

static binding in macromolecular systems. However, the situation in proteins is inherently more complex than in synthetic polymers and unambiguous assessment of the relative importance of site and electrostatic binding remains to be made.

**Acknowledgments.**—The authors wish to thank Professor Charles Tanford for a helpful discussion. The numerical computations were performed with the invaluable aid of Mr. Donald Franz of the Washington University Computer Center.

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## The Helix-Coil Transition in Solutions of Polyglutamic Acid<sup>1</sup>

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Precise potentiometric titration curves have been determined for solutions of poly-L-glutamic acid at various concentrations of sodium chloride between 0.005 and 0.2 *M*. Detailed examination of these data leads to two conclusions. First, the measurements can be used to determine the fraction of amino acid residues in helical form. This constitutes an independent check of helix per cent measurements from optical rotatory dispersion data. The results show that the  $b_0$  parameter of the rotatory dispersion curve is, indeed, a measure of helix content. Second, the results provide a measure of the equilibrium constant for the helix-coil transition and therefore of the free energy change of this reaction. The charge-independent part of the free energy change per amino acid residue is found to vary from 320 cal./residue in water to 120 cal./residue at high concentrations of sodium chloride.

Among the many substances that may be used for studying the helix-random coil transition, polyglutamic acid has attracted particular interest because of the anticipated predominance of internal hydrogen bonding and side-chain charge effects in dictating the molecular configuration. This molecule has therefore been used as a model for studying the effects of these interactions on the helix-coil transition.<sup>3</sup> Recently, Wada showed that this transition appears very clearly in titration studies of polyglutamic acid.<sup>4</sup> We report here the results of titration experiments that are, in part, a repetition of the work of Wada, except that the data were obtained using smaller increments of ionic strength and more precise instrumentation. We have also carried out a more detailed analysis of the results to obtain information on the nature of the transition.

We conclude from this analysis that: (1) Potentiometric titration data may be used to calculate the fraction of amino acid residues in helical form, thus offering an independent check on determinations of this parameter by optical rotatory dispersion or deuterium exchange measurements. (2) The data provide a measure of the charge-independent part of the standard free energy change of the helix-coil transition.

### Experimental

**Materials.**—Poly-L-glutamic acid was provided as the sodium salt by Dr. E. R. Blout of the Childrens Cancer Research Foundation, Boston, Mass.<sup>5</sup> The molecular weight of the sample (58,000 in the acid form) was deduced from the intrinsic viscosity (1.25 dl./g.) in 0.2 *M* NaCl at pH 7.3, using the calibration determined by Doty, *et al.*<sup>3,4</sup>

The sample was dissolved in water and deionized with a mixed bed ion-exchange resin, Amberlite MB-1. The free acid is not soluble in water, but the solution remains clear for several hours after deionization, sufficient time to carry out a titration experiment. The use of an ion-exchange resin to avoid precipitation of the acid may be one cause of the improved accuracy of the data over that of earlier work. The salt concentration was brought to the desired value by addition of a measured volume of a 1 *M* NaCl solution. Experiments were performed with solutions containing 0.0188 monomer mole/l., at starting NaCl molarities of 0.200, 0.100, 0.0500, 0.0200, 0.0100, and 0.00500. To check the effect of polymer concentration, one experiment was carried out using a solution containing 0.0342 monomer mole/l.

(1) This investigation was supported by PHS Research Grant RG-5488 from the Division of General Medical Sciences, Public Health Service.

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(3) P. Doty, A. Wada, J. T. Yang, and E. R. Blout, *J. Polymer Sci.*, **23**, 851 (1957).

(4) A. Wada, *J. Mol. Phys.*, **3**, 409 (1960).

(5) E. R. Blout and M. Idelson, *J. Am. Chem. Soc.*, **78**, 497 (1956).

and 0.0200 *M* NaCl. These solutions were titrated in a nitrogen atmosphere at  $25 \pm 1^\circ$ , using 0.1000 *M* NaOH delivered with a Gilmont microburet.

**pH Measurements.**—The Radiometer pH M4 instrument was used with Radiometer standard buffer (pH  $6.50 \pm 0.02$ ). The sensitivity of this instrument is 0.001 pH unit.

**Optical Rotatory Dispersion.**—Measurements were made as a function of pH on a solution containing 0.341% polymer and 0.200 *M* NaCl. The instrument used was the Rudolph recording spectropolarimeter with a high-pressure Xenon lamp and a 50-mm. cell. The instrument covers the wave length range 2700–6000 Å., but because of the low concentrations necessitated by the poor solubility of the polymer at low pH, the rotations could only be precisely measured in the region 2700–4000 Å. The results were plotted as

$$[\alpha]\lambda(3/n^2 + 2)(M_0/100)(\lambda^2 - \lambda_0^2) \text{ vs. } (\lambda^2 - \lambda_0^2)^{-1}$$

where  $[\alpha]\lambda$  is the specific rotation at the wave length  $\lambda$ ,  $n$  the solvent refractive index,  $M_0$  the monomer molecular weight (129), and  $\lambda_0$  is taken as 2130 Å.<sup>5</sup> This plot was found to be linear, and the best straight line was drawn through the data by eye. According to the empirical equation commonly used in studies of optical rotatory dispersion of proteins and polypeptides,<sup>6</sup> the slope of this line is designated  $b_0\lambda_0^4$ . The quantity  $b_0$  is frequently used as a measure of helix content.<sup>6–8</sup> In our experiments the value of  $b_0$  could be obtained with a precision of  $\pm 5\%$ .

