The Effect of Protein Denaturants on the Stability of the α Helix[1]

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Abstract: By analysis of titration curves of poly-L-glutamic acid by the method of Zimm and Rice, values have been obtained for $\Delta F_{hel}°$, the free energy of helix formation, in 0.1 *M* KCl of various urea molarities up to 8 *M* and in 20 and 40% ethanol. It is found that urea destabilizes the helix at low concentrations. If one attributes the decrease of $-\Delta F_{hel}°$ by urea to binding of urea to the peptide NH and CO groups of the random coil, one obtains $K_B = 0.15\ M^{-1}$, which compares favorably with the equilibrium constant for dimerization of urea ($K = 0.04\ M^{-1}$). However, $-\Delta F_{hel}°$ decreases much less rapidly with the urea concentration at higher molarities. Thus, the polyacid is still largely helical in 8 *M* urea, which also turns out to be a solvent for the polyacid. This has enabled us to obtain a thermal denaturation curve of the polyacid in this solvent by studying the rotation at 233 mμ, and the novel possibility existed of comparing these data and the free energies obtained by titration, with the theory of the helix–coil transition. The agreement was quite satisfactory. (Unfortunately, the accuracy of the experimental data was, for a number of reasons, not as high as desirable for this purpose.) Values of -100 cal/mole for $\Delta H_{hel}°$, the enthalpy of helix formation, and of 5.10^{-5} for σ, the equilibrium constant for initiating the polyacid helix (in 8 *M* urea), were obtained. The effect of ethanol is to increase the stability of the α helix considerably, presumably by strengthening the hydrogen bonds. These results, together with data in the literature showing that proteins are more easily denatured in the presence of ethanol, constitute a simple but compelling proof of the importance of hydrophobic bonding in proteins.

Most proteins can be denaturated by changing the temperature or the pH. These effects are often reversible.[3] Protein denaturants are those substances which when added to water greatly facilitate the denaturation; *i.e.*, they lower the transition temperature[4] and bring the pH at which denaturation occurs closer to 7.

Because of the reversibility of these phenomena, it should be possible to analyze the effects in terms of thermodynamic parameters, *i.e.*, the free energy (and its temperature dependence) for the denaturation reaction and the dependence of the free energy on denaturant concentration. Analyses based on the assumption of a two-state model for the denaturation equilibrium have been given.[5,6] However, it is not certain if the denaturation of protein molecules may be described as a two-state equilibrium, or if one should assume the presence of a multitude of partly denatured intermediate forms as one does, for example, in analyzing the helix–coil transition observed in synthetic polypeptides.[7]

The analysis being much more complex in the case of multistate transitions, it might be felt that it would be more difficult to obtain the free energy of helix formation and its dependence on the concentration of denaturants for synthetic polypeptides. In fact, curves describing the melting of the helix as a function of temperature cannot very well in themselves yield this information. Also, there are only a few water-soluble uncharged helical polypeptides which can be studied in this manner,[8,9] and these are not necessarily typical.

However, the problems introduced by the presence of many intermediates and by the insolubility of uncharged helical polypeptides may be circumvented in the case of ionizable polypeptides such as poly-L-glutamic acid and poly-L-lysine. For these, a change in pH will allow the transformation of the randomly coiled charged molecule into a helical uncharged molecule, and an analysis of the titration curve may give one the free energy of the reaction uncharged coil to uncharged helix, $\Delta F_{hel}°$.[10a,b] The method has already been applied successfully to obtain the dependence of $\Delta F_{hel}°$ on the concentration of salt,[11] on the leucine content of L-leucine, L-glutamic acid copolymers,[12] and on the temperature.[13] We have, therefore, performed titrations of poly-L-glutamic acid in the presence of urea and of ethanol and analyzed the data in the manner of Zimm and Rice to obtain values of $\Delta F_{hel}°$.

