity data of Figs. 1 and 2 and a partial specific volume, $\bar{v}$ = 0.758 cm.^{3}/g.[6]

$$\beta \equiv Ns[\eta]^{1/3}\eta/M^{2/3}(1 - \bar{v}\rho) \qquad (1)$$

In eq. 1, N is the Avogadro number, s the sedimentation constant at infinite dilution, $[\eta]$ the intrinsic viscosity, η the viscosity of the solvent, M the unhydrated molecular weight and ρ the density of the solution. The calculation yields β = (3.00 ± 0.10) × 10^6, corresponding to a prolate effective hydrodynamic ellipsoid with an axial ratio of 55 ± 15. The expected axial ratio for a polymer with the above molecular weight is between 70 and 80, assuming the Cowan–McGavin coördinates[6] (see Table I). It should be noted that small deviations in the experimental data affect β to an extent that leads to a relatively large deviation in the axial ratios calculated. There is, however, little doubt that the poly-L-proline molecules (sample B4, Form II) are highly asymmetric. An analogous calculation to that given above for Form I yielded an axial ratio of 50 ± 15.

Attempts to calculate the axial ratios from viscosity data alone, using the equation of Simha[30] (Eq. 2), where ν is the well known shape factor, and

$$[\eta] = \frac{N\nu}{100} \cdot \frac{V_e}{M} \qquad (2)$$

tentatively assuming that the specific volume, $M\bar{v}/N$, in acetic acid or propionic acid equals the volume of the effective hydrodynamic ellipsoid, V_e, yielded axial ratios of 37 and 40 for Forms I and II of sample B4, respectively. Since these ratios are close to those derived above from the rigorous Scheraga and Mandelkern equation, it was felt that a rough estimate of the axial ratios of the prolate effective hydrodynamic ellipsoids of other poly-proline samples may be obtained, for comparison purposes, from viscosity data alone by making use of the assumption $N\bar{v}/M \backsimeq V_e$. The axial ratios thus calculated for a number of poly-L-proline samples, as Form II, are given in Table I. All the samples tested showed rather high viscosities suggesting the existence of rod-like solute molecules. However, the axial ratios of the equivalent ellipsoids derived from the intrinsic viscosities were markedly smaller than those calculated for rod like particles possessing the Cowan–McGavin configuration.[6] This discrepancy becomes larger with increasing molecular weight. A low molecular weight polymer (B2, see Harrington and Sela[11]) gave an axial ratio of 19 as compared with the calculated one of 20. The difference between the found and calculated axial ratios indicates that if poly-L-proline (Form II) helices persist in solution, they possess a certain degree of flexibility. This point will be further elaborated in the discussion.

A comparison of the hydrodynamic data in the different solvents tested shows that the viscosity

(29) H. A. Scheraga and L. Mandelkern, THIS JOURNAL, **75**, 179 (1953).

(30) R. Simha, *J. Phys. Chem.*, **44**, 25 (1940).

(Table I) of poly-L-proline (Form II) in water is lower than that in organic acids, while the sedimentation in water was as a rule faster than in propionic or acetic acid. Preparation B4, for example, gave $S^{w}_{20} = 0.72$ at $c = 0.5\%$ in water and $S^{w}_{20} = 0.52$ at $c = 0.5\%$ in propionic or acetic acid.

Although significant differences are observed in the intrinsic viscosity of Form II in organic acids and in water, optical rotatory characteristics show little difference either with respect to molecular weight or solvent. When appropriate correction for the index of refraction of the medium is made, $[\alpha]^{25}D$ is about $-540°$ for samples of poly-L-proline II of different molecular weights, in the various solvents mentioned in Table I.

Some of the average molecular weights given in Table I have been estimated from osmotic measurements in aqueous 0.1 *M* sodium acetate, carried out with Forms II of the corresponding polypeptides. Typical plots of π/c *versus* c for several of these polymers are reproduced in Fig. 3.

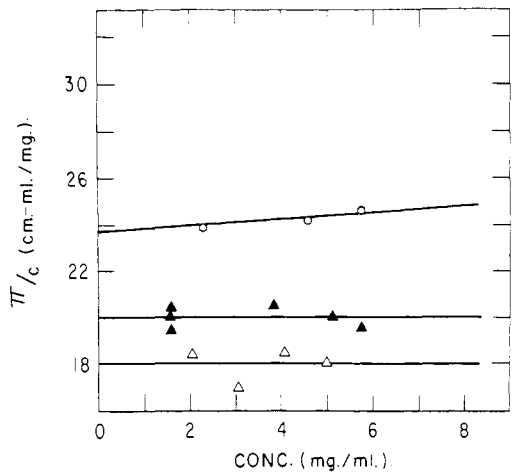


Fig. 3.—The reduced osmotic pressure, π/c, of three poly-L-proline samples as a function of concentration at 30°: O-O, sample P8; ▲-▲, sample P2; Δ-Δ, sample B4. Measurements were carried out in 0.1 *M* aqueous sodium acetate. Pressure π is given in cm. of decalin.

Viscosity measurements of polyproline samples in Form I seem to indicate that the polypeptide possesses marked molecular asymmetry also in this configuration. From the intrinsic viscosity of sample B4, Form I, ($[\eta] = 0.78$ in propionic acid, see Fig. 2) an axial ratio of 37 was derived by making use of eq. 2 and tentatively assuming that the effective hydrodynamic volume may be derived from the partial specific volume as described above. A similar calculation for sample P8 with $[\eta] = 0.56$ (in propionic acid) gave an axial ratio of 29. If it is assumed that polyproline I has a right handed helical structure suggested by Harrington and Sela,[11] and the molecular axial ratios are calculated from the molecular dimensions, values of 39 and 27 are obtained for samples B4 and P8, respectively. The apparent agreement which seems to hold for the low molecular weight samples does not hold, however, for high molecular weight samples. Thus a sample of mol. wt. 52000 (D33, Blout and Fasman[10]) in Form I showed an intrinsic viscosity of 0.99 in acetic acid corresponding to an axial ratio of 44, whereas the calculated value for this degree of polymerization is 116.

Kinetics of Forward Mutarotation. A. In Acetic Acid.—The general form of the change in optical rotation on mutarotation of Form I into Form II has been described in a previous communication.[8] In this section we wish to examine the kinetics of these optical rotatory changes.

The change in the specific optical rotation of poly-L-proline I (sample B1) in glacial acetic acid at 25° at three different concentrations is given in Fig. 4. The course of the reaction is seen to be practically independent of concentration in the range studied (0.25 to 2.0 g./100 ml.). This independence on concentration also was observed in other solvent systems and with other polymer samples. Similar results were obtained in the studies of the reverse mutarotation. These findings justify the use of $[\alpha]$ as the variable in the presentation of the kinetic experiments discussed below.

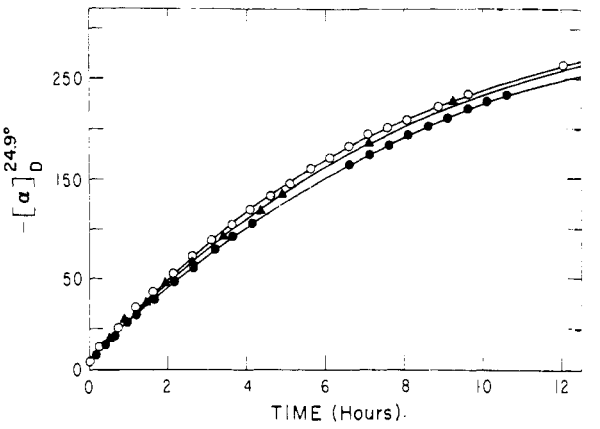


Fig. 4.—The forward mutarotation of poly-L-proline (sample B1, form I) in glacial acetic acid at 24.9° at three different concentrations: ●-●, 2 g. per 100 ml.; ▲-▲, 0.5 g. per 100 ml.; O-O, 0.25 g. per 100 ml.

In order to evaluate the apparent order of the forward mutarotation in acetic acid, log $d[\alpha]_t/dt$, taken from an experiment performed at 44°, was plotted against log $([\alpha]_t - [\alpha]_\infty)$. A straight line with a slope of 4/3 was obtained.

The course of the reaction may thus be represented by

$$-\frac{d[\alpha]_t}{dt} = k([\alpha]_t - [\alpha]_\infty)^{4/3} \qquad (3)$$

where k is constant. Integration of this equation between the appropriate limits gives

$$([\alpha]_t - [\alpha]_\infty)^{-1/3} = \frac{kt}{3} + ([\alpha]_0 - [\alpha]_\infty)^{-1/3} \qquad (4)$$

The plot of $([\alpha]_t - [\alpha]_\infty)^{-1/3}$ *versus* time is given in Fig. 5. The experimental points fall on a straight line throughout the whole range of the mutarotation.

In the experiments described above, in which the mutarotation was studied at different polymer concentrations, c_0, it was found that $\frac{d\alpha}{dt}$, *i.e.*, the measured rate of change in optical rotation, is, at any given $[\alpha]$, proportional to c_0. This would

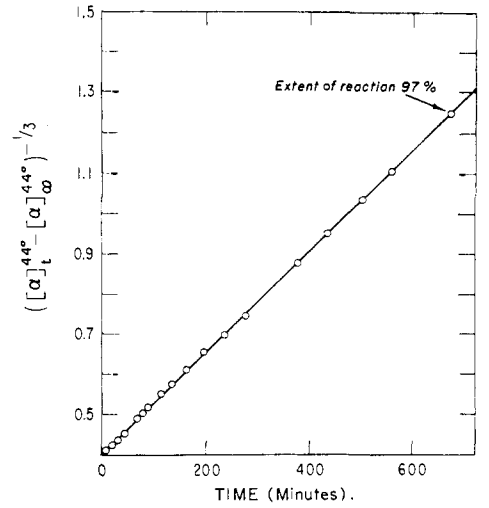


Fig. 5.—The kinetics of forward mutarotation of poly-L-proline (sample B4, c = 1.5 g. per 100 ml.) in glacial acetic acid at 44°; for explanation, see text.

indicate that the mutarotation reaction obeys first order kinetics. On the other hand, we demonstrated that in any single experiment, *i.e.*, when c_0 is constant, the change in $[\alpha]$ with time seems to proceed according to an order of 4/3. This seeming contradiction stems from the fact that the observed mutarotation is the result of a series of intramolecular configurational changes in every single macromolecule. This point will be considered in detail in the discussion.