### Results

**Potentiometric Titration Curves.**—The apparent ionization constant of a polymeric, weak acid changes with the degree of ionization because the ionization of an acid group must be accompanied by electrostatic work done against the charges already present on the molecule. In general, the pH of such an acid is given, in terms of the change of electrostatic free energy accompanying the ionization process, by the expression<sup>9,10</sup>

$$\text{pH} = \text{p}K_0 - \log((1 - \alpha)/\alpha) + (0.434/RT)(\partial G_{el}/\partial Z) \quad (1)$$

where  $\text{p}K_0$  is the negative logarithm of the intrinsic dissociation constant of a carboxylic acid group,  $\alpha$  is the fraction of the ionizable groups which are ionized,  $G_{el}$  is the electrostatic free energy of the polymer, and  $Z$  is the number of fundamental charges on the polyion. In Fig. 1, a few examples of  $[\text{pH} + \log((1 - \alpha)/\alpha)]$  vs.  $\alpha$  curves are shown. As Wada pointed out,<sup>4</sup> region B contains the titration curve of completely helical poly-

(6) P. Doty, *Rev. Mod. Phys.*, **31**, 107 (1959); P. Urnes and P. Doty, *Advan. Protein Chem.*, **16**, 401 (1961).

(7) C. Cohen and A. G. Szent-Györgyi, *J. Am. Chem. Soc.*, **79**, 248 (1957).

(8) J. Schellman and C. Schellman, *J. Polymer Sci.*, **49**, 129 (1961).

(9) A. Katchalsky and J. Gillis, *Rec. trav. chim.*, **68**, 879 (1949).

(10) A. Arnold and J. Overbeek, *ibid.*, **69**, 192 (1950).

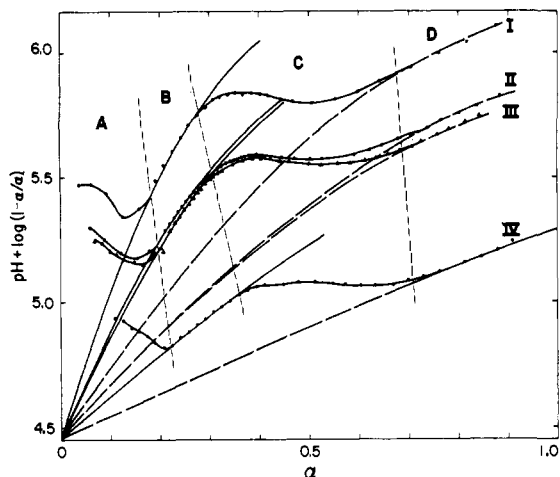


Fig. 1.—Examples of the potentiometric titration curves: I, 0.00500 *M* NaCl, 0.0188 *N* PGA; II, 0.0200 *M* NaCl, 0.0188 *N* PGA; III, 0.0200 *M* NaCl, 0.0342 *N* PGA; IV, 0.200 *M* NaCl, 0.0188 *N* PGA. Extrapolations of coiled regions are dashed lines, of helical regions, solid lines. Regions A, B, C, and D are explained in the text.

glutamic acid, region D that of the completely coiled molecule, and region C that of the mixture of helix and coil. As Wada suggested, the deviations observed in the region A are probably due to aggregation preceding precipitation.

Equation 1 requires that the extrapolation of the data of regions B and D to  $\alpha = 0$  (*i.e.*, to the state of noncharged helix or coil) gives  $pK_0$ , which must be essentially independent of the ionic strength of the solution.<sup>11</sup> Indeed, it is observed that all the curves of Fig. 1 can be brought to the same intercept,  $pK_0 = 4.45$ . Slightly curved lines were chosen for the extrapolation. This is not only because the curved lines appear to be natural extensions of the trend of the data, but also because the linear relationship between  $[pH + \log((1 - \alpha)/\alpha) - pK_0]$  and  $\alpha$ , which is obtained from the Debye-Hückel approximation,<sup>12</sup> holds only for polyions which have very low charge density, that is, only for  $[pH + \log((1 - \alpha)/\alpha) - pK_0] < 0.434$ . The plots of  $[pH + \log((1 - \alpha)/\alpha) - pK_0]$  vs.  $\alpha$  in regions B and D were found to be in good agreement with the electrostatic potential calculated by an electronic computer without the Debye-Hückel approximation.<sup>13</sup> The calculated curves are very similar to the extrapolated lines in Fig. 1. The detailed comparison, however, will be reported in a separate paper.

Comparison of curves II and III shows that the results are virtually independent of polymer concentration, as is usually the case for polyelectrolytes in systems containing added salt. The bulk of the deviation of the two curves at large  $\alpha$  is caused by the greater change in salt concentration that occurs in the more concentrated polymer solution during the course of the titration.