It is interesting to note that much information about the helix–coil transition can be obtained by combining values of $\Delta F_{hel}°$ from titration curves with thermal denaturation data. One may obtain a rather precise value for the equilibrium constant for initiation of the helix, and one may even perform a check on the validity of the theory of the helix coil transition if the titration is done at several temperatures.[14] Of all the mixed solvents studied by us, only 8 *M* urea fulfilled all the prerequisites for doing this. We have observed the helix–coil transition of uncharged poly-L-glutamic acid in this solvent as a function of temperature by following the optical rotation and have performed titrations at two different temperatures. (Unfortunately the temperature dependence of $\Delta F_{hel}°$ in this solvent turns out

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(3) See, for example, J. Hermans, *Methods Biochem. Analy.*, **13**, 81 (1965).

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(8) R. K. Kulkarni and E. R. Blout, *J. Am. Chem. Soc.*, **84**, 3971 (1962).

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(10) (a) B. H. Zimm and S. A. Rice, *Mol. Phys.*, **3**, 391 (1960); (b) S. A. Rice and M. Nagasawa, "Polyelectrolyte Solutions," Academic Press Inc., New York, N. Y., 1961, Section 7,7.

(11) M. Nagasawa and A. Holtzer, *J. Am. Chem. Soc.*, **86**, 538 (1964).

(12) W. G. Miller and R. E. Nylund, *ibid.*, **87**, 3542 (1965).

(13) J. Hermans, *J. Phys. Chem.*, **70**, 510 (1966).

(14) B. H. Zimm in "Polyamino Acids, Polypeptides and Proteins," M. A. Stahmann, Ed., University of Wisconsin Press, Madison, Wis., 1962, p 229.

to be quite small, and the comparison between theory and experiment is not as rigorous as might have been hoped for.)

Experimental Section

Materials. The sodium salt of poly-L-glutamic acid was procured from Sigma (Lots No. 74B-1720 and 75B-0400, both with reported mol wt 70,000). Urea was a reagent grade Baker product.

Titrations. The titrations were carried out as described before,[11] with the following two changes. In the first place, the glass electrode used was of the type Corning 476020. In the second place, because of the high blank titration of the urea solutions, the titrations were all done by adding to 5-ml aliquots of a stock solution of polyglutamate in 0.2 *M* KCl, 5 ml of urea solution, *c.q.*, weighed urea (or 2 or 4 ml of ethanol), and the calculated volume of water to make the final volume 10 ml. In this manner, the total volume of 1 *N* HCl needed to titrate all the polyglutamate was known precisely from the titration in the absence of urea, and this value could be used with confidence in calculating the degree of ionization, α, of the polyglutamic acid in all solutions.

Optical Rotation. Optical rotatory dispersion curves were obtained with a Cary Model 60 spectropolarimeter, temporarily put at the disposal of this department by the Applied Physics Corp. Sales Division. The solutions had polymer concentrations of *ca.* 0.02 mg/ml and were studied in regular 1-cm cells and in the heatable 1-cm cell. The temperature of the solution inside this cell was determined as a function of the temperature of the circulating water bath used, and the temperatures reported have been corrected according to this calibration.

Results

Titrations. The customary plots of pK_{app} = pH − log $[\alpha/(1-\alpha)]$ as a function of the degree of ionization α[10] have been obtained from titrations at a number of urea concentrations up to 8 *M*. Some of these plots are shown in Figure 1. The pK^0 of the carboxyl groups in the coil (extrapolation to $\alpha = 0$ of lines with smallest slope) increases with the urea concentration, although not as much as the p*K* of acetic acid.[15] The pK^0 values of the carboxyl groups of helical molecules (extrapolation to $\alpha = 0$ of lines with greatest slope) are significantly smaller than those of the randomly coiled molecules at urea concentrations greater than 1 *M*.[16] From an inspection of the curves at low values of α, it is seen that aggregation of the polyacid occurs at all urea concentrations below 8 *M*. In fact, in the course of the titrations, precipitation is observed in all these cases. The polyacid is soluble in 8 *M* urea. We have been able to put the solubility in this solvent, in which the helix is not very stable, to use in following the *thermal* helix–coil transition as a function of temperature with the spectropolarimeter (see below).

