In addition, the required data should be obtained by means of Rayleigh line-broadening measurements, since it is not yet clearly established that diffusion constants obtained by other methods are identical with results from line broadening. (For polystyrene in cyclohexane at 35.0° at concentrations under 2%, values of the diffusion constants obtained in this laboratory[2,11] are 15–25% lower than those obtained by Cantow[8] from ultracentrifuge experiments on the same system.)

Analysis of the data for NBS-706 polystyrene in cyclohexane reported above yield a single diffusion constant of $1.89 \times 10^{-7}$ $cm^2$ $sec^{-1}$ at a concentration of 0.54% polymer by weight. A plot of log $D$ against log $M$ using data obtained previously for the polystyrene–cyclohexane system[2] at about the same polymer concentration can be used to determine an estimated value of the diffusion constant of the species having the weight-average molecular weight. For $M$ equal to 257,800 ($M_w$ from light-scattering), the value of $D$ estimated in this fashion is approximately $2.16 \times 10^{-7}$ $cm^2$ $sec^{-1}$; for $M$ equal to 288,100 ($M_w$ from sedimentation equilibrium) the estimated value of $D$ is $2.07 \times 10^{-6}$ $cm^2$ $sec^{-1}$. The experimentally determined ratio $D_c/D_w$ is thus 0.87 or 0.91, depending on whether the value of $M_w$ from sedimentation or light scattering, respectively, is used. These values compare very favorably with the computed value of 0.82 given in Table I for $x = 0.1$, $\alpha = 0.5$, $z = 1$. The agreement can probably be considered excellent when it is taken into account that all the data used in calculating the experimental value of $D_c/D_w$ were obtained at a fairly high concentration.

(11) T. F. Reed, Ph.D. Thesis, The University of Akron, 1970.

**Summary**

The experimental results reported here confirm two predictions of theory for the spectrum of light scattered from polydisperse samples of random-coil polymers of relatively low molecular weight. The spectrum from samples with fairly broad distributions of molecular weight will be close to a single Lorentzian, and discrepancies from the Lorentzian form will be detectable only by measurements of very high precision. Second, the angular dependence of the spectral half-width for such samples will be very nearly proportional to $\kappa^2$. These results point out the relative insensitivity of linewidth studies to sample polydispersity; this should be taken into account in interpretation of diffusion studies by this technique. On the other hand, this insensitivity may be beneficial in studies of other variables (*e.g.*, intramolecular motion) because of the lack of complication due to polydispersity.

Finally, additional experimental work will be necessary to allow quantitative comparison of theory for polydisperse materials with experimentally observed apparent diffusion constants. Particular emphasis should be placed on obtaining reliable extrapolated values at zero concentration and on firm establishment of the relationship between diffusion constants obtained by spectral determinations and those obtained by other methods.

**Acknowledgments.** This work was supported in part through National Science Foundation Grant GP-8328. Acknowledgment is also made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this research.

# A Nuclear Magnetic Resonance Study of Poly(L-proline) in Aqueous and Aqueous Salt Solutions

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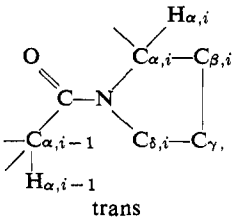
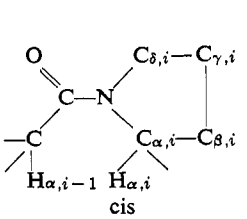
ABSTRACT: The isomerization of poly(L-proline) I has been followed in $D_2O$ using 220-MHz nmr. It was found that the reaction follows zero-order kinetics with an activation enthalpy of *ca.* 23 kcal/mol. It is suggested, on the basis of these results and the behavior during the reaction of the clearly observed $\alpha$-trans and $\alpha$-cis proton resonances, that in $D_2O$ isomerization begins at the carboxyl end of the polymer and proceeds in a stepwise fashion down the chain. The effects of concentrated solutions of KI, NaSCN, $CaCl_2$, and LiBr on the structure of poly(L-proline) I and II were also examined using 220-MHz nmr. Resonances corresponding to both $\alpha$-trans and $\alpha$-cis protons were found in all these solutions. It is therefore concluded that the disruption of the ordered poly(L-proline) structure in these solutions is primarily due to the presence of both trans and cis peptide bonds randomly distributed along the polymer chain.

The conformational isomerization of poly(L-proline) in water and organic solvents and the effects of high concentration of salts on the structure of poly(L-proline) have been the subject of many investigations employing optical and hydrodynamic techniques. A detailed discussion of this work will not be given since several recent reviews summarizing and evaluating these results are available.[1-3] One conclusion resulting from previous work is that when poly(L-proline) I, containing only cis peptide bonds, is dissolved in water, it undergoes an isomerization at the peptide bonds and is converted to the all-trans form II. The geometry of the peptide bond in the trans and cis conformations is shown. The *i*th



trans



cis

(1) J. P. Carver and E. R. Blout in "Treatise on Collagen," Vol. I, G. N. Ramachandran, Ed., Academic Press, New York, N. Y., 1967, p 441.
(2) L. Mandelkern in "Biological Macromolecules," Vol. I, G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1967, p 675.
(3) P. H. Von Hippel and T. Schleich in "Biological Macromolecules," Vol. II, S. N. Temasheff and G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1969, p 571.