In order to evaluate the enthalpy of activation, the mutarotation in acetic acid was carried out at several temperatures between 30 and 45°. Each of the curves given in Fig. 6 may be described by

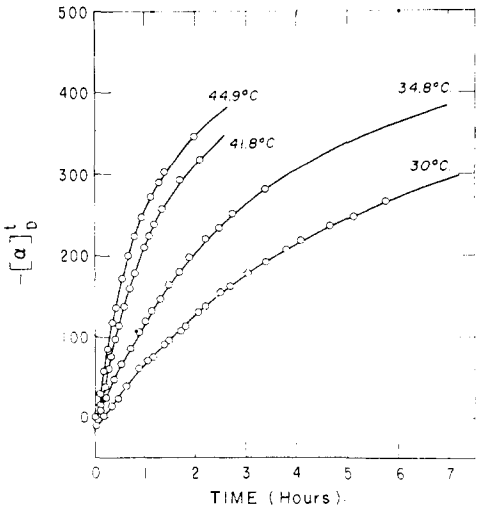


Fig. 6.—Forward mutarotation of poly-L-proline (sample B4) in glacial acetic acid at different temperatures. Practically identical curves were obtained at each temperature with concentrations of 1 g. per 100 ml. and 0.25 g. per 100 ml.

eq. (4). Up to 30% conversion, however, the experimental points deviate so little from the first order equation

$$-\frac{d[\alpha]}{dt} = k^0'([\alpha]_t - [\alpha]_\infty) \qquad (5)$$

that the apparent constants, k', could be evaluated from the linear plots of log $([\alpha_t - [\alpha]_\infty)$ *versus* time. This was done in order to facilitate comparison with the first order reverse mutarotation to be described below. An enthalpy of activation $\Delta H^* = 20.6$ kcal. per mole peptide bond was obtained from the usual Arrhenius plot of log k' *versus* the reciprocal of the absolute temperature (Fig. 7).

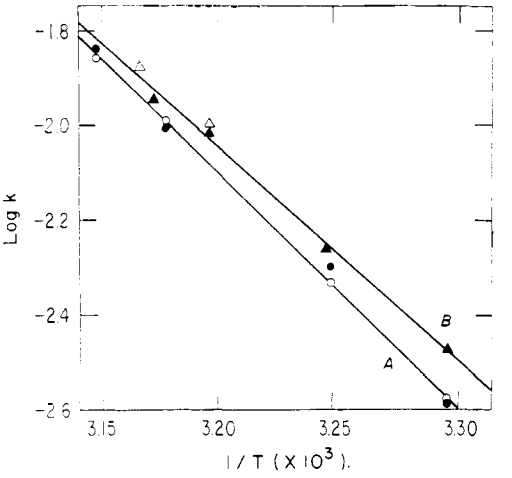


Fig. 7.—Arrhenius plots of forward mutarotation (●-●, c = 0.25 g. per 100 ml.; O-O, c = 1 g. per 100 ml. in glacial acetic acid) and of reverse mutarotation (▲-▲, c = 0.25 g. per 100 ml.; Δ-Δ, c = 0.5 g. per 100 ml. in acetic acid–propanol, 1:9 v./v.); for explanation, see text.

The procedure adopted seems to require some clarification since the relation between optical rotation and concentration of the reacting kinetic unit has not been defined. It will be seen that for the present purpose such a definition is unnecessary.

As has been mentioned previously we assume that the forward mutarotation involves a *cis–trans* conversion of the peptide bonds. Denoting by c the concentration of the unreacted *cis* bonds, the rate of reaction may be represented by

$$-\frac{dc}{dt} = f(c)e^{-\Delta H^*/RT} \qquad (6)$$

where $f(c)$ is a function of c whose dependence on temperature may be neglected in comparison with the exponential term. Assuming the general relation

$$[\alpha] = \phi(c,c_0) \qquad (7)$$

and differentiating (7) with respect to time at any given value of c_0, one obtains

$$\frac{\partial[\alpha]}{\partial t} = \frac{\partial[\alpha]}{\partial c}\frac{\partial c}{\partial t} \qquad (8)$$

Equations 8 and 6 yield

$$-\left(\frac{\partial[\alpha]}{\partial t}\right)_{c_0} = f(c)e^{-\Delta H^*/RT} \cdot \frac{\partial[\alpha]}{\partial c} \qquad (9)$$

The data presented in Fig. 4 show that the partial derivative $\left(\frac{\partial[\alpha]}{\partial t}\right)_{c_0}$ is practically in-

dependent of c_0. It may, therefore, be substituted by the total derivative $\frac{d[\alpha]}{dt}$. Hence

$$-\frac{d[\alpha]}{dt} = f(c)e^{-\Delta H^*/RT} \cdot \frac{\partial[\alpha]}{\partial c} \qquad (10)$$

Equation 11 is obtained finally by taking the logarithm of eq. 10, differentiating with respect to $1/T$ and neglecting the temperature dependence of ln $f(c)$ and ln $\frac{\partial[\alpha]}{\partial c}$

$$\left(\frac{\partial \ln\left(-\frac{d[\alpha]}{dt}\right)}{\partial(1/T)}\right)_{[\alpha]} = -\frac{\Delta H^*}{R} \qquad (11)$$

Obviously at $[\alpha]$ = constant, eq. 11 can be written as

$$\frac{\partial \ln k'}{\partial\left(\frac{1}{T}\right)} = -\frac{\Delta H^*}{R} \qquad (12)$$

B. In Acetic Acid–Water (7:3 v./v.).—The course of forward mutarotation in this solvent mixture is different from that in glacial acetic acid (see Fig. 8). In this case the rate of mutarotation is practically constant up to two thirds of the reaction, slowing down markedly at its later stages.

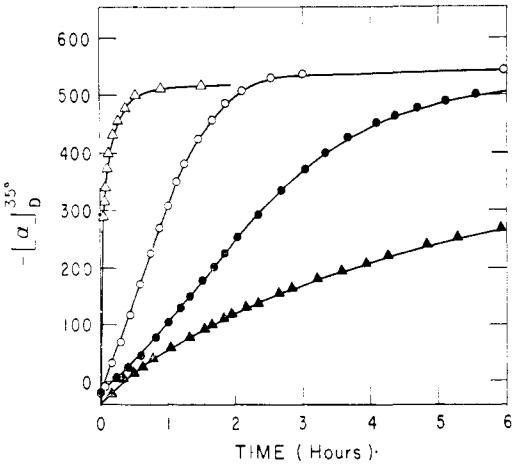


Fig. 8.—Effect of perchloric acid on the forward mutarotation of poly-L-proline (c = 0.5 g. per 100 ml.) in glacial and aqueous acetic acid at 30°: ▲-▲, glacial acetic acid; Δ-Δ, 2.6 × 10^{-3} N $HClO_4$ in glacial acetic acid (0.052 mole $HClO_4$ per mole peptide bond); ●-●, acetic acid–water (7:3 v./v.); O-O, 0.1 N $HClO_4$ in acetic acid–water (7:3 v./v.), (2 moles $HClO_4$ per mole peptide bond).

A zero order reaction might be expected when the configurational changes underlying the corresponding changes in the optical rotation start at unique points in the molecule, being propagated along the peptide chain. Such unique points could be either the ends of the macromolecule or peptide bonds involving hydroxyproline, an amino acid which has been shown to occur in poly-L-proline samples prepared from commercial L-proline.

In the first case proportionality between the rate of reaction and the number of polyproline molecules per unit volume would be expected. At equal weight concentrations the rate should be inversely proportional to the molecular weight of the sample. It was found, however, that two samples of poly-L-proline, B4 and P8, with average molecular weights of 19,000 and 12,000, respectively, mutarotated at equal rates in the acetic acid–water solvent.

In the second case the rate of mutarotation should be proportional to the hydroxyproline content of the poly-L-proline samples tested. However, it was found that a copolymer containing proline and hydroxyproline in a molar ratio of 88 to 12 mutarotates in acetic acid–water with the same rate as sample B4, which contains proline and hydroxyproline in a molar ratio of 98 to 2.

It seems, therefore, that the problem of zero order kinetics is connected with other deviations from the expected first order kinetics and will accordingly be dealt with later.

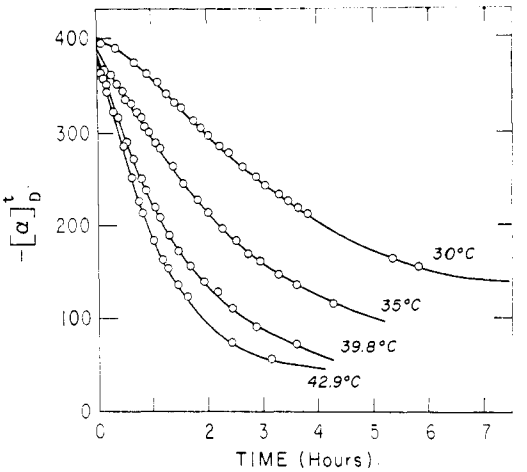


Fig. 9.—Reverse mutarotation of poly-L-proline (sample B4) in acetic acid–propanol, 1:9 v./v. at different temperatures.

An estimate of the enthalpy of activation of the forward mutarotation in acetic acid–water was derived from kinetic measurements at 30 and 41°. By making use of eq. 11, a value of ΔH^* = 24 kcal./mole was obtained.