Plots of the titration data in 20 and 40% ethanol, 0.1 *M* KCl, are shown in Figure 2. Precipitation was found to occur in these solvents at low α. The lowest ethanol concentration in which the polyacid is soluble is 50%. From the change in the position of the curves of Figure 2 with ethanol concentration one would expect that in 50% ethanol the curve would be shifted so far to the right that the polyion (at $\alpha = 1$) would have to be partly helical. Interestingly, this is also the ethanol concentration above which the polyion is no longer soluble.

(15) J. W. Donovan, M. Laskowski, and H. A. Scheraga, *J. Mol. Biol.*, 1, 293 (1959).

(16) By studying the rotation of solutions in 8 *M* urea as a function of pH, we have been able to show experimentally that the observation of a difference in these p*K*'s is not due to faulty extrapolations. It is observed that the helicity is *less* in 1 *N* HCl than at pH 4 where $\alpha \sim 0.1$. This type of behavior is expected only if $pK_{hel}^0 < pK_{coil}^0$.

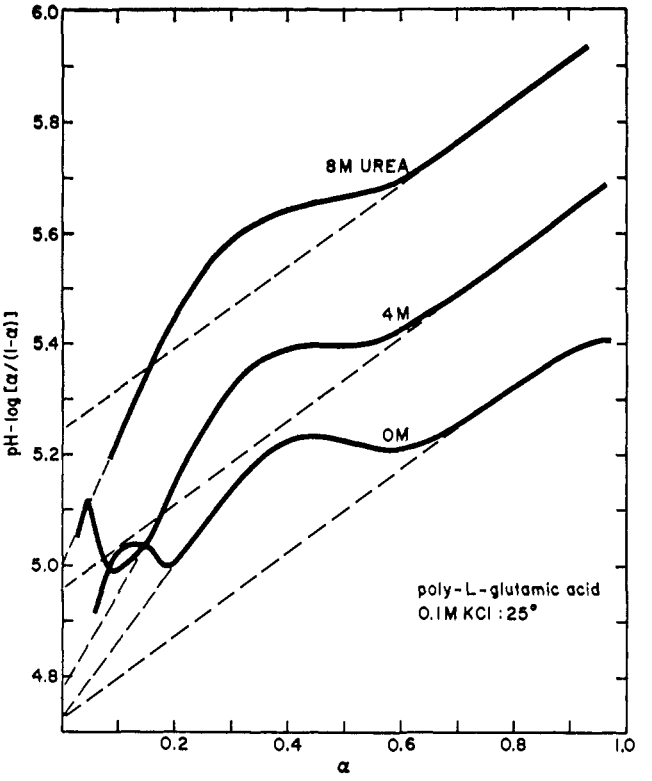

Figure 1. Plots of pH − log $[\alpha/(1-\alpha)]$ as a function of the degree of ionization, α, for poly-L-glutamic acid in 0, 4, and 8 *M* urea. Solid curves have been drawn in lieu of the experimental points. Extrapolations for the pure helix (greatest slope) and pure coil (smallest slope) are shown as dashed lines.

From the curves of Figure 2, it is clearly quite difficult to determine which is the position of the (extrapolated) titration curves for the randomly coiled polypeptide in 20 and 40% ethanol. In fact, the slope of the plots over the short interval in which the polymer is fully randomly coiled seem to be considerably greater than in water. However, the pK^0 of the helical molecules varies with the ethanol concentration in very much the same way as the p*K* of acetic acid determined in our laboratory by potentiometric titration: from 4.62 in 0.1 *M* KCl to 4.83 and 5.23 in 0.1 *M* KCl–20% ethanol and 0.1 *M* KCl–40% ethanol. Thus, there is apparently little interaction between uncharged carboxyl groups in the helix. While it is possible that there is such interaction in the randomly coiled molecule, this would have the effect of decreasing the dependence of pK_{app} on α for the pure random coil. Thus, it is reasonable to assume that pK^0 is the same for both helix and coil in 20 and 40% ethanol.

