JOURNAL

OF THE AMERICAN CHEMICAL SOCIETY

VOLUME 86, NUMBER 4 FEBRUARY 20, 1964

PHYSICAL AND INORGANIC CHEMISTRY

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND THE ADOLPHUS BUSCH III LABORATORY OF MOLECULAR BIOLOGY, WASHINGTON UNIVERSITY, ST. LOUIS, MO.]

The Use of the Debye–Hückel Approximation in the Analysis of Protein Potentiometric Titration Data[1]

BY MITSURU NAGASAWA[2] AND ALFRED HOLTZER

RECEIVED JUNE 10, 1963

Numerical solutions to the nonlinearized Poisson–Boltzmann equation have been obtained, using an electronic computer. These results are used to calculate the electrostatic free energy of several macro-ions as a function of charge and ionic strength. The computations are compared with available experimental (potentiometric titration) determinations of electrostatic free energies for two globular proteins (β-lactoglobulin and conalbumin) and with our own data for a synthetic helical polypeptide (poly-L-glutamic acid) and a synthetic linear polyampholyte (2-dimethylaminoethyl methacrylate–methacrylic acid). The results show clearly that deviations from the behavior predicted by the theory of Linderstrøm-Lang (for spheres) or Hill (for rods) need not be a result of ion binding as is often assumed, but may be caused by failure of the smeared charge, or Debye–Hückel approximations, or both. Independent experiments on ion binding by measurement of ion activity coefficients or shifts in isoionic pH are shown to be subject to the same ambiguity.

Since proteins are polyelectrolytes, often having many charges per particle, the dissociation constant of a given type of ionizable group depends on the net electric charge of the molecule. Furthermore, proteins are polyampholytes, a given molecule bearing both positively and negatively charged groups. Thus, even when the net charge is close to zero, there are many charged groups on the molecule and the electrostatic interaction among these ionized groups may not be zero. The first problem has been discussed extensively in connection with studies of linear polyelectrolytes and proteins. Except in rare instances, however, research on polyampholytes has been restricted to proteins.

Theoretical treatment of the problem of potentiometric titration of polyelectrolytes having a uniform distribution of ionizable groups of one kind leads to an expression for the pH of the solution[3,4]

$$\mathrm{pH} = \mathrm{p}K_0 + \log\left(\frac{\alpha}{1-\alpha}\right) + \frac{0.434}{kT}\frac{\partial G_{el}}{\partial \nu} \qquad (1)$$

where $\mathrm{p}K_0$ is the intrinsic ionization constant of the group, α the degree of ionization, ν the number of groups per molecule that are already ionized, and G_{el} is the electrostatic free energy of the molecule.

Since the last term represents the work done in removing the νth proton from the molecule to infinity, eq. 1 may also be written[5,6]

$$\mathrm{pH} = \mathrm{p}K_0 + \log\left(\frac{\alpha}{1-\alpha}\right) - 0.434\,\frac{\epsilon\Psi_b}{kT} \qquad (2)$$

ϵ being the magnitude of the electronic charge, and Ψ_b the electrostatic potential at the point on the molecule from which the proton is removed.

For a spherical molecule having a large number of charges on its surface, Ψ_b may safely be assumed to be the surface electrostatic potential of the sphere and must be calculated by solving the Poisson–Boltzmann equation

$$\nabla^2\Psi = \frac{-4\pi\epsilon}{D}\,C_0[e^{-\epsilon\Psi/kT} - e^{\epsilon\Psi/kT}] \qquad (3)$$

where C_0 is the concentration of added 1–1 electrolyte and D is the dielectric constant of the medium. If the electrostatic potential is sufficiently low everywhere, *i.e.*, if $\epsilon\Psi/kT << 1$, then the Debye–Hückel approximation may be applied and the resulting solution to 3 leads to a simple expression for the surface potential[7,8]

$$\Psi_b = \frac{Z\epsilon}{D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) = \left(\frac{2kT}{\epsilon}\right)wZ \qquad (4)$$

$$\kappa^2 = (8\pi\epsilon^2/DkT)C_0$$

where Z is the net valence of the molecule, b is the radius of the sphere, and a the distance of closest approach of small ions to the macro-ion.