Kinetics of Reverse Mutarotation.—When a solution of poly-L-proline II in formic, acetic or propionic acid is diluted tenfold with 1-propanol, the specific rotation decreases over several days, at room temperature, from $[\alpha]_D$ = − 370° to a final value in the neighborhood of $[\alpha]_D$ = − 20°.[13,31] The initial decrease in specific rotation from $[\alpha]_D$ = − 540° to $[\alpha]_D$ = − 370° is essentially instantaneous and cannot be explained by the index of refraction correction (about 5%) in transferring the polymer to the alcoholic medium. Dilutions of 20-fold and 40-fold (propanol–formic acid) give identical initial values of $[\alpha]_D$ = − 370°, though in these solvents reverse mutarotation appears to be significantly more rapid. Indeed an instantaneous decrease in rotation is observed in a variety of organic solvent systems as is shown in Table II. On the other hand, if the polymer is precipitated with ether from solution, immediately after diluting with 1-propanol, and redissolved in acetic acid, the specific rotation is found to have returned to the initial value, *i.e.*, $[\alpha]_D$ = − 540°.

(31) I. Z. Steinberg, *Bull. Research Council of Israel*, **7A**, 97 (1958).

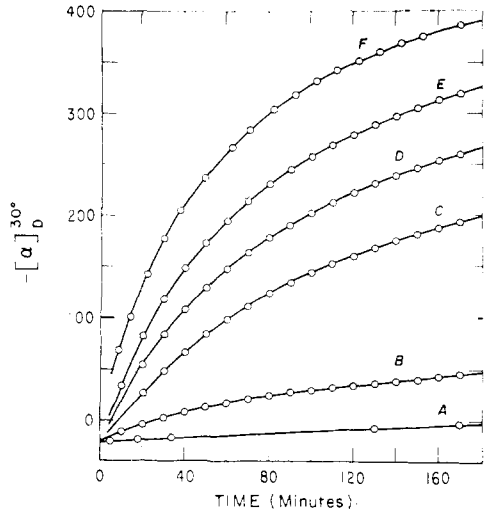


Fig. 10.—Forward mutarotation of poly-L-proline (sample B4, c = 0.5 g. per 100 ml. at 30°) in propionic acid with the addition of different amounts of perchloric acid, given in moles $HClO_4$ per mole peptide bond: curve A, 0.023; curve B, 0.027; curve C, 0.031; curve D, 0.035; curve E, 0.0388; curve F, 0.0465.

The time dependent decrease in optical rotation following dilution of a solution of poly-L-proline II (sample B4) in acetic acid by 1-propanol, at various temperatures, is shown in Fig. 9. The solvent was acetic acid–1-propanol (1:9 v./v.) and the polymer concentration was 0.25%. Similar data were obtained with 0.5% solutions. When log $([\alpha]_t - [\alpha]_\infty)$ for the various curves was plotted against time, straight lines were obtained up to about 90% of the reaction. First order kinetics is thus obeyed to a good approximation in this system.

The Arrhenius plot of log k *versus* $1/T$ in Fig. 7 gives $\Delta H^* = 20.2$ kcal./mole peptide bond demonstrating that the forward and reverse mutarotation reactions have essentially identical activation energies. Interconversion of Form II into Form I occurs also in a solvent consisting of water–1-propanol (1:9 v./v.). The reaction is significantly accelerated by the addition of perchloric acid. No apparent difference in the rate of reverse mutarotation was observed between a polymer of a molecular weight 19,000 ($k = 3.84 \times 10^{-3}$ min.$^{-1}$) and one of molecular weight 11,000 ($k = 3.58 \times 10^{-3}$ min.$^{-1}$), when the

TABLE II

SPECIFIC ROTATION OF POLY-L-PROLINE II IN VARIOUS SOLVENT SYSTEMS

Solvent system (v./v.)	Initial $[\alpha]_D^{30}$	Initial[a] $[\alpha]_D^{30}$ (corr.)
Formic acid (10%)–methanol (90%)	−480°	−479°
Formic acid (10%)–ethanol (90%)	−480	−470
Formic acid (10%)–propanol (90%)	−410	−395
Formic acid (10%)–butanol (90%)	−400	−382
Acetic acid (10%)–propanol (90%)	−370	−357
Propionic acid (10%)–propanol (90%)	−370	−356
Acetic acid (20%)–propanol (80%)	−385	−372
Acetic acid (40%)–propanol (60%)	−385	−372

[a] Corrected for index of refraction of solvent.

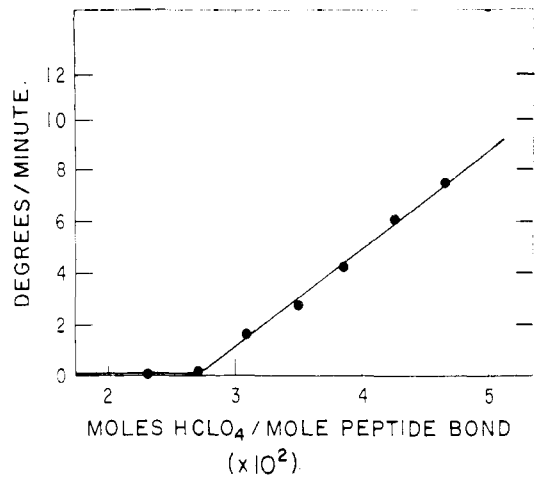


Fig. 11.—The rate of forward mutarotation of poly-L-proline (sample B4) at $[\alpha]_D^{30}$ −80° (corresponding to 20% conversion) as a function of moles $HClO_4$ per mole peptide bond; temperature 30°.

mutarotation was followed in acetic acid–1-propanol (2:8 v./v.) at 40°.

Acid Catalysis of Mutarotation.—The rate of forward mutarotation in aliphatic acids is greatly increased by minute quantities of strong mineral acids such as perchloric acid. The effects of $HClO_4$ in amounts from 0.023 to 0.047 mole per mole peptide bond on the forward mutarotation in propionic acid is demonstrated in Fig. 10. In Fig. 11 the rate of mutarotation at 20% conversion, corresponding to $[\alpha]_D = -80°$, is plotted against the molar ratio of perchloric acid to peptide bonds. It can be seen that amounts of perchloric acid from 0.027 to 0.047 mole per mole peptide bond caused a linear increase in the velocity of mutarotation. Larger amounts of acid caused precipitation. The addition of $HClO_4$ up to an amount of 0.027 mole per mole peptide bond had no catalytic effect. This was found to be due to the presence of basic groups (0.0275 mole per mole peptide bond as determined by potentiometric titration of the polymer in propionic acid by means of 0.04 N $HClO_4$ in propionic acid). These groups probably consist of the terminal imino groups (0.005 mole per peptide bond) and traces of pyridine retained by the polymer from the polymerization medium.

The strong catalytic effect of $HClO_4$ on the forward mutarotation is also observed in glacial acetic acid solution (see Fig. 8). In this case 0.052 mole of $HClO_4$ per mole peptide bond reduced the half-life time of the reaction from 345 to 4 minutes. The catalytic effect of strong acid also was observed in aqueous acetic acid (Fig. 8), though to a lesser extent. A concentration of 0.1 N $HClO_4$ in acetic acid–water (7:3 v./v.), corresponding to 2 moles $HClO_4$ per mole peptide bond was necessary to reduce the half-life of the reaction from 130 to 52 minutes. This is due undoubtedly to the fact that water is a more basic solvent than acetic acid.

The reverse mutarotation was found to be similarly catalyzed by $HClO_4$. The change of optical rotation of poly-L-proline II with time in water–propanol (1:9 v./v.) in the presence and absence

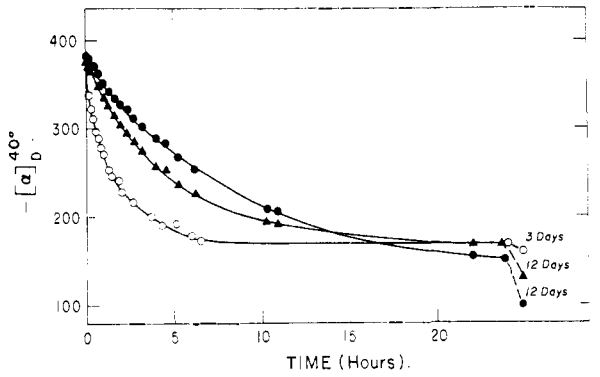


Fig. 12.—The effect of mineral acids on the reverse mutarotation of poly-L-proline (sample B4, c = 0.5 g. per 100 ml.) in water–propanol (1:9 v./v.) at 40°: ●-●, in water–propanol; ▲-▲, 0.1 M HCl in water–propanol; O-O, 0.15 M $HClO_4$ in water–propanol.

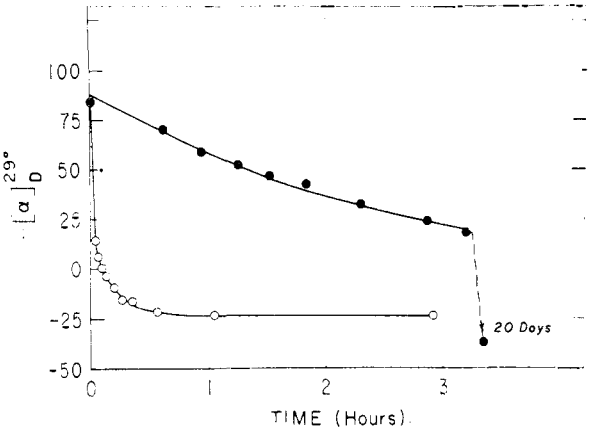
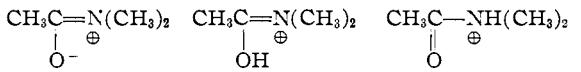


Fig. 13.—The effect of perchloric acid on the reverse mutarotation of poly-O-acetylhydroxy-L-proline (c = 0.5 g. per 100 ml.) in acetic anhydride at 29°: ●-●, in acetic anhydride; O-O, 7.1 × 10^{-4} M $HClO_4$ in acetic anhydride (0.022 mole $HClO_4$ per mole peptide bond).

of perchloric acid (0.15 M) is given in Fig. 12. The half-life times of the non-catalyzed and catalyzed reaction are 7.4 and 1 hr., respectively. It should be noted that in the reverse mutarotation the final value of $[\alpha]_D$ in the presence of perchloric acid differs from the corresponding value in the absence of catalyst. This indicates, as will be explained below, that in the presence of $HClO_4$ the transformation of poly-L-proline II to I is incomplete, reaching an intermediate equilibrium stage.