The free energies of formation of the uncharged helix from the uncharged random coil were obtained from the areas between the curve and the extrapolations in the plots of pH − log $[\alpha/(1-\alpha)]$ *vs.* α as described before.[13] These values are shown in Figure 3 and in the inset in Figure 2.[17] It should be pointed out that all these values should be corrected. When ΔF_{hel}° is large and negative, that is, when the fraction of helix is very close to unity, the experimentally

(17) As was pointed out in the preceding paragraph, the position of the extrapolation for the random coil is not obvious from the data shown in Figure 2. To calculate the values of ΔF_{hel}° we have assumed that the extrapolations are straight lines with pK^0 the same as for the coil and coinciding as best as possible with the titration curve at high values of α.

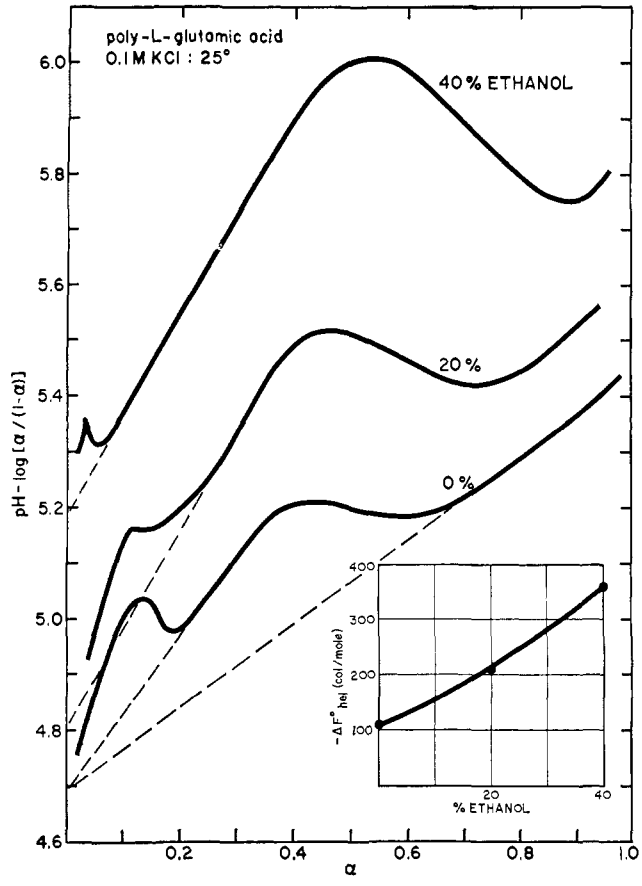

Figure 2. Plot of pH − log $[\alpha/(1 - \alpha)]$ as a function of α for poly-L-glutamic acid in water and 20 and 40% ethanol. The extrapolations for the helical molecules and the random coil in water are shown as dashed lines. The inset shows values of $\Delta F_{hel}°$, the free energy of formation of the uncharged helix from the uncharged coil, as a function of ethanol concentration. These numbers are proportional to the areas bounded by the extrapolations and the curve and are expressed in calories per mole of monomer unit. For the solutions containing ethanol it was assumed that the titration curve for the coil extrapolated to the same ordinate at $\alpha = 0$ as for the helix.

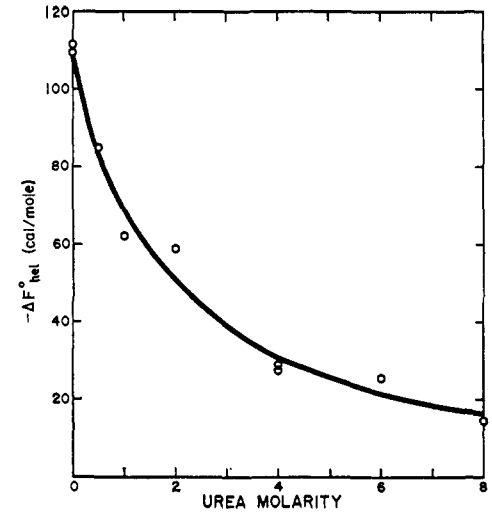

Figure 3. Free energy of formation of the mixture of helix and random coil existing in the solvent when $\alpha = 0$, from the pure random coil at $\alpha = 0$ as a function of urea concentration. The curve has been drawn to fit the points.