Substitution of 4 into 2 leads to the well-known equation

$$\mathrm{pH} = \mathrm{p}K_0 + \log\left(\frac{\alpha}{1-\alpha}\right) - 0.868wZ \qquad (5)$$

For rod-like molecules, the Debye–Hückel approximation leads to the similar result[9]

$$\Psi_b = \frac{2Z\epsilon}{DL}\left[\frac{K_0(\kappa a)}{(\kappa a)K_1(\kappa a)} + \log\left(\frac{a}{b}\right)\right] = \left(\frac{2kT}{\epsilon}\right)wZ \qquad (6)$$

where b is the radius of the rod, L its length, and the K's represent modified Hankel functions.

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

(2) Department of Synthetic Chemistry, Nagoya University, Chikusa-ku, Nagoya, Japan.

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

(4) A. Arnold and J. Th. G. Overbeek, *ibid.*, **69**, 192 (1950).

(5) J. Th. G. Overbeek, *Bull. soc. chim. Belges*, **57**, 252 (1948).

(6) A. Katchalsky, N. Shavit, and H. Eisenberg, *J. Polymer Sci.*, **13**, 69 (1954).

(7) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **15**, No. 7 (1924).

(8) J. Edsall and J. Wyman, "Biophysical Chemistry," Academic Press, Inc., New York, N. Y., 1958.

(9) T. L. Hill, *Arch. Biochem. Biophys.*, **57**, 229 (1955).

Equation 5 has been used extensively and successfully in the interpretation of data from potentiometric titration studies of globular proteins.[8] The method of treatment has been to recognize that pH and α are experimentally measurable and, if it is assumed that the charge is given by the number of protons bound, so is Z. Consequently, if the experimental values of pH $-$ log $(\alpha/1 - \alpha)$, determined in the course of the titration at a given ionic strength, are plotted *vs.* Z, a straight line should be obtained, the intercept at $Z = 0$ providing pK_0 and the slope giving the value of w. In the absence of complications, experiments at different ionic strengths should extrapolate to the same value of pK_0 at $Z = 0$, since the effect of ionic strength on the pH at zero charge can be shown to be rather small; furthermore, the dependence of w on ionic strength should follow simply from the ionic strength dependence of κ, as expressed in eq. 4.

In practice, deviations from this ideal behavior are often observed. In one case, in which the plots are nonlinear indicating a variation of w with charge as well as ionic strength, the discrepancy is ascribed to an expansion of the radius b of the protein with increasing charge.[10] This interpretation has been amply confirmed by a variety of means. In other cases, less striking, but real, deviations have been found in both the slopes and intercepts of such plots. The intercepts are often found to vary with ionic strength, and the slopes to depend on ionic strength and apparent charge in a manner not given by 4.[11,12] Ordinarily, it is assumed that both of these deviations are caused by the binding of ions other than hydrogen, making it incorrect to compute the net charge on the macroion from proton binding alone. The deviations mentioned are then used to obtain information about the nature and extent of the ion binding. This method of study of ion binding has been widely used; indeed, the measurement of the effect of salts on the isoionic pH, which is essentially a direct experimental determination of the $Z = 0$ intercept, has become almost a routine method of assessing ion binding in proteins.[13]

In spite of the success of this approach, however, it is necessary to keep in mind that deviations from an approximate theory may as easily stem from the approximations themselves as from the presence of additional physical phenomena (such as ion binding) requiring further description. In this connection, then, it is important to examine closely the approximations of the theory to see if they can lead to apparent deviations that may be misinterpreted.

Two particular approximations that require such scrutiny are the use of the smeared charge model and of the Debye–Hückel approximation. Both Linderstrøm-Lang's theory for spherical molecules and Hill's for cylinders assume that the charge distribution is uniform and continuous. Consequently, effects of interaction among charged groups at the isoionic point (where $Z \cong 0$) cannot be discussed on this basis; it is possible that effects of added salt on the isoionic pH may exist that the theory is incapable of describing, but that may be caused by specific conformations of discrete charges rather than by ion binding. Furthermore, both theories employ the Debye–Hückel approximation, thus requiring $\epsilon\Psi/kT << 1$ and their use should therefore certainly be confined to the region $\epsilon\Psi_b < 1$.