The effect on $HClO_4$ on the reverse mutarotation of poly-O-acetylhydroxy-L-proline in acetic anhydride is given in Fig. 13. This polymer undergoes reverse mutarotation in this solvent (initial $[\alpha]_D$ = − 84°, final $[\alpha]_D$ = + 38°) with a half-life time of about 174 minutes. On the addition of a small amount of perchloric acid (0.022 mole $HClO_4$ per mole peptide bond) the reverse mutarotation is accelerated to a half-life time less than 2.5 minutes. At high perchloric acid concentrations the rate of reverse mutarotation is too rapid to measure.

The experiments on acid catalysis are reported here in considerable detail since they provide important evidence for the occurrence of *cis–trans* isomerizations of the peptide bonds during mutarotation. The absence of free rotation about the C–N bond in amides, due to its partial double bond character, is well established.[32,33] The mechanism of protonation and its effect on the rotation of the C–N bond in N-methylacetamide and N,N-dimethylacetamide has been investigated recently by Berger, Loewenstein and Meiboom[34] using the nuclear magnetic resonance technique. The absence of free rotation[33,35] about C–N bond in N,N-dimethylacetamide in neutral aqueous solution is indicated by the presence of two N-methyl lines in the n.m.r. spectra. This doublet results from the non-equivalence (*cis* and *trans*) of the two N-methyl groups. On acidifying aqueous solutions of this amide the doublet collapses into a single line indicating the onset of free rotation. It was demonstrated that the acid catalysis of free rotation takes place according to a mechanism involving an equilibrium between the three species of which the



last one is capable of free rotation. We may infer that in the polymers of proline, protonation results in a "loosening" of the peptide bonds allowing *cis–trans* isomerization to occur.

Support for the mechanism outlined above is obtained from several experiments demonstrating the strong binding of acid by polyproline and related polymers.

Polyproline precipitates from its solution in acetic acid on the addition of anhydrous solutions of perchloric acid.[18] The precipitates obtained with either polyproline I or polyproline II contained (after repeated washing with acetic acid followed by drying *in vacuo* over potassium hydroxide at 75°) from 0.26 to 0.31 mole perchloric acid per mole peptide bond. When the washed perchloric acid precipitates derived from either form were redissolved in water, the specific rotation was that of Form II. Precipitates similar to the above also were obtained on the addition of sulfuric, hydrobromic and periodic acids to solutions of polyproline in acetic acid.

Proton binding in solution by peptides containing imide bonds could be demonstrated potentiometrically. Wimer[28] has shown that in solvents of low basic strength, such as acetic anhydride, amides can be titrated as bases by means of perchloric acid. We have observed that the imide nitrogens of the pyrrolidine rings may be similarly titrated. As poly-L-proline I and II are insoluble in acetic anhydride, the titration study was carried out with poly-O-acetylhydroxy-L-proline.

The potentiometric titration procedure used is described under Materials and Methods. In order to ascertain whether the proline–proline peptide bond can be accurately titrated under these conditions, *p*-toluenesulfonylprolylproline and carbo-

(32) L. Pauling, "The Nature of the Chemical Bond," 2nd Ed., Cornell University Press, Ithaca, New York, N. Y., 1948, p. 207.

(33) H. S. Gutowsky and C. H. Holm, *J. Chem. Phys.*, **25**, 1228 (1956).

(34) A. Berger, A. Loewenstein and S. Meiboom, THIS JOURNAL, **81**, 62 (1959).

(35) W. D. Phillips, *J. Chem. Phys.*, **28**, 1363 (1955).

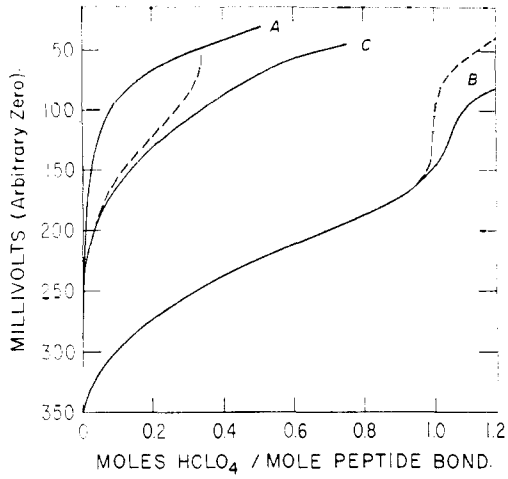


Fig. 14.—Titration of imide bonds in acetic anhydride: curve A, titration of solvent; curve B, titration of *p*-toluenesulfonyl-L-prolyl-L-proline; curve C, titration of poly-O-acetylhydroxy-L-proline; ———, experimental titration curves; - - - -, corrected curves. For details see Materials and Methods.

benzoxyprolylproline were titrated. These titrations gave 1.05 and 0.97 equivalents of imide group, respectively, with very sharp end points after correction for titration of the solvent (Fig. 14). A typical titration curve of poly-O-acetylhydroxyproline in acetic anhydride is shown in the same figure. It will be observed that the end point of the corrected titration curve corresponds to about 37% of the number of imide nitrogens present in the polymer. Studies have been made on two different samples of polymer at concentrations of 2 to 4% w./v., when 0.35 to 0.37 mole per mole peptide bond were titrated. It is of interest that this average value of 35% is close to the amount of perchloric acid bound by poly-L-proline, when precipitated by means of perchloric acid from acetic acid solutions.

In connection with the above it is pertinent to note that the rate of forward mutarotation of poly-L-proline I in formic, acetic and propionic acids is roughly comparable to the acidity of these acids as measured by conductivity. Thus at 25°, the conductivities of the three acids are 6.4×10^{-5}, 11.2×10^{-9} and 1×10^{-9} mho, respectively, while the half-life times of mutarotation are approximately 1, 1000 and 17,000 minutes. Addition of a small amount of formic acid to acetic acid markedly accelerates the mutarotation rate in this solvent. Thus in a solvent consisting of 10% formic acid and 90% acetic acid the half-life time decreases from 690 minutes (in acetic acid) to 69 minutes ($t = 25°$). The velocity of mutarotation in propionic acid is similarly increased by addition of a small amount of formic acid. At a volume ratio of 1:24 formic to propionic acid, the half-life time equals 1770 minutes at 25°, whereas at a volume ratio of 1:9 it is 385 minutes. On the other hand the addition of base to aliphatic acids markedly decreases the rate of forward mutarotation. The effect of sodium formate on the course of the mutarotation in formic acid is given in Fig. 15. A similar effect is observed when the half-life

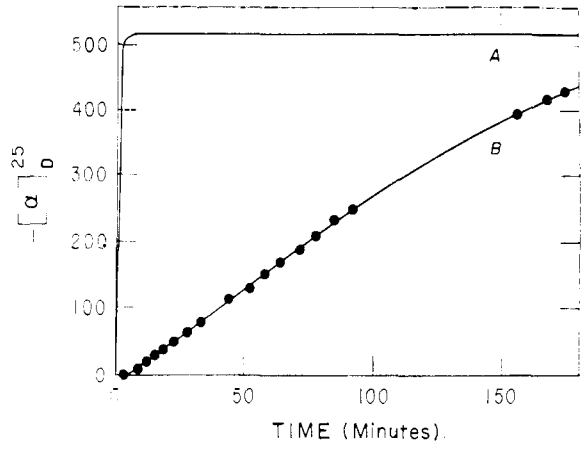


Fig. 15.—The effect of sodium formate on the forward mutarotation of poly-L-proline (sample B4, $c = 0.5$ g. per 100 ml.) in formic acid 20.5°: A, in formic acid; B, 1.32 *M* HCOONa in formic acid.

time of mutarotation at 53.7° in acetic acid (half-life time 23.5 minutes) is compared with that in 0.1 *M* sodium acetate in acetic acid (48.2 minutes).

Effect of Strong Acid on the Equilibrium State.—In the case of the acid-catalyzed reverse mutarotation it was noticed that the final specific optical rotation, $[\alpha]_D$, differed from that of Form I. The effect of perchloric acid on $[\alpha]_D$ in the case of poly-O-acetylhydroxy-L-proline in acetic anhydride was investigated in some detail. In this system equilibrium is established rapidly and small amounts of acid suffice to produce marked effects.

As mentioned above, poly-O-acetylhydroxy-L-proline is stable in its dextrorotatory Form (Form I, $[\alpha]_D = +40°$) in acetic anhydride. However, form II ($[\alpha]_D = -130°$) becomes the stable one in the presence of perchloric acid at concentrations exceeding 0.3 mole $HClO_4$ per mole peptide bonds. With lesser amounts of acid, intermediate $[\alpha]_D$ values are obtained as illustrated in Fig. 16.

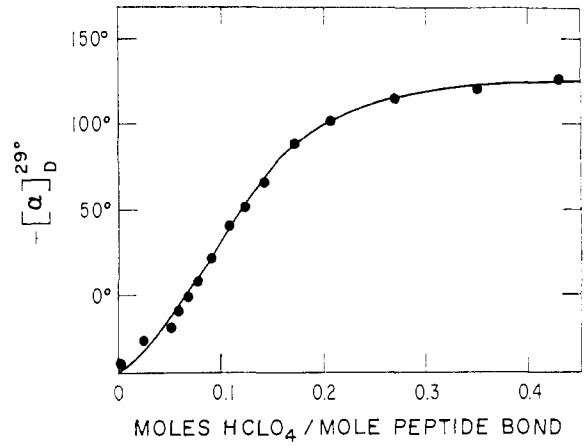


Fig. 16.—Equilibrium values of $[\alpha]_D$ of poly-O-acetylhydroxy-L-proline in acetic anhydride ($c = 0.5$ g. per 100 ml.) as a function of moles $HClO_4$ per mole peptide bond; temperature 29°.