**Calculation of the Degree of Helix.**—Region C of Fig. 1 contains the potentiometric titration curves of mixtures of helices and random coils. Therefore, it is possible to calculate the degree of helix in this region, if we can use the assumption that the potentiometric

(11) If there is appreciable binding of small ions other than  $H^+$  to specific sites on the polymer, this statement will not be true. However, since the polymer is either neutral or negatively charged, binding of  $Na^+$  is the only relevant possibility. Association of  $Na^+$  with carboxylate anions is generally conceded to be negligibly small. Electrostatic "binding" of  $Na^+$  of the Bjerrum type, which surely does occur, will not affect the value of  $pK_0$ .

(12) K. Linderström-Lang, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **15**, No. 7 (1924); T. Hill, *Arch. Biochem. Biophys.*, **57**, 229 (1955).

(13) L. Kotin and M. Nagasawa, *J. Chem. Phys.*, **36**, 873 (1962).

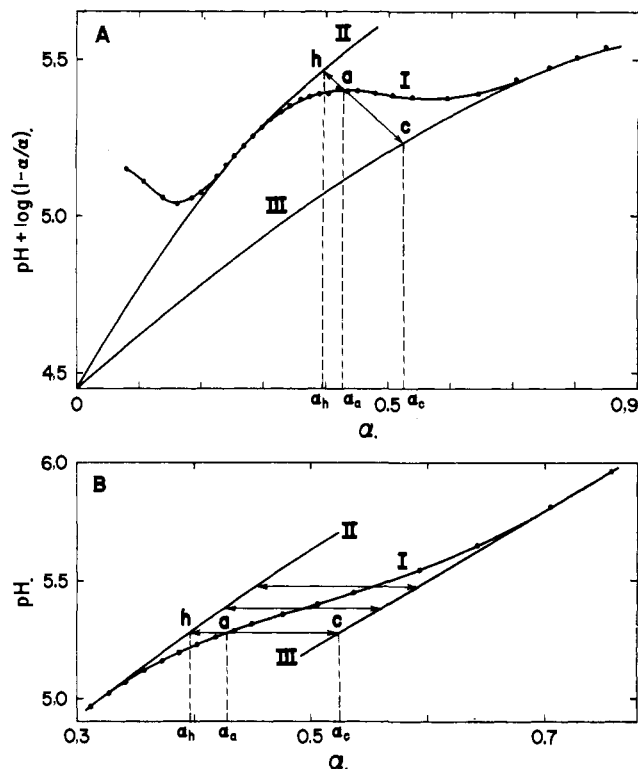


Fig. 2A-B.—Method for determining the degree of helix. Experimental points are for a solution of 0.0500 *M* NaCl and 0.0188 *N* PGA. Horizontal arrows represent solutions in which  $C_c/C_h = 1/3$  (bottom), 1 (middle), and 3 (top).

titration curves of helical and coiled molecular sections existing in the region C are the same as those of completely helical and coiled molecules, respectively. This assumption is probably valid except for very low degree of helix or coil, since it is well known that the potentiometric titration of polyelectrolytes is independent of degree of polymerization.

In Fig. 2, one example of this calculation is shown. Data for a solution 0.05 *M* in NaCl and containing 0.0188 *N* polyglutamic acid have been used. For convenience, the data are shown both as  $pH + \log((1 - \alpha)/\alpha)$  vs.  $\alpha$  (Fig. 2A), and simply as  $pH$  vs.  $\alpha$  (Fig. 2B). Curve I is the actual titration curve; curves II and III are the presumed titration curves of completely helical and coiled molecules, respectively, as obtained from extrapolation on the plot of Fig. 2A. The actual molecule at the point "a" must have some helical and some coiled portions, and those helical or coiled parts must have the same degree of neutralization as the completely helical or coiled molecules would have if they were alone in solutions of this same pH. Thus, it can be seen from Fig. 2B that the system at "a" (at pH 5.28) has an average degree of neutralization  $\alpha_a$  and consists of a mixture of helical chains having degree of neutralization  $\alpha_h$ , and randomly coiled chains having degree of neutralization  $\alpha_c$ . In this manner, for any actual degree of neutralization, the degree of neutralization of helical and coiled chains in the same solution may be determined. The fraction of residues in the helical form is then simply  $(\alpha_c - \alpha_a)/(\alpha_c - \alpha_h)$ .

In this system, then, it is possible to check the validity of experiments employing the optical rotatory dispersion parameter  $b_0$  as a measure of helix content. In Fig. 3, the degree of helix determined by the method described above is plotted vs.  $-b_0$ . The data fall on a straight line which passes through the origin. This indicates clearly that  $b_0$  is, indeed, proportional to the helix content. It should be noted that the value of  $b_0$

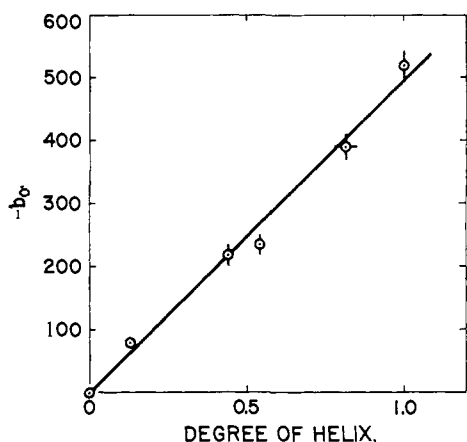
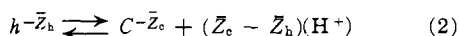