$\alpha$ proton is said to be a trans $\alpha$ proton if $C_{\alpha,i}$ is trans to $C_{\alpha,i-1}$. The term cis $\alpha$ proton is defined in analogous fashion.

Detailed kinetic studies[4,5] of the isomerization have been carried out by following the change in $[\alpha]_D$ which occurs on dissolving poly(L-proline) I in solvents which support form II. Although the relationship between $[\alpha]_D$ and the fraction of the chain that has isomerized is not known, it was found possible to obtain the activation enthalpy (*ca.* 20–24 kcal/mol) of the reaction. However, a simple kinetic law describing the extent of isomerization as a function of time was not found. Previous nmr studies[6,7] of poly(L-proline) have shown that $\alpha$-trans and $\alpha$-cis protons have significantly different chemical shifts. At 220 MHz we find that resonances corresponding to the $\alpha$-trans and $\alpha$-cis protons can be clearly distinguished in $D_2O$ and that therefore the rate of cis–trans isomerization can be directly measured. In this paper the nmr data so obtained are reported; they indicate that in $D_2O$ the conformational isomerization of poly(L-proline) (DP *ca.* 25) follows zero-order kinetics and that resonances corresponding to structures different from either form I or form II are present during the reaction.

Previous studies of poly(L-proline) in aqueous solution containing high concentrations of salts such as LiBr, NaSCN, KI, or $CaCl_2$ have led to the conclusion[5,8–13] that the polymer loses its regular structure in such solvents. However, general agreement as to the precise nature of the disordered structure has not yet been attained. It has been proposed[5,10–12] that high concentrations of these salts induce a collapse in the ordered poly(L-proline) structure by increasing the accessible range of the $C_\alpha$—C=O angle, $\psi$, while all peptide bonds remain trans (*i.e.*, $\omega = 0°$). In an early paper, Harrington and Sela concluded that isomerization at the peptide bonds was responsible for the loss of regular structure. More recently, however, Kurtz and Harrington[9] have ascribed the loss of regular structure to both increased freedom of rotation about $\psi$ and to small deviations of $\omega$ from a value of 0°. The nmr data we have obtained provide strong evidence for the presence of cis and trans peptide bonds in individual polymer chains and indicate that isomerization about the peptide bond is the principal cause of disorder in the polymer in concentrated salt solutions.

## Experimental Section

All experiments were carried out using a single sample of low molecular weight poly(L-proline)[14] which was kindly provided by Dr. C. M. Deber and Professor E. R. Blout of the Harvard Medical School. The synthesis of this material is described in the literature,[7] and the sample was found to have DP *ca.* 25.[15] The salts were purchased from commercial sources and dried in a vacuum oven to remove as much $H_2O$ as possible. Diaprep "100.0%" $D_2O$ was used to prepare all solutions. *tert*-Butyl alcohol-$d_1$ was purchased from Merck, Sharp and Dohme and the *tert*-butyl group at $\tau$ 8.77[16] was used as an internal reference.

Nmr spectra were obtained using the Varian HR-220 spectrometers at these laboratories and at Rockefeller University. A Fabri-Tek time-averaging computer (CAT) with 1024 channels was used to improve the signal-to-noise ratio of the spectra of some of the salt solutions. The spinning side bands of the HDO resonances have been deleted from all the spectra for purposes of clarity.

## Results

**Isomerization of Poly(L-proline) in $D_2O$.** Portions of the nmr spectra of poly(L-proline) I in $D_2O$, measured at successive times after dissolving the sample, are presented in Figure 1. Changes in the $\alpha$- and $\delta$-proton resonances during isomerization are clearly evident. The resonances at $\tau$ 5.6 and 5.7 are assigned to the $\alpha$-cis protons. The area of the cis-proton resonance at $\tau$ 5.6 is seen to decrease with time, while the area of the $\alpha$-trans resonance, which appears at $\tau$ 5.3, increases. Significantly, the cis resonance at $\tau$ 5.7 corresponds to about one $\alpha$ proton per chain and maintains an approxi-