The stabilization of Form II at relatively high $HClO_4$ concentrations is most likely connected with the protonation of the peptide chain. Elec-

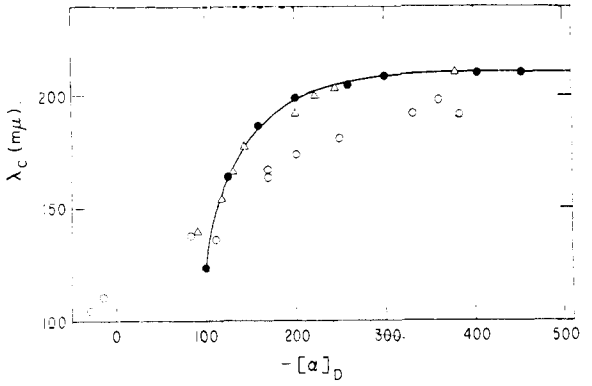


Fig. 17.—The rotatory dispersion constant, λ_c, of poly-L-proline as a function of the specific rotation $[\alpha]_D$: ●-●, data taken during forward mutarotation in glacial acetic acid; ▲-▲, data taken during reverse mutarotation in formic acid–1-butanol (1:9 v./v.); for comparison (Harrington and Sela), data taken during forward mutarotation in water (O) are included.

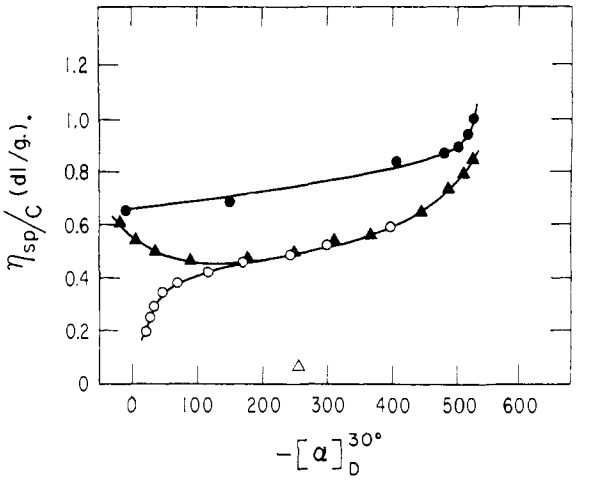


Fig. 18.—Reduced viscosity (c = 1.0 g. per 100 ml.) at 30° as a function of $[\alpha]^{30}_D$: ●-●, data taken during forward mutarotation in glacial acetic acid; ▲-▲, data taken during forward mutarotation in acetic acid–water (7:3 v./v.); O-O, data taken during reverse mutarotation in acetic acid propanol (2:8 v./v.); △, value in aqueous 12 M LiBr.

trostatic repulsion forces in the charged chain favor the extension of the molecule. It will be remembered that Form II, as represented by the all *trans* Cowan–McGavin helix,[6] is the most extended form which the polypeptides under discussion can attain. It should be noticed further that maximum extension coincides with maximum protonation (see Fig. 14).

The states characterized by the specific optical rotation values between $[\alpha]_D$ = + 40 and − 130° (Fig. 16) are true equilibrium states, since the $[\alpha]_D$ values in this region were found to be determined uniquely by the ratio $HClO_4$/peptide bond. The same $[\alpha]_D$ values were reached either by adding the necessary amount of acid to a solution of lower levorotation or by partially neutralizing the acid (with sodium acetate) of a solution with higher levorotation. The experiments described here suggest that we are dealing with an equilibrium between *cis* and *trans* peptide bonds in each macromolecule, the ratio of which is determined by the extent of protonation.

An attempt was made to evaluate the enthalpy of the *cis–trans* isomerization reaction. The optical rotation of a solution containing poly-O-acetylhydroxy-L-proline and 0.106 mole $HClO_4$ per mole peptide bond in acetic anhydride was measured at several temperatures in the range from 19 to 46°. The value of $[\alpha]_D$ (−42°) did not change within the limits of experimental error. It is thus concluded that the ΔH of isomerization in the system investigated does not exceed 0.5 kcal. per mole peptide bond. The above conclusion seems self-evident since the enthalpies of activation for the forward and reverse mutarotation were found to be essentially the same. It should be recalled, however, that these enthalpies were derived from experiments carried out in different solvents.

Optical Rotatory Dispersion Studies during Mutarotation.—Optical rotatory dispersion measurements during the conversion of poly-L-proline I into poly-L-proline II in water have been described in an earlier paper.[11] Values of the optical rotatory dispersion constant, λ_c, obtained from linear plots of $\lambda^2[\alpha]_\lambda$ *versus* $[\alpha]_\lambda$ during forward mutarotation in water, changed appreciably with time. Dispersion measurements have now been obtained during the forward mutarotation in glacial acetic acid at 35°, as well as the reverse mutarotation in formic acid–1-butanol (1:9 v./v.) at 25°. Because of the speed of the mutarotation it was impossible to measure the $[\alpha]_\lambda$ values at five different wave lengths fast enough to be able to relate them to a single $[\alpha]_D$ value, *i.e.*, to a defined degree of reaction. A group of curves of $[\alpha]_\lambda$ *versus* time for each of five wave lengths (λ = 5890, 5000, 4500, 4000 and 3750 Å.) was, therefore, drawn from the data recorded in a single mutarotation experiment. The five $[\alpha]_\lambda$ values at any given time could be read off the graph and used for the construction of the plots of $\lambda^2[\alpha]_\lambda$ *versus* $[\alpha]_\lambda$. These plots were non-linear between $[\alpha]_D$ = + 50° and $[\alpha]_D$ = − 100°. The λ_c values derived from the slopes of the linear plots in the range of $[\alpha]_D$ = − 100° to $[\alpha]_D$ = − 500° are given in Fig. 17 as a function of $[\alpha]_D$. In the same figure λ_c *versus* $[\alpha]_D$ is also given for a reverse mutarotation run in formic acid–1-butanol (1:9 v./v.). Anomalous dispersion, *i.e.*, a rotatory dispersion which cannot be represented by a simple one term Drude equation, also was found in this system in the range of $[\alpha]_D$ = − 100° to $[\alpha]_D$ = − 20°. The relation between λ_c and $[\alpha]_D$ is the same for the forward and reverse mutarotation, both conducted in non-aqueous media. This relation differs, however, from that reported previously[11] for the forward mutarotation in water (see Fig. 17). It might be noted that the water-soluble sample of poly-L-proline I (B2, DP = 50) used by Harrington and Sela[11] showed normal dispersion in aqueous solution. The same sample when investigated in propionic acid exhibited anomalous rotatory dispersion. This and the data given above are in accord with the findings of Blout and Fasman,[10] who report anomalous rotatory dispersion of poly-L-proline I in acetic acid.

Viscosity Changes During Mutarotation.—The viscosity of poly-L-proline in form I is always less than that of form II in a given solvent at the same concentration. Forward and reverse mutarotations are, therefore, necessarily accompanied by changes in viscosity.

Fig. 18 shows these changes during mutarotation under three different conditions. During forward mutarotation in acetic acid the viscosity increases in a monotonic fashion. It should be noted, however, that at the first stages of the reaction marked changes in specific rotation are accompanied by a rather small increase in viscosity. On the other hand, at the later stages of the mutarotation, when the reaction is practically complete, the viscosity continues to increase to its final value. These findings indicate that changes in the molecular configuration of the poly-L-proline molecule are reflected in a different manner in its hydrodynamic and rotatory properties. This can also be observed in the two other cases recorded in the figure.

During forward mutarotation in acetic acid–water (7:3 v./v., at 30°) the viscosity decreases at first, passes through a shallow minimum and increases during the later stages of the reaction. A difference in the kinetics of mutarotation in acetic acid on the one hand and in acetic acid–water on the other was mentioned above in the section on optical rotation. A similar viscosity minimum was observed previously[11] during the forward mutarotation of a low molecular weight sample of poly-L-proline in water.

Viscosity changes during the conversion of poly-L-proline II into I in acetic acid–propanol (1:9 v./v.) also are recorded in Fig. 18. The three mutarotation experiments described graphically in the figure demonstrate clearly that a poly-L-proline molecule may attain different shapes, as reflected by different hydrodynamic properties, but still possess the same optical rotation.

Optical Rotatory Properties of Low Molecular Weight Proline Derivatives.—The optical rotatory behavior of two low molecular weight proline-containing compounds, *p*-toluenesulfonyl-L-prolyl-L-proline and L-proline anhydride in a number of solvent systems are given in Table III. It will be seen that solvents which produce large optical rotatory changes in poly-L-proline have little effect on these simple proline-containing substances. This finding is thus in agreement with our proposal that the optical rotatory behavior of poly-L-proline in these same solvents results entirely from configurational alterations along the polypeptide chain.

TABLE III

OPTICAL ROTATORY CHARACTERISTICS OF TWO L-PROLINE DERIVATIVES

p-Toluenesulfonyl-L-prolyl-L-proline	$[\alpha]^{25}D$	λ_c (mμ)
Acetic acid	−147°	220
Acetic acid–1-propanol (1:9) v./v.)	−147	220
L-Proline anhydride		
H_2O	146.8	204
Acetic acid	143.3[a]	201[a]
Acetic acid–1-propanol (1:9 v./v.)	131.9[a]	210[a]
Saturated aqueous LiBr	135.1	186

[a] Not significantly changed, when the readings were repeated after 20 days.

The Effect of Neutral Salts on the Configuration of Poly-L-proline.—In the previous sections we have considered configurational changes in the polyproline molecule which apparently involve *cis–trans* isomerizations at the peptide bonds. Now we wish to describe a series of experiments involving configurational changes in poly-L-proline which cannot be explained readily in terms of this mechanism. The only other bond open to isomerization is the C_α–$\overset{O}{\overset{\|}{C}}$ bond. We have shown[11] that the specific optical rotation of poly-L-proline II ($DP = 50$) approaches a value of $[\alpha]D = -240°$ in concentrated aqueous lithium bromide, while the intrinsic viscosity falls from a value of 0.22 (in water) to 0.075 dl./g. in this salt solution. Since the imide nitrogen of polyproline is devoid of a hydrogen atom, the observed optical rotatory and viscosity behavior cannot be attributed to the effect of this salt in promoting the formation of peptide hydrogen bonds as was observed in the case of two unfolded polypeptide chains, clupein and oxidized ribonuclease.[36] Destruction of a homogeneous asymmetric configurational pattern seems the most likely explanation. This view is strengthened by an examination of the effect of LiBr on the viscometric properties of poly-DL-proline. The intrinsic viscosity, $[\eta]$, of a poly-DL-proline sample of molecular weight 9700 was found to be 0.05 dl./g. in 0.1 *M* KCl and 0.05 dl./g. in 6 *M* LiBr. The low viscosity of this polymer in water may be taken as evidence that the polymer has no regular configurational pattern, and that, therefore, the intrinsic viscosity is unaffected by lithium bromide.