Fig. 3.—Relationship between  $b_0$  of optical rotatory dispersion and degree of helix from titration. Data for 0.341% PGA in 0.200 M NaCl.

found in this study for the completely helical chain ( $-520$ ) is somewhat less negative than has been reported previously for this substance ( $-610$ ),<sup>14</sup> and for fully helical molecules in general ( $-650$ ).<sup>15</sup> We attribute this to the lower range of wave length used in our experiments. It is not unexpected that data fitted to an empirical relationship should yield values of the constants that vary somewhat with the precise conditions of the measurements. This result points up the danger of altering the wave length range without determining the absolute value of  $b_0$  corresponding to completely helical molecules in that range. We have consistently found that measurements in this lower wave length region yield values of  $b_0$  for molecules completely helical (e.g., paramyosin and tropomyosin) that are less negative ( $-570$ ) than found in a region of greater average wave length.<sup>16</sup>

**Determination of the Free Energy of the Helix-Coil Transition.**—In order to simplify the discussion of the helix-coil transition, we will first assume that the process is of an all-or-none character, *i.e.*, that a given molecule in the equilibrium mixture is either fully helical or fully coiled. This cannot be true, as has been shown both theoretically<sup>17</sup> and experimentally<sup>6</sup>; but it has also been shown theoretically, that this alters the over-all thermodynamic properties of the transition only slightly, and that the effect is extremely small at large values of the degree of polymerization.<sup>17</sup> In our case, with a degree of polymerization of 450, the transition should behave in a manner very closely all-or-none. It should be apparent that by proceeding in this manner, we are deliberately avoiding the elegance that characterizes an earlier effort to obtain similar information from similar data.<sup>18</sup>

Making the all-or-none assumption, then, the solutions of polyglutamic acid are assumed to contain species of helical molecules having charges from zero to the maximum possible charge ( $N$ ) in equilibrium with a distribution of coiled molecules having the same range of charge, but a different average charge. The "helix-coil transition" in this system involves the participation of all molecular species in a complex series of equilibria. The equation written for the average species is but a sample



(14) E. R. Blout in "Optical Rotatory Dispersion," by C. Djerassi, McGraw-Hill Book Co., Inc., New York, N. Y., 1960, p. 248.

(15) C. Tanford, P. De, and V. G. Taggart, *J. Am. Chem. Soc.*, **82**, 6028 (1960).

(16) M. Noelken, Ph.D. Thesis, Washington University, 1962.

(17) J. Schellman, *J. Phys. Chem.*, **62**, 1485 (1958).

(18) B. H. Zimm and S. A. Rice, *J. Mol. Phys.*, **3**, 391 (1960).

where  $\bar{Z}_h(\bar{Z}_c)$  is the average number of fundamental charges on helical (coiled) molecules in the solution. The standard free energy change of this reaction will, of course, involve electrostatic effects as well as contributions of hydrogen bonding, configurational entropy, and charge-independent solvation. However, it is of great interest in the theory of denaturation to determine separately the charge-independent contribution in aqueous media. This quantity is involved, alone, in one of the many microscopic equilibria mentioned above, namely that between undissociated acid forms of helix and coil



The standard free energy change of this reaction is given by

$$\Delta G^\circ = -RT \ln [C_c^0/C_h^0] \quad (4)$$

and involves only charge-independent factors. This free energy change may be assessed if the concentrations of undissociated acid forms of helix and coil are known.

Since the individual titration curves of helix and coil are available (as shown above), it is possible to obtain these concentrations from the theory of protolytic equilibria of polyvalent acids. This analysis provides the concentration of species of any charge in the form (written for helical molecules)<sup>19</sup>

$$C_{h^{-z}} = C_h^0 \left( \frac{K_0}{a_{H^+}} \right)^z \left[ \frac{N!}{(N-Z)!Z!} \right] e^{-(G_{h,z}/RT)} \quad (5)$$

where  $C_{h^{-z}}$  is the concentration of all helical molecules having net valence  $-Z$ , and  $a_{H^+}$  is the activity of hydrogen ions. Similar equations hold for the randomly coiled form.