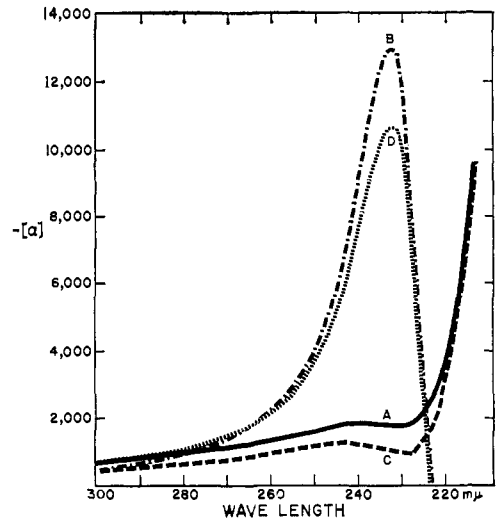

Figure 4. Optical rotatory dispersion curves for poly-L-glutamic acid: A, in 0.1 *M* KCl, pH 7; B, in 0.1 *M* KCl, pH 4.4; C, in 0.1 *M* KCl, 8 *M* urea, pH 7; D, in 0.1 *M* KCl, 8 *M* urea, pH 3.5.

determined $-\Delta F_{hel}°$ is too small by an amount $\Delta F_{in}°/n$, where $\Delta F_{in}°$ is the large and positive free energy needed to initiate a helix and n is the degree of polymerization.

On the other hand, when $\Delta F_{hel}°$ is close to or greater than zero, that is when the fraction of helix is significantly smaller than unity and the average length of the helical stretches is less than the length of a molecule, the experimentally determined free energy is that for the conversion of random coil to the equilibrium mixture of helix and coil, all at $\alpha = 0$, and is less than $\Delta F_{hel}°$. (In fact, in the extreme case where $\Delta F_{hel}° >> 0$, we will be titrating the random coil at *all* values of α, and the observed free energy will be zero.) In this case also, the magnitude of the difference between the experimental free energy and $\Delta F_{hel}°$ will be determined by $\Delta F_{in}°$.[14] An estimate of $\Delta F_{in}°$ will be obtained in the discussion where we shall take this whole problem up in more detail.

Optical Rotatory Measurements. The optical rotatory dispersion curves of poly-L-glutamic acid were measured in water at pH 7 and pH 4.4, and in 8 *M* urea at pH 7 and pH 3.5 in the wavelength range from 210 to 300 mμ. The data obtained are shown in Figure 4. The presence of the urea apparently does not affect the curves for helical polyglutamic acid very much. (It should be realized that the polymer is only from 70 to 80% helical in 8 *M* urea at pH 3.5. See below.) The curves for the randomly coiled polyion are qualitatively more diverse. This behavior is not unexpected, since the chromophoric groups and the solvent molecules can interact quite easily in this case.

Measurements of the rotation at 233 mμ as a function of temperature were carried out on the polyacid in 50% ethanol, 0.1 *M* KCl, and 0.1 *M* HCl. We observed only a very small temperature dependence between 25 and 70°. (In view of the large value of $-\Delta F_{hel}°$ in this solvent the degree of helicity is unity at all temperatures in this interval.) In contrast, the temperature dependence of the rotation of the polyacid in 8 *M* urea 0.1 *M* KCl, and 1 *N* HCl was found to be strong (Figure 5). Reversibility is demonstrated by the results of measurements 1, 2, and 3 which were done in that order and are indicated by filled circles in Figure 5.