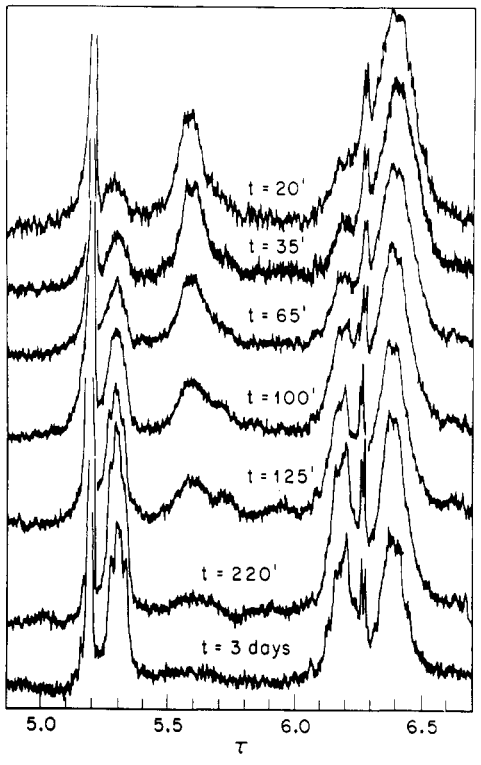



Figure 1. 220-MHz spectrum of the $\alpha$-trans ($\tau$ *ca.* 5.3), $\alpha$-cis (*ca.* 5.6–5.7), and $\delta$ protons (*ca.* 6.2–6.4) of poly(L-proline) at successive times after dissolving a form I sample in $D_2O$ at 22°. HDO resonance at 5.2. Methyl ester resonances[14] at 6.25 and 6.27.

(4) A. R. Downie and A. A. Randall, *Trans. Faraday Soc.*, **55**, 2132 (1959).
(5) I. Steinberg, W. F. Harrington, A. Berger, M. Sela, and E. Katchalski, *J. Amer. Chem. Soc.*, **82**, 5263 (1960).
(6) F. Conti, M. Piatelli, and P. Viglino, *Biopolymers*, **7**, 441 (1969).
(7) C. M. Deber, F. A. Bovey, J. P. Carver, and E. R. Blout, *J. Amer. Chem. Soc.*, **92**, 6191 (1970).
(8) W. F. Harrington and M. Sela, *Biochim. Biophys. Acta*, **27**, 24 (1958).
(9) J. Kurtz and W. F. Harrington, *J. Mol. Biol.*, **17**, 440 (1966).
(10) M. L. Tiffany and S. Krimm, *Biopolymers*, **6**, 1379 (1969).
(11) T. Schleich and P. H. Von Hippel, *ibid.*, **7**, 861 (1969).
(12) P. H. Von Hippel and T. Schleich, *Accounts Chem. Res.*, **2**, 257 (1969).
(13) W. L. Mattice and L. Mandelkern, *Biochemistry*, **9**, 1049 (1970).
(14) The presence of a sharp singlet at $\tau$ 6.27, which remains on lyophilization from $D_2O$, indicates that the sample is the methyl ester of poly(L-proline). This conclusion is confirmed by the presence of a methyl resonance at $\tau$ 6.25 in the spectrum of *N*-acetyl-L-proline methyl ester in $D_2O$. Also, addition of base to solutions of the polymer or model compound results in loss of the methyl ester peak with the appearance of the methyl resonance of methanol at $\tau$ 6.67. The polymer also contains a low molecular weight methyl ester impurity identified by a singlet at $\tau$ 6.25 which disappears on lyophilization and shifts to $\tau$ 6.67 (MeOD) in the presence of base. All methyl resonances have been edited from the salt solution spectra.
(15) The number average molecular weight of the polymer was determined by nmr in the following way. Poly(L-proline) II was dissolved in TFA. In this solvent the $NH_2^+$ protons on the N-terminal proline residue exchange slowly with the TFA proton and are observed as a broad but distinct resonance at $\tau$ *ca.* 3. The area of the $\alpha$-proton resonance was found to be about 12–14 times the area of $NH_2^+$ resonance. From this result it follows that a polymer chain contains about 25 $\alpha$ protons per $NH_2^+$ group.
(16) Relative to DSS in $D_2O$ and in concentrated salt solutions.

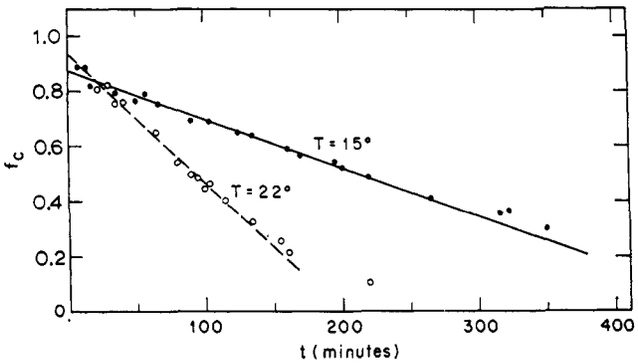



Figure 2. Fraction of cis peptide bonds in samples of form I dissolved in $D_2O$ plotted as a function of time.

mately constant area until the isomerization is almost complete.

The data shown in Figure 1 were obtained at 22°. Owing to the presence of the HDO peak near the α-trans resonance it is difficult to determine if an additional small trans resonance is also present. For this reason the reaction was also observed at 8°. At this temperature the HDO peak is located more than 50 Hz downfield from the α-trans resonance, and it can be clearly seen that only one α-trans resonance is present. As these changes take place in the α region, the δ protons, both of which have nearly the same chemical shift in form I and appear as a broad resonance at τ *ca.* 6.4, gradually separate into two distinct resonances of almost equal area at τ 6.2 and 6.4 in the fully isomerized sample.