Since the total concentration of helix is

$$C_h = \sum_0^N C_{h^{-z}}$$

we have

$$C_h/C_h^0 = \sum_{z=0}^N \left( \frac{K_0}{a_{H^+}} \right)^z \left( \frac{N!}{(N-Z)!Z!} \right) e^{-(G_{h,z}/RT)}$$

and thus

$$\frac{d \log (C_h/C_h^0)}{d \text{pH}} = \frac{\sum_0^N |Z| C_{h^{-z}}}{\sum_0^N C_{h^{-z}}} = |\bar{Z}| \quad (6)$$

The concentration of un-ionized helix or coil may therefore be calculated by integration of the titration curve ( $\alpha$  vs. pH)

$$\int_0^{\log (C_h/C_h^0)} d \log (C_h/C_h^0) = \int_{|\bar{Z}|=0}^{|\bar{Z}|=N\alpha} |\bar{Z}| d \text{pH}$$

or

$$\log (C_h/C_h^0) = \int_{|\bar{Z}|=0}^{|\bar{Z}|=N\alpha} (N\alpha) d \text{pH} \quad (7)$$

The free energy change per residue is then

$$\frac{\Delta G^\circ}{N} = \frac{-RT}{N} \ln (C_c^0/C_h^0) = -2.3RT \left[ \int_{|\bar{Z}|=0}^{\text{pH}} \alpha_h d \text{pH} - \int_{|\bar{Z}|=0}^{\text{pH}} \alpha_c d \text{pH} + (1/N) \log (C_c/C_h) \right] \quad (8)$$

The last term inside the bracket on the right-hand side of 8 involves the total concentrations of helix and coil, which may be evaluated as in the previous section. The first two terms inside the bracket are calculated by integration of the individual titration curves from very acid pH to the pH in question. The latter computation is illustrated in Fig. 4; the shaded area represents the net value of the first two terms at pH 5.5. In practice these areas were evaluated by cutting them out and

(19) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. I, Academic Press, Inc., New York, N. Y., 1958, p. 515.

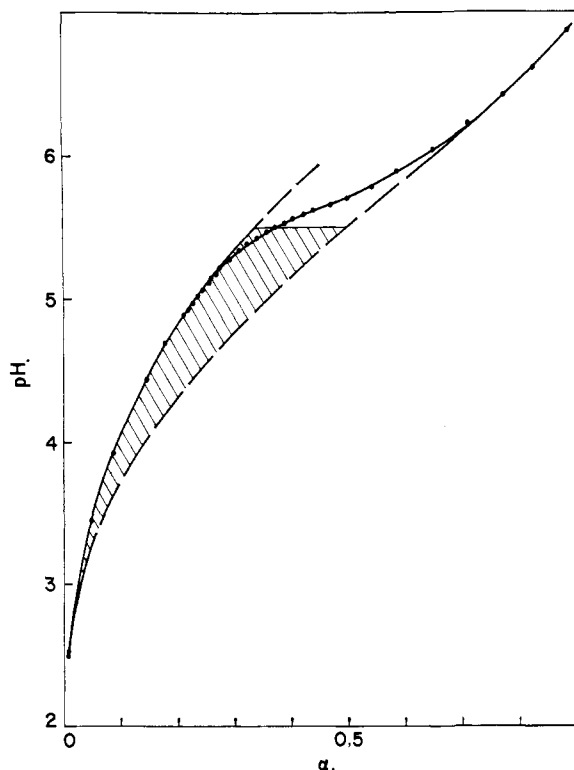


Fig. 4.—Titration curve of 0.0188 *N* PGA in 0.0100 *M* NaCl. Shaded area represents the net value of the first two terms inside the bracket on the right-hand side of eq. 8.

weighing the paper. An alternative method of evaluating  $\Delta G^\circ/N$  is described in Appendix I.

It is found that the values obtained using eq. 8 are slightly dependent on pH at a given ionic strength (*i.e.*, slightly dependent on extent of reaction). This may arise from ambiguity in the value of *N*, the size of the denaturing unit, here assumed equal to the degree of polymerization. However, it is evident from eq. 8 that the measurements for  $C_c/C_h = 1$  must provide the correct value of  $\Delta G^\circ/N$  whether or not the all-or-none assumption is rigorously valid.

A plot of these values of the free energy change per residue *vs.* square root of salt concentration is shown in Fig. 5. Although it is apparent that  $\Delta G^\circ/N$  is independent of over-all concentration of polymer, it appears that it falls from about 320 cal./residue in water to about 120 cal./residue at higher concentrations of sodium chloride.

#### Discussion

**The All-or-None Hypothesis.**—As Schellman has shown,<sup>17</sup> the removal of the all-or-none restriction introduces a basic asymmetry into the course of the denaturation reaction: the onset of the transition is sharper in proceeding from coil to helix than from helix to coil. The effect, however, diminishes with increasing degree of polymerization, and, for long chains, the behavior is indistinguishable from the all-or-none case. The asymmetry appears in theoretical plots of helical fraction *vs.* free energy of unfolding.<sup>20</sup> The difficulty in relating the latter parameter to experimental variables (*e.g.*, pH) has been emphasized,<sup>17</sup> but, ignoring the inexactitude of the comparison, we show in Fig. 6 a graph of degree of helix *vs.* pH for data at two ionic strengths. No asymmetry is evident, and we believe this supports the argument that, in this case, the results

(20) Actually, the variable used by Schellman is  $\Delta G_{\text{pep}}$  related to the free energy of unfolding by  $\Delta G_{\text{unf}} = n' \Delta G_{\text{pep}} + C$ , where  $n'$  is the number of residues involved and  $C$  accounts for some atypical residues at the helix ends.