It was suggested in an earlier paper[11] that destruction of the helical pattern of poly-L-proline II in concentrated aqueous solutions of LiBr might occur through *cis–trans* isomerization about the peptide bonds. Several considerations stemming from our recent work, however, point to the possibility that the mechanism of action of LiBr, NaSCN, $CaCl_2$ and similar salts, in collapsing the poly-L-proline II structure, may have a different origin:

(1) The effect of these salts on the specific rotation and viscosity of Form II is immediate at room temperature, the optical rotation and viscosity falling to their terminal values within the time interval required for measurement. Yet, as we have demonstrated, reverse mutarotation in the acid–alcohol solvents takes several hours.

(2) The intrinsic viscosity of solutions of poly-L-proline II in concentrated aqueous LiBr is significantly lower than the viscosity of this polymer in the solvents favoring reverse mutarotation. This is true at all stages of mutarotation (see Fig. 18).

(3) In 6 *M* LiBr the optical rotation is $[\alpha]D = -400°$. On dilution at room temperature (20 fold) with water the specific levorotation immediately increases to $[\alpha]D = -520°$. No further changes are then observed. On the other hand forward mutarotation in water over this same interval in specific rotation requires many hours.

(36) W. F. Harrington and J. A. Schellman, *Compt. rend. Lab. Carlsberg, Sér. chim.* **30**, 167 (1957).

A more detailed investigation of the changes in optical rotation observed on dilution from concentrated aqueous solutions of lithium bromide seemed warranted. It was found that an extremely rapid mutarotation occurs under these conditions which could be measured in the temperature range 0–12°.

A 5% solution of poly-L-proline II in 12 *M* LiBr ($[\alpha]^{25}D \cong -250°$) was cooled to 0.15° and diluted 50 fold with water which had been precooled to the same temperature. The solution was immediately transferred to a jacketed 2 dm. polarimeter tube and the rotation followed over a period of time. At a wave length of 313 mμ the specific levorotation was observed to increase continuously from a value near $[\alpha]_{313} = -2100°$ ($[\alpha]D = -400°$) to $[\alpha]_{313} = -2645°$ ($[\alpha]D = -540°$) with a half-life of about 29 minutes. Similar experiments were carried out at 1.7, 2.3, 9.0 and 11.8°. Time dependent changes in $[\alpha]_{313}$ for the two extreme temperatures, 0.15 and 11.8°, are presented in Fig. 19. Plots of log $([\alpha]_t - [\alpha]_\infty)$ *versus* time were found to be quite linear over about three half lives for each reaction studied. Logarithms of the specific velocity constants obtained from these linear plots are given as a function of the reciprocal of absolute temperature in Fig. 19 yielding an enthalpy of activation, $\Delta H^* = 20.6$ kcal./mole prolyl residue.

The value of ΔH^* for the mutarotation reaction discussed above is close to that estimated for the enthalpy of activation for the forward and reverse mutarotation reaction. On the other hand one can calculate from the Arrhenius plot of Fig. 19 that the half life of this reaction at 30° approximates 0.69 minute, which is about 10^3 times faster than the acid catalyzed reaction at this temperature. From the rate constants the entropy of activation for the two reactions may be estimated from the Eyring equation as $\Delta S^* = -12.5$ for the acid catalyzed reaction and $\Delta S^* = -0.84$ for the dilution reaction, demonstrating that fundamentally different mechanisms must be involved in the two processes.

When a solution of the polymer in 10 *M* LiBr was diluted at 1.7° to concentrations of 0.1, 0.24 or 1.2 *M* LiBr, it was found that all the diluted solutions exhibited an initial specific rotation, $[\alpha]D = -400°$. These solutions mutarotated (at 1.7°) with specific velocity constants of $k = 2.3 \times 10^{-2}$ and 2.8×10^{-2} and 1.8×10^{-2} min.$^{-1}$ respectively. Thus it appears that LiBr has no significant catalytic effect on this mutarotation.

One dilution study also was made on a solution of poly-L-proline II in 5 *M* potassium thiocyanate at 1.7°. Final concentration of this salt after dilution was 0.1 *M*. Essentially the same effect was observed as in the experiments using LiBr. In this case the specific rotation increased from $[\alpha]_{313} = -1990°$ to $[\alpha]_{313} = -2550°$ with a velocity constant $k = 1.9 \times 10^{-2}$ min.$^{-1}$.

It is of interest that in all of the dilution experiments the initial specific rotation following dilution was about $[\alpha]D = -400°$. The specific rotation in 10 *M* LiBr and 5 *M* KSCN salt medium approximates $[\alpha]D = -250°$. Thus it would

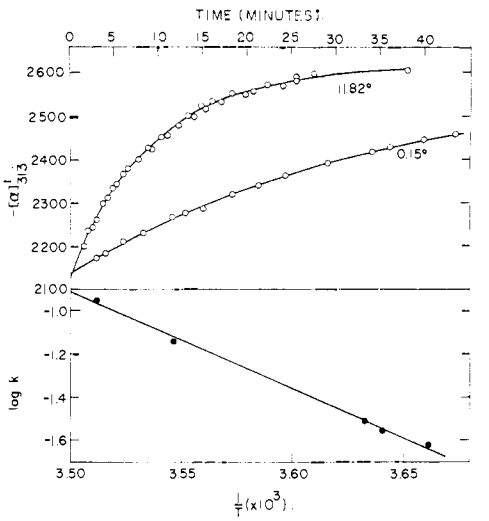


Fig. 19.—Kinetics of rotation following dilution from 12 *M* to 0.2 *M* LiBr at low temperature: upper, mutarotation following dilution at 0.15 and 11.82°; lower, Arrhenius plot of kinetics following dilution at various temperatures.

appear that an essentially instantaneous rotatory change (from $[\alpha]D = -250°$ to $[\alpha]D = -400°$) precedes the time dependent changes which have been demonstrated above.

Independent evidence that the effect of LiBr, NaSCN and similar salts does not involve *trans–cis* isomerization at the peptide bonds comes from an examination of the nuclear magnetic resonance spectra of N,N-dimethylacetamide. As it was shown earlier, the n.m.r. spectrum of dimethylacetamide in water exhibits a characteristic doublet. In concentrated aqueous solutions of LiBr or NaSCN this doublet pattern in the n.m.r. spectrum remains unchanged, demonstrating that these salts, unlike strong acids, have no observable effect in "unlocking" the peptide bonds.

The Effects of Some Solvents on Rotatory Properties of Collagen and Gelatin.—The effect of neutral salts on the optical rotatory properties of gelatin has been mentioned earlier.[17] For example, the specific rotation of ichthyocol gelatin is lowered between 70–80° in the presence of a variety of neutral salts at high concentration, while the optical rotatory dispersion constant, λ_c, in these solutions remains at 204 mμ.[16,17] Optical rotatory studies on the effect of LiBr on the specific rotation of three different invertebrate collagens reveal that in each case there is a decrease in λ_c on addition of LiBr, while the specific levorotation (D line) decrease is between 250–300°. In view of the immediate drop in $[\alpha]D$ of poly-L-proline II (paralleling that observed in aqueous LiBr), when acetic acid solutions of this polymer are diluted with 1-propanol, it was of interest to determine whether such solvents, which favor reverse mutarotation, would have any effect on the optical rotatory properties of gelatin and collagen.

A solution of ichthyocol collagen in formic acid was diluted eightfold with 1-propanol. Immediately after dilution $[\alpha]D$ was found to be −36°. The optical rotatory dispersion constant could not be obtained, however, since the solution became

turbid shortly after dilution. However, the above specific rotation should be compared to that of ichthyocol collagen in water ($[\alpha]_D = -330°$) and in 12.5 *M* LiBr ($[\alpha]_D = -56°$). In a second experiment bovine gelatin was dissolved in 98% formic acid and the solution diluted eightfold with 1-propanol. Immediately after diluting, $[\alpha]_D = -59.8°$, and the specific rotation remained unchanged thereafter. A plot of $\lambda^2[\alpha]_\lambda$ *versus* $[\alpha]_\lambda$ obtained from dispersion measurements of optical rotation gave $\lambda_c = 203$ mμ. These values of $[\alpha]_D$ and λ_c should be compared with bovine gelatin in 8.5 *M* LiBr, where $[\alpha]_D = -60°$ and $\lambda_c = 198$ mμ, and in water, where $[\alpha]_D = -147°$ and $\lambda_c = 217$ mμ.[16] The data suggest that both LiBr and the alcoholic–acid mixtures affect the optical rotatory properties of gelatin through similar mechanisms of action.

Optical Rotatory Properties of Polymers Related to Poly-L-proline.—The effect of lithium bromide on the optical rotatory properties of polyhydroxy-L-proline is qualitatively similar to that observed for poly-L-proline II. Thus the specific rotation of the sodium D line is lowered from $-384°$ to $[\alpha]_D = -168°$ when lithium bromide is added to an aqueous solution of the polymer (final concentration of LiBr = 6 *M*). Moreover, the optical rotatory dispersion constant, λ_c, falls from $\lambda_c = 206$ mμ to $\lambda_c = 191$ mμ, a decrease paralleling that observed for poly-L-proline II under these same conditions ($\lambda_c = 202$ mμ in water: $\lambda_c = 185$ mμ in 8 *M* LiBr). These results support the idea that the polyhydroxy-L-proline chain possesses an asymmetric structure in aqueous solution essentially similar to that of poly-L-proline II.