In general, the assignments made here are in agreement with those found in the literature.[6,7] However, as indicated in Figure 1, our spectra exhibit two features previously unreported: (1) the presence of two resonances in the α-cis region of the spectrum (at 5.6 and 5.7) during isomerization, (2) the resolution of the spin–spin fine structure of the α-trans resonance. It was found that the resolution of the fine structure of the α-trans resonance improved as the isomerization advanced.

The kinetics of the isomerization were determined by measuring the areas of the cis and trans α-proton resonances as a function of time. Defining the fraction of cis peptide bonds as

$$f_c = \frac{\text{area of } \alpha\text{-cis resonances (at } \tau \text{ 5.6 and 5.7)}}{\text{area of } \alpha\text{-cis resonances} + \text{area of } \alpha\text{-trans resonance}}$$

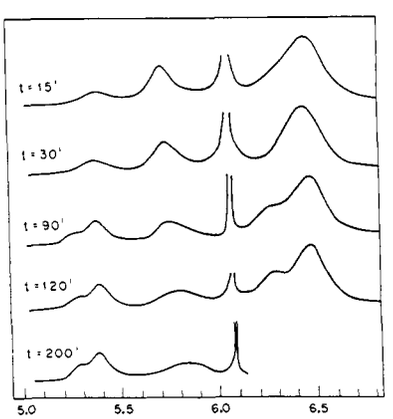



Figure 3. 220-MHz spectrum[18] of the α-trans (τ *ca.* 5.3–5.4), α-cis (*ca.* 5.7–5.9), and δ protons (*ca.* 6.2–6.5) of poly(L-proline) at successive times after dissolving a form I sample in 5 *M* KI at 22°. HDO resonance at 6.1.

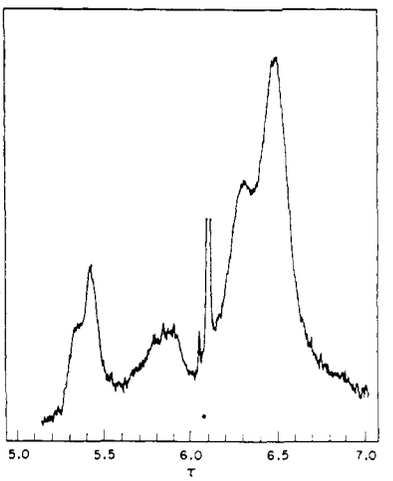



Figure 4. 220-MHz spectrum (86 CAT scans accumulated) of the α-trans (τ *ca.* 5.3–5.4), α-cis (*ca.* 5.7–5.9), and δ protons (6.2–6.5) of poly(L-proline) equilibrated in 5 *M* KI at 22°. HDO resonance at 6.1.

one finds that $f_c$[17] decreases linearly with time, indicating that the reaction follows zero-order kinetics. The results obtained at 15 and 22° are presented in Figure 2, which shows that the rate constant increases by a factor of 2.7 on raising the temperature from 15 to 22°. From this result an activation enthalpy of 23 kcal/mol is calculated, in approximate agreement with the values of $\Delta H^{\ddagger}$ found previously[4,5] using optical data.

**Poly(L-proline) in Aqueous Salt Solutions.** The spectral changes observed when poly(L-proline) I is dissolved in 5 *M* KI are shown[18] in Figure 3. An accumulated spectrum (86 CAT scans) of the sample equilibrated in the KI solution appears in Figure 4. The changes occurring during isomerization in the salt solution are similar to those found in Figure 1, and the half-life for isomerization in salt solution and in $D_2O$ is about 90 min at 22°. However, the spectrum of the equilibrated sample in the salt solution differs from that found in $D_2O$ in several important respects. The α-cis resonance, while broadened and reduced in area, is still present when equilibrium is attained, the ratio of trans to cis bonds being about 2:1. Also two separate resonances are clearly seen in the α-trans region of the spectrum and the upfield δ resonance has about twice the area of the downfield δ resonance.

Spectral changes observed when poly(L-proline) I is dissolved in 4.5 *M* NaSCN are very similar to those shown in Figure 3. Again, a half-life of about 90 min is observed for isomerization. An accumulated spectrum (128 scans) of poly(L-proline) equilibrated in 4.5 *M* NaSCN, shown in Figure 5, is seen to have essentially the same features found for the polymer dissolved in 5 *M* KI. The same final spectrum is obtained whether form I or II is initially dissolved in the salt solution. However, form I requires several hours to attain equilibrium whereas form II equilibrates within the time re-

(17) When the isomerization of form I solutions with polymer concentrations greater than *ca.* 1% were observed it was found that a small resonance (with an area equal to *ca.* 10% of the area of the α-proton resonance) persisted with constant area at τ 5.6 for weeks after the polymer was dissolved. Since the resonance disappeared after heating the sample to 60° it appears probable that it corresponds to α protons in a form I aggregate of the type reported previously.[8] The area corresponding to the aggregate was not included in the calculation of $f_c$.