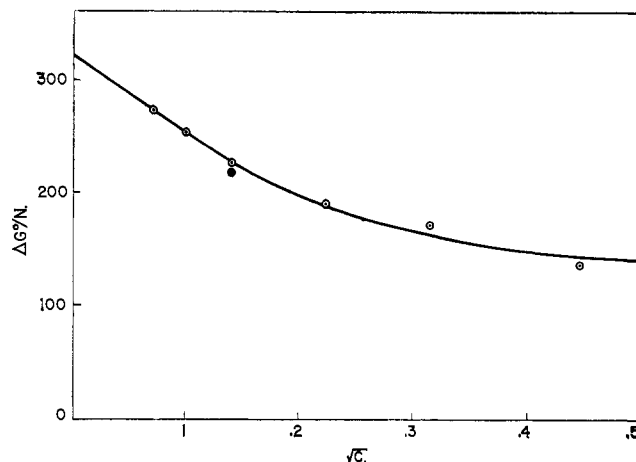


Fig. 5.—Dependence of  $\Delta G^\circ/N$  on concentration of NaCl. Filled circle is for a solution 0.0342 *N* in PGA; others are 0.0188 *N* PGA.

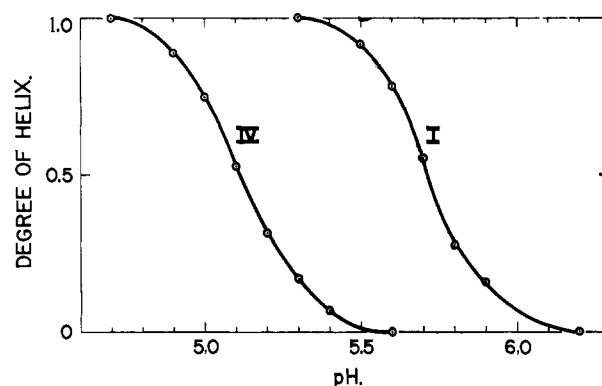


Fig. 6.—Relationship between degree of helix from titration curve and pH. Roman numerals refer to data of Fig. 1: I, 0.00500 *M* NaCl, 0.0188 *N* PGA; IV, 0.200 *M* NaCl, 0.0188 *N* PGA.

of the all-or-none treatment will not be materially altered by a more rigorous approach.

**The Numerical Value of  $\Delta G^\circ/N$ .**—The charge-independent part of the standard free energy change of the polypeptide helix-coil transition in water is an essential parameter in the development of the theory of denaturation. An early estimate of this quantity, based on the use of thermodynamic data in the urea-water system, yielded a value of about 300–600 cal. per residue at 25°.<sup>21</sup> Later experiments, using *N*-methylacetamide as a peptide model, suggest that association of peptide-like groups is of little consequence in aqueous solution, the process of dimerization being unfavorable to the extent of approximately 3000 cal./residue.<sup>22</sup>

In the case of polyglutamic acid, of course, the secondary structure may contain other sources of stabilization in addition to interpeptide hydrogen bonds; the presence of side-chain carboxyl-carboxyl hydrogen bonds is a likely possibility. There is some independent evidence that such bonding contributes to the stability of the  $\alpha$ -helix in proteins.<sup>16</sup> The result of the experiments reported here is that the sum of all the charge-independent interactions, both peptide and side chain, provide a total stabilization of about 200 cal./residue. This quantity represents the sum of contributions of hydrogen bonding, configurational entropy, and charge-independent solvation of both peptide

(21) J. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **29**, 223 (1955).

(22) I. M. Klotz and J. S. Franzen, *J. Am. Chem. Soc.*, **84**, 3461 (1962).

groups and side chains. It will be seen below that this value is probably an upper limit to the free energy of stabilization per residue.

There are two sources of ambiguity in this method of determination of  $\Delta G^\circ/N$ . First, there is obviously considerable difficulty in determining the proper choice of  $pK_0$  for the random coil. The extrapolation is long and has been arbitrarily brought to the same point as for the helix. If there is a considerable amount of carboxyl-carboxyl hydrogen bonding in the helix, then the intrinsic ionization constant of helix and coil may differ, and this will affect the area measurement involved in the determination of  $\Delta G^\circ/N$ . It may be possible in the future to determine the value of  $pK_0$  appropriate to the random coil by titration of either small-molecular model compounds or poly-DL-glutamic acid, which would be expected to remain randomly coiled to lower charge densities and yet would be expected to have the same  $pK_0$  as the coiled form of poly-L-glutamic acid.

A second source of difficulty is revealed by the examination of titration data of typical, randomly coiled polyelectrolytes. While numerical integration of the Poisson-Boltzmann equation gives results in agreement with experiment at high degree of neutralization (and hence over the entire range in which the coil appears in our experiments), deviations are observed between experimental data and theoretical curves (such as III of Fig. 2A) at lower charge. In detail, the experimental curve, at some low value of  $\alpha$ , rises above the theoretical line, reaches a maximum, and then falls again, reaching  $\alpha = 0$  at the same point as the theoretical. This effect is caused by the tighter coiling of the molecule that occurs at low charge.<sup>13</sup> Quantitative correction for an effect of this type is impossible to make, but would serve to diminish the area of Fig. 4, and thus to decrease the numerical value of  $\Delta G^\circ/N$ , and perhaps also to introduce an artifactual dependence of this quantity on ionic strength. It is for this reason that our determination of this quantity should probably be viewed as an upper limit.