The consequences of the first approximation, the use of the smeared charge model, have been thoroughly investigated by Tanford and Kirkwood.[14a,b] These authors computed the electrostatic free energies of spherical macromolecules having various, fixed conformations of positively and negatively charged groups, each fixed charge being surrounded by an ionic atmosphere of the Debye–Hückel type. It was found that the effect of the ionic atmosphere on the slope of the pH $-$ log $(\alpha/1 - \alpha)$ *vs.* Z curve is the same as that obtained from the smeared charge model only at very low ionic strengths. For higher concentrations of salt, the results of the two theories are quite different and show clearly that purely electrostatic effects can alter the slopes markedly. In addition, the intercepts at $Z = 0$ are found to depend on the ionic strength in the discrete charge case, and the value of the intercept may be larger or smaller than the true pK_0, depending on the disposition of the discrete charges on the molecular surface. The results of Tanford and Kirkwood thus reveal a serious ambiguity in the method of interpretation of potentiometric titration data described above. Mazur, Silberberg, and Katchalsky have also pointed out that the isoelectric point of polyampholytes can be affected by nearest neighbor interactions.[15]

The possible effect of the second important approximation in the Linderstrøm-Lang theory, the use of the Debye–Hückel ion atmosphere, has not been explored in detail, and it is our purpose in this paper to do so. Two consequences of the Debye–Hückel approximation are immediately apparent. First, the theory should not be used where $\epsilon\Psi_b/kT > 1$, that is, if the quantity pH $-$ log $(\alpha/1 - \alpha) - pK_0$ is greater than 0.434. Second, the use of this approximation always leads to a linear dependence of the experimental quantity pH $-$ log $(\alpha/1 - \alpha)$ on Z at a given ionic strength; that is, w depends on ionic strength, but not on charge. Both features are common to the equations of Linderstrøm-Lang, Hill, or Kirkwood and Tanford, all of which involve the Debye–Hückel ion atmosphere.

To avoid the use of the Debye–Hückel approximation we have calculated the electrostatic potential surrounding a spherical macroion (with smeared charge) by solving the Poisson–Boltzmann equation (3) by numerical integration using an electronic computer. These results are compared with data on the potentiometric titration of globular proteins. Similar computations for rod-like molecules have already been described and compared with experiments on linear polyelectrolytes[16a]; such computations have also been made for spheres for use in a different context, over a different range of conditions.[16b]

Our examination of existing data for globular proteins shows that the Linderstrøm-Lang theory has, in some cases, been used beyond the point where the Debye–Hückel approximation can be valid. The resulting curvature of the experimental plots of pH $-$ log $(\alpha/1 - \alpha)$ *vs.* Z and the magnitude of the deviation of the slopes and intercepts from those given by Linderstrøm-Lang's equation can be satisfactorily explained by the computer results without the introduction of the *ad hoc* assumption of ion binding.

In the present paper we also compare these calculations with the experimental results for the rod-like

(10) C. Tanford, S. A. Swanson, and W. S. Shore, *J. Am. Chem. Soc.*, **77**, 6414 (1955).

(11) (a) Y. Nozaki, L. G. Bunville, and C. Tanford, *ibid.*, **81**, 5523 (1959), (b) R. K. Cannan, A. H. Palmer, and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(12) A. Wishnia, I. Weber, and R. C. Warner, *J. Am. Chem. Soc.*, **83**, 2071 (1961).

(13) G. Scatchard and E. S. Black, *J. Phys. Chem.*, **53**, 88 (1949).

(14) (a) C. Tanford and J. G. Kirkwood, *J. Am. Chem. Soc.*, **79**, 5333 (1957); (b) C. Tanford, *ibid.*, **79**, 5340 (1957).

(15) J. Mazur, A. Silberberg, and A. Katchalsky, *J. Polymer Sci.*, **35**, 43 (1959).

(16) (a) L. Kotin and M. Nagasawa, *J. Chem. Phys.*, **36**, 873 (1962); (b) A. L. Loeb, J. Th. G. Overbeek, and P. H. Wiersema, "The Electrical Double Layer Around a Spherical Colloid Particle," M.I.T. Press, Cambridge, Mass., 1961.