(18) For purposes of clarity, the noise present in the original spectra has been eliminated by smoothing. Since these spectra are time dependent, it was not feasible to improve the signal-to-noise ratio by computer averaging.

quired to obtain the spectrum (10 min). These results are similar to those obtained from optical measurements.[5,9]

It was not possible to follow the isomerization of poly(L-proline) I in $CaCl_2$ or LiBr solutions due to the low solubility of the polymer in concentrated solutions of these salts. Form II was sufficiently soluble to allow spectra to be obtained. The spectrum of form II as a function of $CaCl_2$ concentration[19] is shown in Figure 6 along with the form II spectrum in $D_2O$ for purposes of comparison. As the concentration of salt is increased the $\alpha$-trans resonance at $\tau$ 5.3 decreases in area relative to the broad shoulder at $\tau$ *ca.* 5.2–5.3 and is finally replaced by a single broad resonance in 4 *M* $CaCl_2$. In the cis $\alpha$-proton region a broad resonance appears at *ca.* 5.7–5.9 at all salt concentrations. The area of the high field ($\tau$ 6.4) $\delta$ resonance is greater than the area of the low field (6.2) $\delta$ resonance at all salt concentrations and both resonances broaden with increasing salt concentration. Similar spectra were obtained for LiBr and KI solutions when the salt concentrations were increased from 2.5 to 9 *M* and from 1.5 to 5 *M*, respectively.

## Discussion

Figure 1 shows that the spectrum of poly(L-proline) II (all trans bonds) contains a single $\alpha$-proton resonance at $\tau$ 5.3. A more complex spectrum is expected for a polymer which contains a sequence of cis bonds for one part of its length and a sequence of trans bonds over the remainder. Counting bonds from the N-terminal and denoting the *i*th cis bond by $c_i$ and the *i*th trans bond by $t_i$, such a chain can be represented by the sequence

$$c_1 \ldots c_{i-1}\ c_i\ t_{i+1}\ t_{i+2} \ldots t_n \qquad \text{(A)}$$

In such a sequence the trans protons should exhibit a single resonance with about the same chemical shift found in poly(L-proline) II, since, as in form II, every trans $\alpha$ proton is attached to an $\alpha$ carbon which is trans to both the $\alpha$ carbon on its right and on its left. Every cis $\alpha$ proton, except the *i*th, is attached to an $\alpha$ carbon which is cis to the $\alpha$ carbon on its right and left. Figure 1 indicates that a resonance corresponding to these protons lies 0.3 ppm upfield from the $\alpha$-trans resonance. Since the *i*th $\alpha$ carbon is unique in that it is cis to the $\alpha$ carbon on its left but trans to the $\alpha$ carbon on its right, the *i*th cis $\alpha$ proton is assigned a chemical shift which is different from that of the other cis $\alpha$ protons. Thus, for a sequence of type A the above analysis predicts the presence of a single trans resonance and two cis resonances, with relative area in the ratio of $(i - 1):1$, in the $\alpha$ proton region of the spectrum. In the case of the sequence

$$t_1 \ldots t_{i-1}\ t_i\ c_{i+1}\ c_{i+2} \ldots c_n \qquad \text{(B)}$$

one cis and two trans resonances are expected.

Extending the above argument to a chain containing a random sequence of cis and trans bonds it is noted that while the chemical shift of the *i*th $\alpha$-trans proton is influenced by the conformation at the $(i + 1)$ peptide bond, conformations at the neighboring but more distant peptide bonds at positions $i - 1$, $i + 2$, etc., are expected to exert a smaller influence on the chemical shift of the proton. In a random chain, many different sequences of peptide bonds can occur in the neighborhood of the *i*th $\alpha$-trans proton, each different sequence producing a slightly different magnetic field at the proton. For this reason, the $\alpha$-trans region of the spectrum will consist of a sum of resonances corresponding to $\alpha$-trans protons having slightly different chemical shifts, resulting in a single broad resonance. The above argument, when applied to the $\alpha$-cis protons in a random chain, predicts that a single broad resonance also occurs in the $\alpha$-cis region of the spectrum.

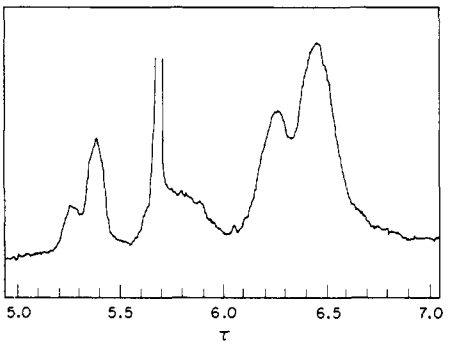



Figure 5. 220-MHz spectrum (128 CAT scans accumulated) of the $\alpha$-trans ($\tau$ *ca.* 5.3–5.4), $\alpha$-cis (*ca.* 5.7–5.9), and $\delta$ protons (6.2–6.5) of poly(L-proline) equilibrated in 4.5 *M* NaSCN at 22°. HDO resonance at 5.7.