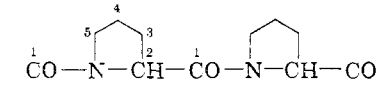
Upon preparation of polyhydroxy-L-proline from poly-O-acetylhydroxy-L-proline by deacetylation in aqueous solution, the strongly levorotatory form is obtained directly. Reverse mutarotation could not be effected since polyhydroxy-L-proline is insoluble in the solvents used for reverse mutarotation of poly-L-proline or poly-O-acetylhydroxy-L-proline. The decrease in levorotation upon dissolving polyhydroxy-L-proline in 6 *M* LiBr is, therefore, the only experimental evidence of changes in the configuration of polyhydroxy-L-proline.

Poly-O-acetylhydroxy-L-proline is insoluble in water but is readily soluble in the simple aliphatic acids. The mutarotation properties of this material have been considered in a previous section, but it was also of interest to examine the effect of LiBr on the optical rotatory properties. In acetic acid the specific rotation has the surprisingly low levorotatory value of $[\alpha]_D = -156°$, whereas $\lambda_c = 204$ mμ. Addition of LiBr (final concentration was 3.2 *M*) results in an *increase* in levorotation giving an $[\alpha]_D = -205°$ and $\lambda_c = 194$ mμ.

Discussion

The considerable amount of experimental and theoretical evidence now available shows that high molecular weight polypeptides may exist in solution in different configurations and that both the helical and random coil conformations are of importance in determining the distinct hydrodynamic and optical rotatory properties of these macromolecules in solution.[37] In discussing the configurational changes which polyproline undergoes in various solvent systems, it is pertinent to consider the possible configurations which this molecule can assume.

In general, the configuration of a chain molecule can be defined by the relative positions of the groups along the chain with respect to rotation around the bonds joining these groups. If rotation is relatively unhindered, then a chain will assume random configurations, changing continuously from one into another. The more restricted rotation becomes, the more probable will it be that certain energetically favored configurations become stable ones. Transition from one configuration to another, if at all possible, will be controlled by the energies of activation involved.



Since in polyproline stabilizing factors such as hydrogen bonds are absent, any stable configurations must be the result of rotational restrictions at all the bonds along the peptide chain. Rotation about the N–C(2) bond is obviously impossible as a result of its position in the pyrrolidine ring. Rotation about the peptide bond N–C(1) is restricted by its partial double-bond character. It has been estimated that rotations about similar CO–NH bonds require an energy of activation of more than 20 kcal./mole.[32] We shall define, as is generally accepted, the peptide bond as "*trans*" when the α-carbons are *trans* to each other,[33,38] *i.e.* in the case of polyproline, the oxygen atom is *trans* with respect to the C(5) carbon.

Rotation about the C(1)–C(2) bond is open to question. Inspection of polyproline-chain models constructed from Catalin (Waltham Abbey, Essex, England) atom models shows that there is definite restriction to rotation about this bond. If the peptide bond is in the "*trans*" configuration, the C(1)–C(2) bond can assume two rotational positions. In the first position the oxygen is *cis* to the C(2)-hydrogen. We shall call this position *cis'*. Rotation around this bond is limited to about 15°. In the second position, which we denote by *trans'*, the oxygen is *trans* to the C(2)-hydrogen and freedom of oscillation is about 60°. When the peptide bond is in the *cis* configuration, the rotation around the C(1)–C(2) bond is also restricted and the bond can in principle assume two positions a *cis'* and a *trans'*. These restrictions severely limit the number of regular configurational patterns which are available to a polymer of proline. When peptide bond groupings are all in the *trans* configuration, two homogeneous structures are possible. The first is a left-handed helix with C(1)–C(2) bonds in the *trans* configuration. This structure is the one proposed by Cowan and McGavin for polyproline II in the solid state. It has three proline residues per complete turn of the helix and a unit translational distance of 3.12 Å. along the longitudinal axis. The other form,

(37) S. J. Leach, *Rev. Pure and App. Chem.* (*Austral.*), **9**, 33 (1959).
(38) R. B. Corey and L. Pauling, *Proc. Royal Soc.* (*London*), **141B**, 14 (1953).

characterized by a *trans–cis'* configuration, would represent a right-handed helix. No indications, however, for its existence as a homogeneous structure were encountered in this investigation.

Turning now to the situation in which all peptide bonds are in the *cis* configuration, it can be seen from an examination of the models that a right-handed helix is obtained when the C(1)–C(2) bonds are in the *trans'* configuration. The helix has $3^1/_8$ residues per turn and a unit translational repeat distance of 1.85 Å. No experimental evidence was found for the existence of the fourth possible combination, the left-handed *cis–cis'* helix.

In addition there may exist an unlimited number of "mixed" configurations in which *cis*, *trans*, *cis'* and *trans'* bonds are arranged along the chain in different proportions and different patterns.

A left-handed helix would be expected to contribute to the inherent optical rotation of the L-prolyl residue ($[\alpha]_D = -200$ to $-250°$; see ref. 11 and discussion below) with a different sign than a right-handed one. Thus we might be justified to assign regular helical configurations to solutions exhibiting extreme values of optical rotation. These are the solutions of polyproline I ($[\alpha]_D = +50°$) and of polyproline II ($[\alpha]_D = -540°$).

On the strength of the following arguments we can now assign the *trans–trans'* structure to polyproline II. When a solid polyproline preparation which shows the characteristic X-ray diffraction pattern of the *trans–trans'* helix is dissolved in water or aliphatic acids, the optical rotation observed is that of polyproline II. On the other hand, solid polyproline samples obtained from polyproline II solutions (*e.g.* by drying, precipitation from acetic acid by means of ether, or heat precipitation from water) show the X-ray powder diagrams of the *trans–trans'* helix. Moreover, the observation that a solution of polyproline of a given molecular weight exhibits the highest viscosity when present in form II, is in agreement with the fact that the *trans–trans'* helix has, as evident from the measurements of molecular models, a larger axial ratio than any other possible form.

The assignment of the configuration of poly-L-proline II in solution which is given above allows us to draw a conclusion of more general interest. It demonstrates that the contribution to the optical rotation due to a helix which is left-handed in the absolute sense is of a negative sign. This is in accord with the theoretical considerations of Fitts and Kirkwood.[11,39]

Of the two possible right-handed helices, the *cis–trans'* and the *trans–cis'*, we assign the former to polyproline I in solution. The main argument for this comes from considerations of a kinetic nature. The slow conversion of form II into form I is catalyzed by acids. As discussed in Results, this acid catalysis has been shown to be associated with *cis–trans* isomerization of the peptide bond, whereas *trans'–cis'* transitions seem to be brought about by neutral salts, *e.g.*, lithium bromide, and are very fast at room temperature. The C–N bonds of form I must therefore be in the *cis* configuration. The hydrodynamic properties of polyproline I seem to confirm this conclusion. Axial ratios as determined by viscosity measurements in samples of molecular weight up to 20,000 are in agreement with the molecular dimensions of the right-handed *cis–trans'* helix. For the other possible right-handed helix, the *trans–cis'* helix, lower values in axial ratio are to be expected.

An estimate of the helix contribution to optical rotation for the two helical structures discussed above requires a knowledge of the optical rotation of the proline residue. Likely values for this residue rotation may be obtained from the specific rotations of low molecular weight proline peptides, of certain proline-containing copolymers and of poly-L-proline in aqueous LiBr. Table IV gives the optical rotation of a number of relatively simple peptide derivatives of proline and glycine. The observed specific optical rotation, corrected for index of refraction of the solvent and the calculated "residue" rotation of proline, assuming the rotatory contribution of glycine to be zero, are included. It will be seen that the residue rotation falls between $[\alpha]_D = -210°$ and $[\alpha]_D = -314°$. Harrington and Sela[11] estimated the residue rotation of proline in a peptide chain to be $[\alpha]_D = -250°$ from the specific rotation of a series of glycine–proline copolymers. Recently, copolymers with higher glycine–proline mole ratios have been synthesized,[40] and it has been found that a copolymer having a glycine–proline mole ratio of 8.8 gives $[\alpha]_D = -256°$ for the residue rotation of proline. We should also note that poly-L-proline II gives $[\alpha]^{25}D = -240°$ and $\lambda_c = 186$ mμ in aqueous solutions containing a high concentration of a number of neutral salts such as LiBr, $CaCl_2$, $LiClO_4$ and NaSCN. These salts effect a very drastic decrease in the intrinsic viscosity of polyproline II solutions which approaches that of globular molecules. It must therefore be assumed that the configurational asymmetry of the form II structure has been eliminated. The preceding evidence, when taken in conjunction, points to an $[\alpha]^{25}D$ of approximately $-250°$ as being the specific rotation of the proline residue. From this value the contribution to specific optical rotation $[\alpha]^{25}D$ of the right-handed helix of poly-L-proline I appears to fall around $+300°$ and that of the

TABLE IV

SPECIFIC RESIDUE ROTATION OF PROLINE IN SOME PEPTIDES

Compound	$[\alpha]_D$	Residue rotation	Ref.
Carbobenzoxy-L-propylglycyl-glycine	$-56.0°$	$-210°$	*a*
Carbobenzoxyglycyl-L-propyl-glycine	-80.9	-302	*a*
Glycyl-L-propylglycine	-108.4	-256	*a*
Glycyl-L-propylglycine ethyl ester hydrochloride	-104.0	-314	*b*
Triglycyl-L-proline ethyl ester hydrochloride	-60.0	-244	*b*

[a] N. C. Davis and E. L. Smith, *J. Biol. Chem.*, **200**, 313 (1953). [b] H. N. Rydon and P. W. G. Smith, *J. Chem. Soc.*, 3643 (1956).

(39) D. D. Fitts and J. G. Kirkwood, *Proc. Natl. Acad. Sci. U. S.*, **42**, 33 (1956).

(40) J. Kurtz, unpublished results.

left-handed helix of poly-L-proline II around $-290°$.