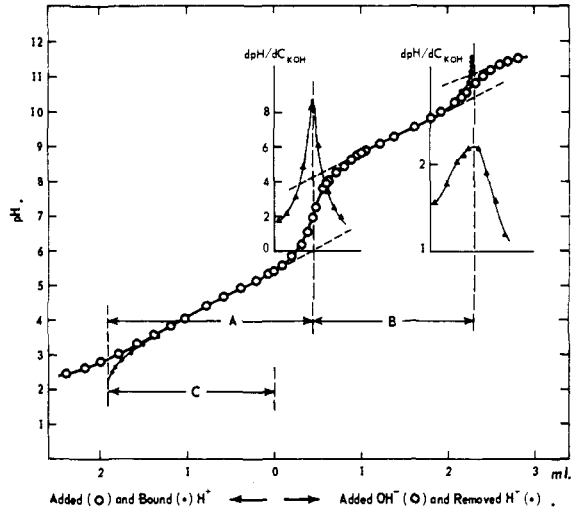

Fig. 1.—Potentiometric titration curve of a copolymer of methacrylic acid and 2-dimethylaminoethyl methacrylate in 0.500 *M* KCl solution at 25 ± 1°. The abscissa represents ml. of 0.100 *M* HCl or 0.100 *M* KOH added to 10.00 ml. of a solution containing 0.500% polymer. The volume of 0.1 *M* reagent required to neutralize the carboxyl groups is shown as A. The volume of 0.1 *M* reagent required to neutralize the amino groups is B or C.

(helix) form of polyglutamic acid and for a linear synthetic polyampholyte.

It is of course true that evidence for the existence of ion binding is not confined to titration experiments. Ionic activity measurements have seemingly provided confirmation. However, the interpretation of activity measurements is by no means unambiguous, and we believe that the experiments can also be explained in terms of purely electrostatic effects.

Finally, it must be stressed that the discussion here is confined to systems containing salts such as alkali halides, which do not ordinarily form complexes with simple carboxyl or amino groups. Ions such as copper, zinc, alkaline earth cations, or complex anions, which often form such complexes, are deliberately excluded from consideration.

Methods

Materials and Potentiometric Titrations.—For the globular proteins, the very precise potentiometric titration data of Tanford, Wishnia, and their co-workers were used.[11,12] These data were reproduced by enlarging photographs of the figures in their papers.

The titration procedure and results for polyglutamic acid are described in an accompanying paper.[17]

A sample of a linear polyampholyte, a copolymer of methacrylic acid and 2-dimethylaminoethyl methacrylate, was kindly provided by Professor P. M. Doty of Harvard University.[18] The potentiometric titration of this sample was carried out essentially as for polyglutamic acid. A sample of the data, the curve obtained in 0.5 *M* NaCl, is shown in Fig. 1. The amount of methacrylic acid in the molecule is determined by the method frequently used for proteins, that is, from the maximum number of bound hydrogen ions, which is the difference between the amount of acid added and the free acid. The concentration of free acid is deduced from the measured pH assuming that the activity coefficient of hydrogen ion is the same as that in a corresponding hydrochloric acid solution containing no polymer. Figure 1 shows that the mole ratio of acidic to basic constituents in the copolymer is 1.23, in agreement with the value obtained from nitrogen analysis by the micro-Kjeldahl method.

The potentiometric titration of polymethacrylic acid was carried out similarly except that a Beckman GS pH meter was used instead of the Radiometer instrument described elsewhere.[17]

Numerical Integration.—The method of numerical integration for the spherical model is virtually identical with the method used previously for cylindrical particles.[16a] The smeared charge model is used throughout. The computations were performed at the Washington University computer center using an IBM 650. The results are obtained in the form of tables of Φ (= $\epsilon\Psi/kT$) and $d\Phi/dX$ *vs.* X (= κr) for various boundary conditions. In practice, for a given ionic strength, and given charge and dimensions of the macro-ion, we can calculate

$$(d\Phi/dX)_{X=\kappa a} = \frac{-2Z\epsilon^2}{DkTL}\left(\frac{1}{\kappa a}\right) \text{ for rods, and} \qquad (7)$$

$$(d\Phi/dX)_{X=\kappa a} = \frac{-Z\epsilon^2}{DkTa}\left(\frac{1}{\kappa a}\right) \text{ for spheres} \qquad (8)$$

The tables are then consulted until an entry is found where the calculated value of $(d\Phi/dX)$ occurs at the chosen X (= κa). The corresponding value of $\Phi_{X=\kappa a}$ (= $\epsilon\Psi_a/kT$) is then read from the same table.