**A Model for the Form I → Form II Transformation in $D_2O$.** In proposing a model for the isomerization of poly(L-proline) in $D_2O$, we are guided by the following results, presented in the previous section. (1) The spin–spin splitting of the $\alpha$-trans proton resonance becomes observable as the isomerization advances. (2) During isomerization one $\alpha$-trans and two $\alpha$-cis resonances appear in the spectrum. (3) The area of the smaller $\alpha$-cis resonance at $\tau$ 5.7 corresponds to about one $\alpha$ proton per polymer chain and maintains a constant area until the reaction is almost over.[20] (4) The cis–trans isomerization of the polymer follows zero-order kinetics.

Observation 1 provides strong evidence that in the early

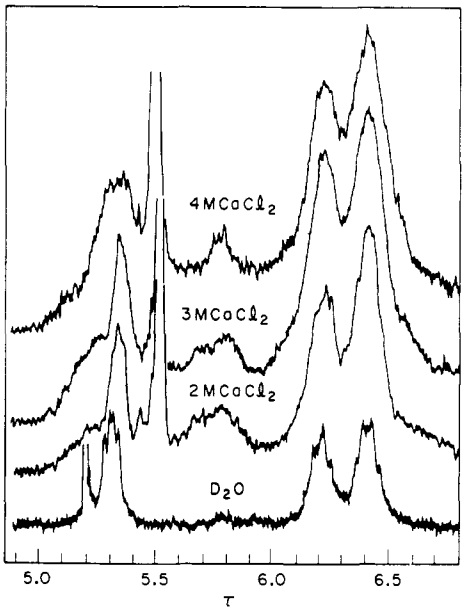



Figure 6. 220-MHz spectra (41, 64, and 15 CAT scans accumulated in 2, 3, and 4 *M* $CaCl_2$, respectively) of the $\alpha$-trans ($\tau$ *ca.* 5.2–5.4), $\alpha$-cis (*ca.* 5.7–5.9), and $\delta$ protons (*ca.* 6.2–6.5) of poly(L-proline) equilibrated in 2–4 *M* $CaCl_2$ at 55 ± 2°. HDO resonance at 5.5. Bottom spectrum, poly(L-proline) II in $D_2O$ at 22°.

(19) The spectra obtained in $CaCl_2$ were run at 55 ± 2°, since at lower temperatures the HDO resonance interfered with observation of the $\alpha$-trans resonance.

(20) The possibility that the $\tau$ 5.7 resonance corresponds to a C- or N-terminal $\alpha$ proton was rejected since the resonance did not shift position on changing the pH from 1.5 to 10.5. At the high pH, the C-terminal methyl ester[14] is converted to the free acid and methanol.

stages of the reaction the $\alpha$-trans proton resonance consists of contributions from protons in different conformational sequences, producing a dispersion of the chemical shifts. On the basis of the discussion presented at the beginning of this section, observation 2 suggests that these intermediate states are peptide sequences of the form A. It would then follow from (4) that isomerization begins at the C-terminal peptide bond[21] and progresses through sequences like A in a stepwise fashion down the chain. This accounts for observation 3.[22]

Several results in the literature provide support for the isomerization model proposed. It has been noted[23] that space-filling models indicate that trans–cis junctions exhibit considerably more steric strain than cis–trans junctions. These qualitative observations have been confirmed by intramolecular energy calculations on poly(L-proline) and prolyl oligomers.[24,25] Thus, type A structures rather than type B or structures containing mixtures of cis and trans bonds would be energetically favored as the intermediate states during isomerization as suggested by the nmr data.

If isomerization begins at one end of the polymer and progresses steadily in a stepwise fashion, it is reasonable to expect $[\alpha]_D$ to be approximately a linear function of time as the reaction progresses,[26] since a given polymer chain will consist of an all-cis sequence followed by an all-trans sequence, with the fraction of trans bonds increasing in a linear fashion with time. When mutarotation is followed in acetic acid, the behavior of $[\alpha]_D$ as a function of time is markedly nonlinear[4,5,27] and the model proposed would not appear to apply in this solvent. In aqueous solution $[\alpha]_D$ was found to change in an approximately linear fashion with time for a sample with DP *ca.* 30[9] but not for a sample with DP *ca.* 50.[8]

The stepwise isomerization model also predicts that the half-life for mutarotation will be inversely proportional to the molecular weight of the polymer.[28] Kurtz and Harrington[9] reported that a sample of DP *ca.* 30 mutarotated in $D_2O$ with a half-life of about 2 hr at 24°, which is about 50% larger than the half-life found for our sample (DP *ca.* 25) at this temperature. However, Harrington and Sela[8] found that a sample with a DP of *ca.* 50 mutarotated with a half-life of about 40 hr in water, which is about ten times the half-life predicted by the model on the basis of the lower molecular weight results.[29]

Thus, the previously published mutarotation data suggest that the stepwise model applies only to low molecular weight poly(L-proline) (DP $\lesssim$ 40) in aqueous solution. However, even in this case there are not sufficient data to provide conclusive support for the model. A study of the isomerization of monodisperse samples in the 1500–5000 molecular weight range is needed to provide a rigorous test of the model.