The experimental data reported in Results suggest that there is little difference between the enthalpies of poly-L-proline I and poly-L-proline II. It is therefore to be expected that solvation may be a decisive factor in determining which of the two forms is the stable one in a given solution. It may be recalled that aliphatic acids, *m*-cresol and water, in which polyproline II is readily soluble, stabilize form II while the addition of alcohols, in which polyproline is insoluble, favors the existence of form I. Similarly, polymerization of N-carboxy-L-proline anhydride in pyridine, in which poly-L-proline cannot be dissolved in either form, yields form I. Also in poly-O-acetylhydroxy-L-proline one observes forward mutarotation in good solvents (aliphatic acids and *m*-cresol) and reverse mutarotation in poor solvents (acetic anhydride and dimethylformamide). A plausible explanation of this situation is that form II, because of the more open character of its helix, is more easily subject to interaction with the solvent, thus gaining stability due to the energy of solvation. The stability of form II of poly-O-acetylhydroxy-L-proline in acetic anhydride in the presence of perchloric acid (Fig. 16) was explained above to be due to electrostatic repulsion between the charged peptide groups of the protonated peptide chain.

The transition of an unstable helical configuration into a stable one is an intramolecular process in which the ratio of the *cis* to *trans* peptide bonds changes gradually toward its final value. These changes are accompanied, as explained above, by various changes in the properties of the solution, the most striking change being that in optical rotation. A kinetic analysis of the configurational changes taking place during mutarotation may be attempted if a plausible correlation can be found between macromolecular conformation and optical rotation. In the following discussion we shall assume that each individual L-proline residue associated with a *cis* or *trans* peptide bond contributes to the specific optical rotation, $[\alpha]$, of the system according to the specific rotations of poly-L-proline I, $[\alpha]_{\mathrm{I}}$, and that of poly-L-proline II, $[\alpha]_{\mathrm{II}}$, respectively. Hence

$$[\alpha] = \frac{C_{cis}}{C_0} \cdot [\alpha]_{\mathrm{I}} + \frac{C_{trans}}{C_0} \cdot [\alpha]_{\mathrm{II}} \qquad (13)$$

where C_{cis} and C_{trans} denote the concentration of *cis* and *trans* peptide bonds, respectively, while $C_0 = C_{cis} + C_{trans}$ is the total concentration of peptide bonds. Eliminating C_{trans} from eq. 13 one obtains

$$\frac{C_{cis}}{C_0} = \frac{[\alpha] - [\alpha]_{\mathrm{II}}}{[\alpha]_{\mathrm{I}} - [\alpha]_{\mathrm{II}}} \qquad (14)$$

In the case of forward mutarotation, the initial specific rotation $[\alpha]_0 = [\alpha]_{\mathrm{I}}$ while the final specific rotation $[\alpha]_\infty = [\alpha]_{\mathrm{II}}$.

Equation 14 shows that $\mathrm{d}[\alpha]/\mathrm{d}t$ measures directly the rate of change in the number of *cis*p eptide bonds, *i.e.*, $\mathrm{d}C_{cis}/\mathrm{d}t$. The proposed picture for the spontaneous *cis*–trans transformation requires at any given instant proportionality between $\mathrm{d}C_{cis}/\mathrm{d}t$ and C_{cis}. Hence

$$-\frac{\mathrm{d}C_{cis}}{\mathrm{d}t} = KC_{cis} \qquad (15)$$

The "reaction constant" K is, however, not necessarily constant during the course of a mutarotation experiment since the ease with which a peptide bond undergoes isomerization may depend on the total configuration of the macromolecule. The "constant" K may, therefore, vary with $[\alpha]$, *i.e.*, with the degree of conversion C_{cis}/C_0, and hence with time. The over-all mutarotation reaction can thus be expressed by the equations

$$-\frac{\mathrm{d}C_{cis}}{\mathrm{d}t} = KC_{cis} \text{ where } K = \mathrm{f}\left(\frac{C_{cis}}{C_0}\right) \qquad (16)$$

or by

$$-\frac{\mathrm{d}C_{cis}}{\mathrm{d}t} = K'C_{cis} \text{ where } K' = \varphi(t) \qquad (17)$$

Equations 16 and 17 express the fact that when the dependence of the rate of mutarotation on the initial concentration C_0 is checked at a given degree of conversion (*i.e.*, when C_{cis}/C_0 = constant or t = constant) the rate is found to be proportional to concentration, thus obeying a first order law. On the other hand, an analysis of the kinetic curve of a single experiment (*i.e.*, when C_0 = constant) may reveal that the rate of mutarotation can be described only by a complicated function of C_{cis}.

The manner in which the reaction constant K' changes with time depends on the ease with which further isomerizations occur at any stage of conversion. This ease is in turn determined by the various intermediate configurations of the macromolecules present in the system at any given instant. The function $K' = \varphi(t)$ (see eq. 17) may be evaluated readily when the conversion, as in the case of forward mutarotation in acetic acid, can be expressed in terms of a reaction of a given order β. In such a case the rate of mutarotation can be expressed by eq. 18 where

$$-\frac{\mathrm{d}C_{cis}}{\mathrm{d}t} = k\left(\frac{C_{cis}}{C_0}\right)^{\beta-1} C_{cis} \qquad (18)$$

$k\left(\frac{C_{cis}}{C_0}\right)^{\beta-1}$ stands for $K'(t)$ in eq. 17, and k is independent of time (or C_{cis}) and defines the initial rate of mutarotation. Integration of (18) within the time limits $t = 0$ to t, corresponding to $C_{cis} = C_0$ to C_{cis}, yields

$$-\int_{C_0}^{C_{cis}} \frac{\mathrm{d}C_{cis}}{C_{cis}^{\beta}} = kC_0^{-(\beta-1)} \int_0^t \mathrm{d}t$$

$$\therefore \left(\frac{C_{cis}}{C_0}\right)^{\beta-1} = \frac{1}{1 + k(\beta - 1)t} \qquad (19)$$

From eq. 19 and 17, we obtain for the particular case under discussion

$$K'(t) = \frac{k}{1 + k(\beta - 1)t} \qquad (20)$$

Equation 20 shows that when $\beta > 1$, $K'(t)$ decreases with time. This case is exemplified by the forward mutarotation in acetic acid where $\beta = 1.33$ and $K'(t) = \frac{k}{1 + 0.33kt}$. When $\beta = 1$, *i.e.*, when the mutarotation proceeds with first order kinetics, as in the case of the reverse mutarotation in acetic acid–propanol, $K'(t)$ is constant throughout the reaction. Finally when $\beta < 1$, the apparent

reaction constant $K'(t)$ increases with time. A special case is found in the first part of the forward mutarotation in acetic acid–water which proceeds with zero order kinetics ($\beta = 0$). Here $K'(t) = k/(1 - kt)$.

The decrease in the apparent reaction constant $K'(t)$ with time might be explained by taking into account that this constant is the reciprocal of the rotational relaxation time of the peptide bond. If the mean hydrodynamic resistance of the two parts of the molecule, between which the rotating bond is located, increases during the reaction, this would cause the relaxation time to become longer. Evidence for a change of the resistance in the postulated direction can be found in the fact that the viscosity of polyproline increases during forward mutarotation in acetic acid. The decrease in viscosity in the first stages of mutarotation in acetic acid–water mixtures, on the other hand, points to a decrease in hydrodynamic resistance of the rotating parts of the molecule. This might be the reason for the observed increase in the reaction constant during mutarotation.

We turn now to a brief consideration of the hydrodynamic properties of poly-L-proline. As was mentioned earlier, the data of Table I demonstrate that the axial ratio of the Form II polymer, as calculated from the coördinates of the Cowan–McGavin helix,[6] for various molecular weights, differs significantly from the axial ratio estimated from the Simha equation (2). This deviation, which is appreciable at molecular weights of 12,000 and above, increases with increasing molecular weight. Since the calculated axial ratio is always larger than that estimated from the intrinsic viscosity and since this difference increases with molecular weight, it appears that the helix, which behaves as a stiff rod at low molecular weight, exhibits increasing flexibility as the contour length of the rod increases. If we assume that the molecule approaches a flexible chain at high molecular weights, the length of a statistical element of the chain may be estimated as follows from the equation of Kirkwood and Riseman (21).[41]

The intrinsic viscosity of a flexible chain may be approximated from the mean-square of the end-to-end distance, $\bar{r}^2$, and the molecular weight, M.

$$[\eta] = \frac{3.62 \times 10^{21} (\bar{r}^2)^{3/2}}{M} \qquad (21)$$

(41) J. G. Kirkwood and J. Riseman, *J. Chem. Phys.*, **16**, 565 (1948).

The root mean square distance may be given in terms of the number of statistical elements of the chain, n, and the length of an element, l, by (22).[42]

$$\bar{r}^2 = nl^2 \qquad (22)$$

Moreover, the molecular weight of a poly-L-proline II helix can be expressed as

$$M = \frac{97.1\ nl}{3.12 \times 10^{-8}} \qquad (23)$$

where 97.1 is the molecular weight of the prolyl residue and 3.12×10^{-8} cm. is the unit translational distance along the chain.

Combining equations 21, 22 and 23 and using $M = 5.2 \times 10^4$ and $[\eta] = 1.43$ dl./g. (sample D-33, Table I), the length of a statistical element, $l = 43$ Å. is obtained. Since the translational distance per proline residue along the axis of the helix is 3.12 Å., each statistical element consists of about 14 prolyl residues, *i.e.*, of approximately 4 turns of the helix. It should be noted that when a similar calculation is made for each of the other samples of poly-L-proline given in Table I, a statistical element of length about 40 Å. is obtained.

In view of the fact that all of the polymers which have been investigated have closely similar values for the specific rotation ($[\alpha]_D \approx -540°$), it can be concluded that while the viscosity is determined primarily by the end-to-end distance of the polymer, the optical rotation is a reflection of the helical structure of relatively short segments. These considerations demonstrate that the factors determining hydrodynamic properties on the one hand and optical rotatory properties on the other hand allow a given molecular species to take up different shapes as measured by viscosity but still possess essentially identical rotatory properties. This situation is clearly seen in Fig. 18.

NOTE ADDED IN PROOF.—The kinetics of mutarotation of poly-L-proline in glacial acetic acid was described recently by A. R. Downie and A. A. Randall, *Trans. Faraday Soc.*, **55**, 2132 (1959). The results which have been obtained are in agreement with those given by us in the corresponding section.

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(42) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 408.