This procedure provides Ψ_a, the electrostatic potential at the radius to which salt ions are excluded. However, for use in eq. 2 we require Ψ_b, the potential at the position of the proton, which is presumably located at the particle surface. This quantity is readily computed from the value of Ψ_a, since there can be no ions between b and a, and we therefore have

$$\left(\frac{\epsilon\Psi_b}{kT}\right) = \left(\frac{\epsilon\Psi_a}{kT}\right) + \frac{Z\epsilon^2}{DkT}\left(\frac{1}{b} - \frac{1}{a}\right) \qquad (9)$$

The difference $a - b$ should represent the radius of the salt ion, *e.g.*, about 2.5 Å. for sodium ions. Strictly speaking, however, the location of the protons may not coincide exactly with the radius of the molecule. The only practical way of proceeding, therefore, is to treat the second term of 9 as an adjustable parameter proportional to the charge; *i.e.*, we write

$$\epsilon\Psi_b/kT = (\epsilon\Psi_a/kT) + BZ \qquad (10)$$
$$B = (\epsilon^2/DkT)(a - b/ba)$$

and choose the value of B that fits the data best. We do expect however, that the value of $a - b$ needed to produce agreement should be roughly of the dimensions of a salt ion.

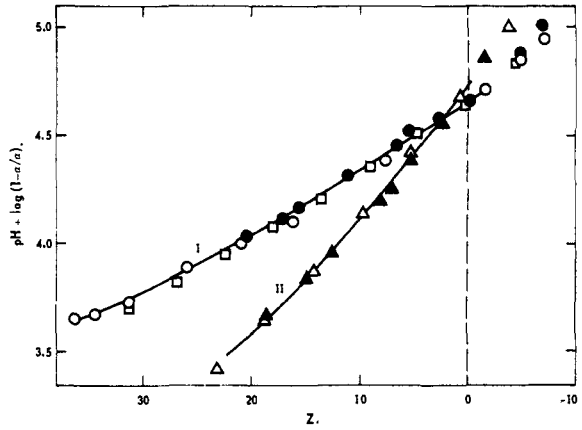

Fig. 2.—Theoretical and experimental titration curves for the carboxyl groups of β-lactoglobulin.[11a,b]

Theor. curve	Solvent	Temp., °C.	*a*, Å.	*b*, Å.	*a* − *b*, Å.
I	0.15 *M* KCl	25	26.2	0.011	1
II	0.01 *M* KCl	25	26.2	0.00	0

Filled (open) circles represent data of Nozaki, *et al.*,[11a] on deionized (nondeionized) sample at ionic strength 0.15. Open squares represent data of Cannan, *et al.*[11b] at ionic strength 0.15. Filled (open) triangles are data of Nozaki, *et al.*[11a] (Cannan, *et al.*[11b]) at ionic strength 0.01.

Comparison of Theory and Experiment

Globular Proteins.—For comparison of theory and experiment we use the results of the very careful potentiometric titration work of Nozaki, Bunville, and Tanford on β-lactoglobulin,[11] and of Wishnia, Weber, and Warner on conalbumin.[12] Plots of the data as pH − log (α/1 − α) *vs.* Z are linear at low charge, as expected from the theory of Linderstrøm-Lang. At larger charge, however, deviations from linearity are observed in all cases. Examination shows that the data lie beyond the region where pH − log (α/1 − α) − pK_0 < 0.434 and therefore beyond the limit of applicability of the Debye–Hückel approximation. Consequently, we do not expect linearity in this range, and the experi-

(17) M. Nagasawa and A. Holtzer, *J. Am. Chem. Soc.*, **86**, 538 (1964).
(18) G. Ehrlich and P. Doty, *ibid.*, **76**, 3764 (1954).

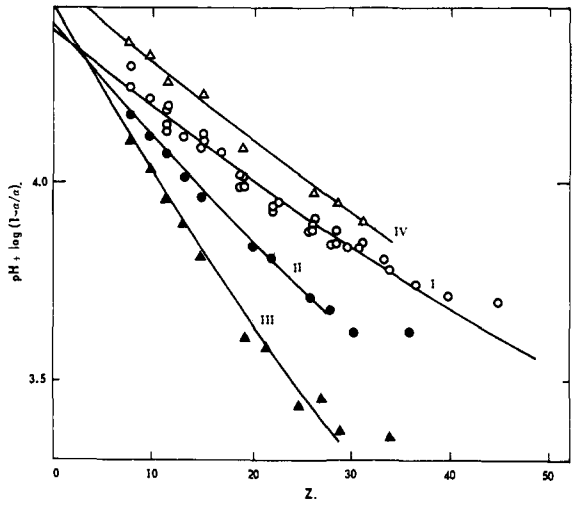

Fig. 3.—Theoretical and experimental titration curves for the carboxyl groups of conalbumin[12]; parameter a = 32.0 Å.