**Discussion of Salt Solution Data.** Optical and nmr results indicate that the conversion of poly(L-proline) I to the disordered conformation in salt solutions proceeds at a much smaller rate than the transformation of form II to the same final state. This result and similar data obtained by diluting a salt solution of partly or fully equilibrated form I samples were taken as evidence[5] that the randomization of the poly(L-proline) structure is caused by increasing the allowed values of the $\psi$ angle, as mentioned previously. If this were the case, the polymer spectra would show a narrowing of the nmr lines in salt solutions due to the increased flexibility of the chain. However, as can be seen in Figures 3–6, adding salts to aqueous poly(L-proline) solutions leads to a broadening of the resonances.[30] Thus, our data do not support the view that the disordered poly(L-proline) structure in salts is due to increasing the range of $\psi$. On the other hand, the appearance of a broad resonance at $\tau$ 5.7–5.9 ($\alpha$-cis) in the salt spectra in Figures 4–6 provides strong evidence for the presence of cis peptide bonds in the disordered polymer structure.[31] Figure 3 shows that the $\alpha$-cis resonance shifts position slightly and broadens markedly as the form I structure is replaced by the disordered salt structure. Such line broadening is expected for reasons given at the beginning of this section if an all-cis sequence is replaced by a random sequence of cis and trans bonds.

Figures 4–6 show that the area of the upfield $\delta$ resonance exceeds that of the $\delta$ resonance at lower field, which is also consistent with the presence of cis peptide bonds in salt solution.

The changes in the $\alpha$-trans resonances in Figure 6 as a function of $CaCl_2$ concentration indicate the manner in which salts disrupt the polymer structure. At moderate $CaCl_2$ concentration (2–3 *M*) two resonances, one "broad" ($\tau$ *ca.* 5.2–5.3) and one "narrow" ($\tau$ *ca.* 5.3) appear in the $\alpha$-trans region. In 2 *M* $CaCl_2$ a spin–spin splitting pattern similar to that obtained for the resonance in form II is just resolved in the $\tau$ 5.3 ($\alpha$-trans) resonance, suggesting that this resonance corresponds to protons in form II regions of the polymer, as yet undisrupted by the salt. As the salt concentration increases, the area of this resonance decreases relative to the area of the broad resonance, indicating that the fraction of form II is reduced relative to that of the disordered structure as the salt concen-

(21) The anomalous cis resonance and zero-order kinetics rule out a highly cooperative process in which the reaction proceeds rapidly after the initial cis–trans isomerization occurs. Random isomerization along the chain is inconsistent with the kinetics, and the $\alpha$-trans line width in Figure 1 is much narrower than expected for a random sequence of cis and trans peptide bonds (see discussion of data in aqueous salts). The kinetic data suggest that the rate of the initial isomerization is at least as large as the rate for subsequent isomerizations. If the initial rate were smaller, the observed kinetics would reflect a combination of zero- and first-order behavior. This may be the case in organic solvents, such as acetic acid.

(22) The initial measurements of the I–II transformation show that one or two trans bonds per chain and a single polymer methyl[14] resonance are present. This result is consistent with the model, since it shows that at the earliest stages of the isomerization all C-terminal prolyl bonds are trans. If cis and trans C-terminal bonds were present, two methyl resonances would appear as is found for *N*-acetyl-L-proline methyl ester ($\tau$ 6.19 and 6.25).

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(26) This statement is not valid for a sample composed of a broad distribution of molecular weights. In such a case all the polymer chains undergo isomerization at the early stages of the reaction but only the high molecular weight chains have cis bonds available for isomerization as time progesses. Thus $d[\alpha]_D/dt$ will show a monotonic decrease as a function of time.

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(28) As before, the statement applies only if the sample contains a narrow molecular weight distribution.

(29) The stepwise isomerization model was rejected in an acetic acid–water (7:3 v/v) solvent system, since samples with $M_n$ = 12,000 and 15,000 mutarotated at the same rate. However, the small range of molecular weights employed in this study[5] does not seem adequate to rule out the model.

(30) The spectrum of poly(L-proline) II in $D_2O$ at 22° indicates that individual lines of the spin–spin multiplets have *ca.* 4-Hz line widths. On doubling the solution viscosity these individual line widths would increase to about 8 Hz, but the total width of a multiplet would increase by only 4–5 Hz. The viscosity of 5 *M* KI and 4.5 *M* NaSCN solutions are respectively 0.9 and 1.5 times the viscosity of water; thus the substantial broadening of the proline resonances observed in these solutions cannot be ascribed to viscosity increases. The same is true for $CaCl_2$ solutions where the solution viscosity (at 55°) increases from 0.9 to 1.8 times that of water (at 22°) on going from 2 to 4 *M* $CaCl_2$.