These data clearly represent a transition from helix to random coil as the temperature is raised. We have

drawn in the values of the rotation of the helix in water at 25° (degree of ionization 0.2, line B) and the coil in 8 *M* urea at 25° (degree of ionization 1.0, line C). Apparently, the upper limit of the data in 8 *M* urea at zero degree of ionization corresponds to the value of line B, while the lower limit is somewhat different from the value of line C. However, we have seen that the rotation of the coil is easily affected by the composition of the solvent, and, therefore, probably also by the degree of ionization. We have found that the rotation of the coil in 8 *M* urea at pH 7 is not dependent on the temperature, and we shall assume that the rotation of both helix and coil in 8 *M* urea, 1 *N* HCl is independent of the temperature.

Finally, we have determined the optical rotatory dispersion of polyglutamic acid in 5 *M* guanidine hydrochloride, 0.05 *M* HCl, this being the lowest concentration of this denaturant in which the polyacid is soluble. Because of the light absorption by guanidinium ion in the ultraviolet, we measured the rotation of a solution at 0.4 mg/ml in the range from 600 to 320 mμ. Comparison between curves for (a) polyion in 5 *M* guanidine, (b) polyacid in 5 *M* guanidine, and (c) helical polyglutamic acid at pH 4.4 in 0.1 *M* KCl showed that the polyacid is 30% helical in 5 *M* guanidine hydrochloride at room temperature.

Discussion

The discussion of these results is conveniently split into two parts. In the first part we shall compare our results on the temperature dependence of the helix–coil equilibrium in 8 *M* urea with the theory of the helix–coil transition. In the second part we shall discuss the dependency of the free energy of helix formation on the concentration of the denaturants and the implication of these findings for the denaturation of proteins by these agents.

Helix–Coil Transition. According to a slightly simplified theory[14,18–21] (the "quadratic approximation"), the helix–coil equilibrium is governed by the equations

$$\theta = \mathrm{d}\ \ln \lambda / \mathrm{d}\ \ln s \tag{1}$$

and

$$\lambda = {}^1/_2\{1 + s + [(1 - s)^2 + 4\sigma s]^{1/2}\} \tag{2}$$

In these, θ is the degree of helicity, s the equilibrium constant for helix formation

$$s = \exp(-\Delta F_{\mathrm{hel}}^\circ / RT) \tag{3}$$

σ a similar equilibrium constant for the initiation of the helix

$$\sigma = \exp(-\Delta F_{\mathrm{in}}^\circ / RT) \tag{4}$$

and λ the partition function per monomer unit for the mixture of helix and coil present. These results were derived for infinitely long polypeptide molecules, but it is generally supposed that they are valid for molecules of degree of polymerization of several hundred, as here studied.

Two consequences of eq 1 and 2 are of particular interest here. In the first place

$$s = 1, \textit{i.e.}, \Delta F_{\mathrm{hel}}^\circ = 0 \text{ at } \theta = 0.5 \tag{5}$$

the midpoint of the transition. In the second place

$$\begin{aligned} (\partial\theta/\partial s)_{s=1} &= 1/4\sqrt{\sigma} \\ (\partial\theta/\partial\sigma)_{s=1} &= 0 \end{aligned} \tag{6}$$

Therefore, the slope of the thermal transition curve at the midpoint, $(\mathrm{d}\theta/\mathrm{d}T)_{s=1}$, depends on two independent parameters: σ and $(\mathrm{d}s/\mathrm{d}T)_{s=1}$, the latter being, of course, equal to

$$(\mathrm{d}s/\mathrm{d}T)_{s=1} = \Delta H_{\mathrm{hel}}^\circ / RT^2 \tag{7}$$

From the curve shown in Figure 5, we have taken $\theta = 0.5$ and $\mathrm{d}\theta/\mathrm{d}T = 1.82 \times 10^{-2}$ at 36.5°, so that

$$\Delta H_{\mathrm{hel}}^\circ / \sqrt{\sigma} = -1.38 \times 10^4 \text{ cal/mole} \tag{8}$$

with the preceding equations. In principle, values for $\Delta H_{\mathrm{hel}}^\circ$ and σ individually can be determined by taking into account the experimental values of θ farther from the transition temperature. However, the shape of the theoretical transition curve is rather insensitive to changes in $\Delta H_{\mathrm{hel}}^\circ$ and $\sqrt{\sigma}$, as long as the ratio of these quantities remains constant. Also, in this particular experiment, the transition is so broad that the limits of the rotation for pure helix and pure coil are uncertain, and this further diminishes the accuracy of such a procedure.