The change in  $\Delta G^\circ/N$  with concentration of salt is, at first, rather surprising. It is often assumed that effects such as hydrogen bonding that exist in the absence of a net charge will not be dependent upon ionic strength. This view is probably superficial.<sup>23</sup> In fact, recent experiments offer a clear demonstration of the important influence of ionic strength in such interactions. Blout and co-workers have succeeded in synthesizing a modified glutamic acid polymer with uncharged, hydrophilic side chains.<sup>24</sup> In one case, they found, by optical rotatory dispersion measurements, that this polymer contains no helical molecules in pure water, but is 30% helical in 2 M NaCl. This result indicates clearly that the free energy of stabilization in the absence of charge may be altered—by an amount at least as great as the amount shown in Fig. 5—by changing the ionic strength. Since, in our case, the direction of the change is opposite to that found by Kulkarni and Blout, the direction as well as the magnitude of the ionic strength dependence apparently depends on the nature of the side chains. It may be possible to learn more about this effect by performing similar titration experiments on other ionizable polypeptides such as polylysine and polyaspartic acid. Such experiments could also serve to test the possibility that the maximum stability observed here (320 cal./residue in water) represents the sum of peptide and side-chain contributions, whereas the limit observed at higher concen-

trations of salt (120 cal./residue) represents the peptide contribution alone.

### Appendix I

**An Alternative Method of Determining  $\Delta G^\circ/N$ .**—Since Zimm and Rice<sup>18</sup> have presented a detailed treatment of this problem from the point of view of statistical mechanics, it is essential that something be said about the relationship of their work to the approach adopted here.

Expressed in our notation, the results of Zimm and Rice can be written

$$\Delta G^\circ/N = (RT/0.434)A \quad (A1)$$

where  $A$  is the area (see Fig. 2A) bounded by the low  $\alpha$  region of the pure helix curve (curve II between  $\alpha = 0$  and  $\alpha \cong 0.25$  in this case), the experimental data (curve I), and the pure coil curve (curve III). If it were not for the upswing of the actual data points at low  $\alpha$ , caused, as noted above, by incipient precipitation, the area could be more simply described as that between the experimental points (which would then exactly follow the helix curve at low  $\alpha$ ) and the coil curve (III).

It is readily shown that eq. A1 can also be obtained by a much simpler, though less fundamental, method than that of Zimm and Rice. It is only necessary to introduce an appropriate modification of eq. 1 to obtain the result. Examination of the derivation<sup>9,10</sup> of eq. 1 immediately reveals that the *electrical* free energy appears simply because it has been assumed that the *only* standard free energy changes (in excess of the intrinsic change embodied in  $pK_0$ ) accompanying ionization of the  $Z$ th carboxyl are electrostatic in origin, *i.e.*, are caused by the electrostatic attraction of the  $Z - 1$  carboxylate ions for the  $Z$ th proton to ionize. If other standard free energy changes are liable to accompany ionization (*e.g.*, those free energy changes attending a configuration change that is brought on by the ionization) then these must be included, and instead of eq. 1, we have

$$pH = pK_0 - \log [(1 - \alpha)/\alpha] + (0.434/RT)(\partial G_{ion}/\partial Z) \quad (A2)$$

where  $G_{ion}$  includes not only the electrostatic free energy but also any other free energy changes that accompany ionization.

It is plain from eq. A2 that integration of an experimental curve (such as in 2A) from  $\alpha = 0$  to  $\alpha = 1$  provides an area  $A_1$  which is

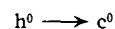
$$A_1 = (0.434/NRT) \int_{\alpha=0}^{\alpha=1} (\partial G_{ion}/\partial \alpha) d\alpha = (0.434/NRT)\Delta G_1$$

where  $\Delta G_1$  represents the total standard free energy change (except that associated intrinsically with carboxyl ionization) accompanying the process:  $h^0 \rightarrow c^{-N} + NH^+$ .

If we imagine returning the system to the un-ionized state *via* the coil curve, we obtain the negative of the area beneath it, given by

$$A_2 = (0.434/NRT) \int_{\alpha=1}^{\alpha=0} (\partial G_{c, ion}/\partial \alpha) d\alpha = (0.434/NRT)\Delta G_2$$

where  $\Delta G_2$  is the total standard free energy change (excepting the intrinsic one) of the process:  $c^{-N} + NH^+ \rightarrow c^0$ . The sum of these two free energy changes is, of course, the standard free energy change for the over-all process



and is also the area described above, *i.e.*

$$A = A_1 + A_2 = (0.434/NRT)(\Delta G_1 + \Delta G_2) = (0.434/RT)(\Delta G^\circ/N)$$

which is eq. A1.

This, of course, represents a method of obtaining  $\Delta G^\circ$  which is, in many respects, more convenient than that embodied in eq. 8; the results are entirely equiv-

(23) J. G. Kirkwood, "Symposium on the Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1954, p. 23.

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

alent when eq. 8 is employed where  $C_c = C_h$ . However, eq. 8 may have the added utility of providing a means of assessing the size of a "denaturing unit," given by the magnitude of  $N$  required to fit the data when  $C_c \neq C_h$ .