(31) On adding any of the four salts to $D_2O$, the $\alpha$- and $\delta$-proton resonances shift upfield by 0.05–0.2 ppm. These shifts are ascribed to solvent effects.

tration increases. At 4 *M* $CaCl_2$ or above a very broad resonance remains in the trans region of the spectrum, indicating that at most only short sequences of form II structure remain at this and higher concentrations. This is in agreement with conclusions of Mattice and Mandelkern,[13] who followed changes in circular dichroism and intrinsic viscosity of poly(L-proline) as a function of $CaCl_2$ concentration.

In an effort to rationalize the stability of a poly(L-proline) chain containing a mixture of cis and trans peptide bonds in the salt solutions, the conformations of structures containing mixtures of cis and trans bonds were examined using CPK models. It was found that carbonyl oxygens in sequences such as trans–cis–trans or trans–cis–cis are favorably positioned to bind a cation. Evidence for $Li^+$ binding to poly(L-proline) in LiBr solutions has been reported by Kurtz and Harrington,[9] who concluded that *partial* rotation about the peptide bonds was due to the formation of a multiresidue $Li^+$ complex. Our picture of the complex indicates that it should contain at most 1 mol of bound salt for every 3 mol of peptide. Kurtz and Harrington found that the complex contained at least 1 mol of $Li^+$/5 mol of proline, which is consistent with the model proposed.

It has long been recognized that the rates for reactions of the type form I $\rightleftharpoons$ form II or form I $\rightleftharpoons$ [disordered state] are much smaller than the rate for the form II $\rightleftharpoons$ [disordered state] reaction. We believe that the results presented above firmly establish the presence of cis peptide bonds in salt solutions of poly(L-proline) and therefore conclude that all these reactions involve cis–trans isomerizations of the peptide bond. The activation enthalpy is about 20 kcal/mol for all these reactions. This implies that there is a large negative entropy of activation in reactions involving the isomerization of poly(L-proline) I but not of form II. Why this is so is not clear, but may be related to the extraordinarily compact structure of form I.

In summary, our data suggest that in $D_2O$, form I poly(L-proline) of low molecular weight isomerizes to form II in a stepwise fashion, starting at the carboxyl end of the chain. Addition of certain salts to aqueous solutions of poly(L-proline) in either form I or form II disrupts the organized polymer structure primarily by producing cis–trans isomerizations at the peptide bonds. At low to moderate salt concentrations the polymer contains ordered sequences of trans bonds and disordered sections containing a mixture of cis and trans bonds. At high salt concentrations the ordered regions are eliminated, leaving a polymer containing mixtures of cis and trans bonds along the entire length of the chain. It is suggested that the resulting disordered structure is stabilized by the formation of a multiresidue cation complex.

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# Proton Nuclear Magnetic Resonance Study of Corticotropins

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ABSTRACT: This study outlines a proton nmr investigation of the hormone fragments of ACTH in neutral and acidic aqueous solution. Difference and addition proton nmr spectral studies of $ACTH^{1-10}$, $ACTH^{11-24}$, and $ACTH^{1-24}$ in the peptide NH and CH regions in acidic aqueous solution indicated the absence of interactions between sequences 1–10 and 11–24 in $ACTH^{1-24}$.

Much activity has been focused over the last few years on the isolation, purification, and synthesis of the adenohypophyseal hormone adrenocorticotropin (ACTH).[1]

$ACTH^{1-24}$

Ser-*Tyr*-Ser-*Met-Glu-His-Phe-Arg-Trp-Gly*-Lys-*Pro*-Val-Gly-
1 5 10
Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro
15 20

The 39 amino acid linear hormone ACTH is believed to stimulate steroidogenesis in the adrenal gland by the specific activation of enzymes. The amino acid residues at the C terminal and at positions 25–33 show species variation. The biologically active fragment $ACTH^{1-24}$ contains the basic amino acid sequence Lys-Lys-Arg-Arg at positions 15–18 which is currently believed to be the binding site for the receptor. The loss of adrenocorticotropic activity on protection of the N-terminal end and the results of amino acid modifications of the positions 1–10 of ACTH have led to the tentative identification of the functional site of the adrenocorticotropic activity with the N-terminal end.

Several approaches to the study of secondary structures of these hormones by physical techniques have recently been reported. Eisinger[2] has studied the efficiency of singlet energy transfer from tyrosine residues at positions 2 and 23 to the tryptophan at position 9 in $ACTH^{1-24}$. The measured intramolecular distances (Tyr-2–Trp 9 = 10 Å, Trp-9–Tyr 23 $\geq$ 19 Å) obtained from an analysis of emission data using Forster's theory suggest some form of loop or helical segment between residues 2 and 9. Edelhoch and Lippoldt[3] have studied the effect of pH, temperature, and guanidine on both the tyrosyl and tryptophan emissions in $ACTH^{1-25}$. They concluded that none of the structural parameters revealed struc-

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