In the present case, we have the novel possibility of using the free energy determined at 10 and 25° by potentiometric titration to determine $\Delta H_{\mathrm{hel}}^\circ$. The values determined from titration curves are ln λ at 10 and at 25°, which are equal to 0.0198 and 0.0105. The following usual assumptions are made regarding the temperature dependence of $\lambda:\lambda$ is related to s by eq 2, in which ln s varies linearly with $1/T$, with a slope $-\Delta H_{\mathrm{hel}}^\circ/R$ and σ is supposed to be independent of temperature.

In order to compare the various results obtained we have calculated s from the experimental values of λ for several values of σ. It turned out that out of the three cases, $\sigma = 10^{-4}$, $\sigma = 5 \times 10^{-5}$, $\sigma = 10^{-5}$, the two values of

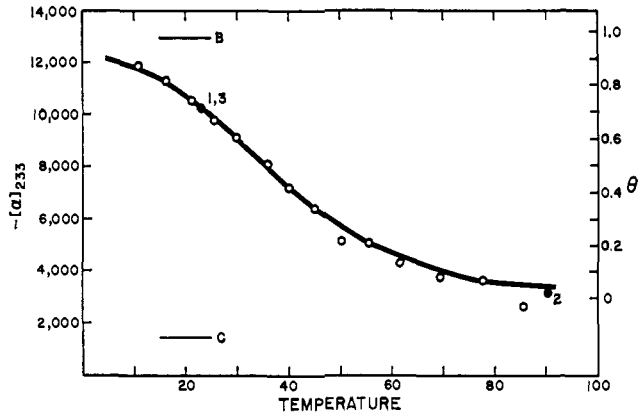

Figure 5. Specific rotation at 233 mμ for poly-L-glutamic acid in 8 *M* urea, 0.1 *M* KCl, 1 *M* HCl as a function of the temperature. The lines B and C represent the value of $[\alpha]_{233}$ for solutions B and C of Figure 4. A theoretical curve of the degree of helicity, θ, as a function of temperature has been fitted to the data by a proper adjustment of the θ scale to the $[\alpha]_{233}$ scale. The derivation of this curve is described in the text.

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ln s calculated at 25 and 10° and the value ln $s = 0$ at the transition temperature (36.5°) lay best on a straight line when plotted *vs.* $1/T$ for $\sigma = 5 \times 10^{-5}$. The slope of this line gave $\Delta H_{hel}^{\circ} = -100$ cal/mole, and the ratio $\Delta H_{hel}^{\circ}/\sqrt{\sigma}$ comes out as -1.4×10^{4}, close to the value derived independently from the slope of the transition curve (eq 8). The agreement is excellent but is probably somewhat fortuitous, the experimental error in the values of λ being such that a larger discrepancy would have been acceptable.

The value for σ is in the same range as the two estimates reported in the literature: 2×10^{-4} for poly-γ-benzyl-L-glutamate in a mixed organic solvent and 10^{-4} for poly-L-glutamic acid in water.[10,14,22] The former estimate was obtained by measuring the helix–coil transition for samples of different molecular weight as a function of temperature, and the latter by an analysis of the titration curve using the theory of the helix–coil transition and the theory of polyelectrolyte solutions. In any case, there is no reason to believe that σ might not vary with the composition of the solvent and the composition of the polymer.

Using the values of the transition temperature, ΔH_{hel}° and σ obtained above, we have calculated θ as a function of temperature. This theoretical curve has been fitted to the data of Figure 5 by a proper choice of the limits of $[\alpha]_{233}$ for completely helical and completely random molecules. The agreement is very good, but, as has been pointed out, this is no guarantee for the correctness of the *individual* values for ΔH_{hel}° and σ used in the calculation, largely because of the relative freedom one has in choosing these limits of $[\alpha]_{233}$.