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## Excluded Volume of Polyelectrolyte in Salt Solutions

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The intrinsic viscosities of sodium poly-(acrylate) having different molecular weights were determined in sodium bromide solutions of various concentrations. The analysis of the data was based on three different theories; Flory and Fox; Kurata, Stockmayer, and Roig; Stockmayer and Fixman. Both K. S. R. and S. F. plots converged at one point, respectively, with different concentrations of sodium bromide, as those theories predicted, whereas F. F. plots did not give the same intercept against the prediction of the theory. From the slopes of K. S. R. and S. F. plots, the mutual excluded volume of segments was determined. The values obtained were compared with the values calculated using the Debye-Hückel theory for the potential of average force between two segments. Large disagreement was observed between the experimental and calculated results, and, moreover, the mutual excluded volume was found to be proportional to the inverse root of sodium bromide concentration, while the calculated result predicts its proportionality to the inverse of sodium bromide concentration. The reason for the discrepancy between theory and experiment is considered to be due to neglect of the counter-ion effect in calculating the mutual excluded volume.

### Introduction

During the past 15 years, great efforts have been devoted to clarify the well known expansion problem of a linear poly-ion in solution both theoretically and experimentally.<sup>1</sup> The origin of the molecular expansion is now accepted to be the electrostatic repulsive force among fixed charges on the polyion. Owing to the electrostatic repulsion between fixed charged groups, the molecular dimension of the poly-ion increases to several times as large as that of the original uncharged polymer and varies markedly with the concentration of added neutral salt, *i.e.*, with ionic strength of solution.

In spite of so many papers published to compute the problem quantitatively, there is still great disagreement between theories and experimental results. In all cases, the calculated expansions of the poly-ion are much larger than the experimental ones if we use the analytical charge density of poly-ion to calculate the repulsive force due to electric charges. The discrepancy is often explained by assuming that the effective charge density of the poly-ion is much smaller than the analytical one due to ion binding or low activity coefficient of the counter-ion in the polymer domain. To explain the discrepancy may be the most important problem to be solved in this field, but before going into the problem it seems necessary to re-examine the theory of hydrodynamic resistance used for polyelectrolytes, considering the recent progress in the theory on excluded volume of nonionic polymers.

In most papers concerning the expansion of a polyion, the hydrodynamic resistance of the poly-ion is computed based on Flory's theory.<sup>2</sup> That is, the polymer coil is practically nondraining for solvent and the intrinsic viscosity is determined by the linear expansion of the polymer coil,  $\alpha_\eta$ , as

$$[\eta] = KM^{1/2}\alpha_\eta^3 (= KM^v) \quad (1)$$

and

$$[\eta]_\theta = KM^{1/2} \quad (2)$$

That is

$$\alpha_\eta^3 = [\eta]/[\eta]_\theta \quad (3)$$

Here,  $[\eta]_\theta$  is the intrinsic viscosity at  $\theta$  temperature where the intrinsic viscosity is linear in the square root of molecular weight,  $M^{1/2}$ , as shown in eq. 2. The intrinsic viscosity  $[\eta]$  is also related to the root-mean-square end-to-end distance  $\langle h^2 \rangle^{1/2}$  by the equation<sup>3</sup>

$$[\eta] = \Phi \frac{\langle h^2 \rangle^{3/2}}{M} \quad (4)$$

where  $\Phi$  is the well known universal constant of Flory.

To relate this expansion factor  $\alpha_\eta$  with molecular constants of polymer and solvent, Flory derived the equation

$$\alpha_\eta^5 - \alpha_\eta^3 = CZ \quad (5)$$

where  $C$  is a numerical constant and  $Z$  is a function defined by

$$Z = \left(\frac{3}{2\pi a^2}\right)^{3/2} \beta \left(\frac{M}{m_s}\right)^{1/2} \quad (6)$$

In eq. 6,  $a$  is the length of a link;  $M$  and  $m_s$ , the molecular weights of polymer and segment, respectively; and  $\beta$  is the binary cluster integral defined by

$$\beta = - \int_0^\infty [1 - \exp(-V(r)/kT)] 4\pi r^2 dr \quad (7)$$

where  $V(r)$  is the potential of average force between two segments as a function of their distance  $r$ . Although  $\beta$  is commonly used in this field, we prefer to use  $B$  converted from  $\beta$  by the relationship

$$B = \beta/2m_s^2 \quad (8)$$

for convenience of our discussion. Combination of eq. 5 and eq. 1 gives the following equation of Flory, Fox, and Schaeffgen<sup>3</sup>

$$\frac{[\eta]^{2/3}}{M^{1/3}} = K^{2/3} + C'B \frac{M}{[\eta]} \quad (9)$$

(1) S. A. Rice and M. Nagasawa, "Polyelectrolyte Solutions," Academic Press, Inc., New York, N. Y., 1961.

(2) P. J. Flory, "Principles of Polymer Chemistry," Cornell University Press, Ithaca, N. Y., 1953, Chapter XIV.

(3) P. J. Flory and T. G. Fox, *J. Am. Chem. Soc.*, **73**, 1904 (1951).