Implications for Proteins. The results of experiments on synthetic polypeptides may only be thought to give information on the properties of globular proteins as long as a number of important restrictions are kept in mind. In the preceding paragraphs we have seen, for example, how the helix–coil transition of polypeptides is described with a model which is quite different from the two-state model which one would like to assume for protein denaturation.[5,6] The most important distinction to make between the poly-L-glutamic acid α helix and proteins in the following part of the discussion, is that the structure of the former is largely stabilized by the presence of N—H···O=C hydrogen bonds,[13] while in proteins stability is provided in roughly equal amounts by hydrogen bonds and hydrophobic bonds.[23,24] Thus, the experiments showing the increase of the stability of the α helix in the presence of ethanol reported here (and the qualitative observation of a similar effect of dioxane noted earlier[25]) are in contrast to the observation of a decrease in the stability of globular proteins in the presence of apolar diluents.[26–28] Indeed, these results constitute a compelling though very simple proof of the importance of hydrophobic bonding for the structure of proteins.

It may well be possible to make a quantitative distinction between the parts of the free energies of formation of native protein molecules contributed by peptide hydrogen bonds and by hydrophobic bonds once methods have been developed for obtaining experimental free energies from thermal transition curves. One would merely have to suppose that the free energy and enthalpy of the transition, and the changes in the free energies under the effect of denaturants can be obtained as sums of terms from hydrogen bonds, hydrophobic bonds, and the loss of rotational freedom.[29] Estimates of each of these contributions are now available, either from experiment[13,29–32] or from theory.[24]

The effect of urea or guanidine on the stability of the α helix is not large. However, the absence of a large change in ΔF_{hel}° is deceptive, since one finds a much larger change in ΔH_{hel}°. This indicates that there is considerable interaction between polymer and urea, but that the effects on ΔF_{hel}° largely cancel one another. The large difference in pK^{0} for the carboxyl group in helical and randomly coiled poly-L-glutamic acid also indicates that specific solvent effects occur. Realizing the existence of these effects, we have nevertheless asked ourselves to what extent it is possible to explain the dependence of ΔF_{hel}° on the urea concentration by assuming that binding occurs between urea molecules and peptide NH and CO groups of the random coil. Since the changes in ΔF_{hel}° are small compared with RT, they should be equal to $RTK_{B}C_{U}$ where K_{B} is a binding constant and C_{U} the urea concentration.[31] This linear relationship is obeyed only to $C_{U} = 1$ M. While some decrease in the rate of change of ΔF_{hel}° with C_{U} would be expected because at higher C_{U} part of the urea molecules are involved in urea–urea hydrogen bonds, the value of the dimerization constant of urea is too low[31] ($4.1 \times 10^{-2} M^{-1}$) than that even the concentration of monomeric urea molecules would vary with C_{U} as is necessary to explain the data of Figure 3, assuming a constant value of K_{B}. Restricting ourselves, then, to the initial slope of this curve, one obtains $K_{B} = 0.15$ M^{-1}. This value is acceptable when one compares it with the value of the dimerization constant. This is very satisfactory, since it would be difficult firstly to admit a predominant stabilization of the α helix by the peptide–hydrogen bond,[13] secondly to explain the dimerization of urea in the same manner[31,32] and then *not* to let urea be a hydrogen bond breaker. However, it turns out that urea is a good hydrogen bond breaker at low concentrations, but a poor one at 8 M concentration. The latter is acceptable, since nothing is known about the properties of the hydrogen bonding groups on the urea molecule at very high concentrations.

The observed lowering of the stability of the α helix in 8 M urea is insufficient to explain the denaturing action of 8 M urea on proteins. However, it is known that apolar molecules are more soluble in 8 M urea than in water[29] and that urea is thus a breaker of hydrophobic bonds as well. From the data obtained here and from data in the literature,[4,30] it would appear that the hydrogen bond breaking effect and the hydrophobic bond breaking effect of 8 M urea on proteins are probably of the same order of magnitude.

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