Theor. curve	Solvent	Temp., °C.	B	$a - b$, Å.
I	0.100 M KCl	25	0.0073	1
II	.030 M KCl	25	.0135	1.8
III	.010 M KCl	25	.0077	1
IV	.100 M KCl	5	.0042	0.5

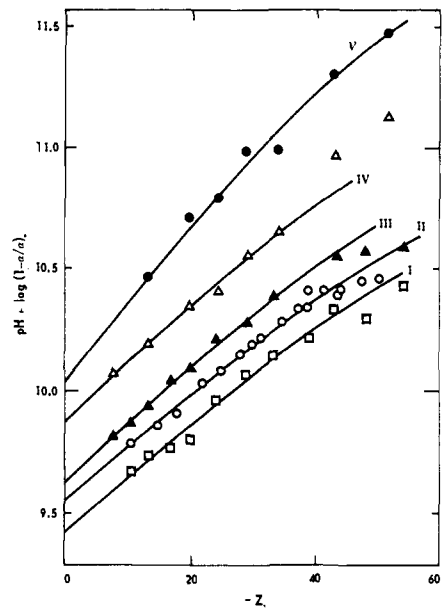

Fig. 4.—Theoretical and experimental titration curves for the noncarboxyl groups of conalbumin in 0.100 M KCl[12]; parameter a = 32.0 Å.

Theor. curve	Temp., °C.	Group titrated	B	$a - b$, Å.
I	25	Phenolic	0.00	0
II	22	Phenolic	.00	0
III	25	ε-Amino	.0029	0.4
IV	5	Phenolic	.0045	0.6
V	5	ε-Amino	.019	2.4

mental results should more properly be compared to solutions of the Poisson–Boltzmann equation that have been obtained without linearization.

In Fig. 2 the data for β-lactoglobulin are so compared, and in Fig. 3 and 4 the comparison for conalbumin is shown. In all cases the calculated curves agree well with the experimental points over the whole range of charge, using a single, reasonable choice of the parameter B. The appearance of curvature in the logarithmic plot at high charge may thus be attributed to the

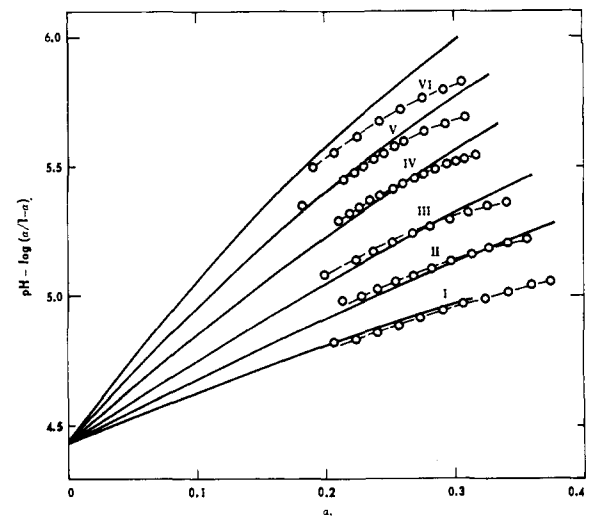

Fig. 5.—Theoretical and experimental titration curves of helical polyglutamic acid. The titration was performed at 25° using NaOH. The parameters employed were: a = 12.5 Å.; B = 0.0; and $a - b$ = 0 Å. The concentrations of salt were:

Theor. curve	I	II	III	IV	V	VI
KCl concn., moles/l.	0.189	0.0947	0.0475	0.0190	0.00954	0.00478

failure of the Debye–Hückel approximation, and it is unnecessary in these cases to invoke any further physical effects such as molecular swelling. The implications of these results for ion binding are discussed below.

It must be emphasized, however, that the computer solutions to the Poisson–Boltzmann equation do not show curvature of anywhere near the magnitude revealed in experiments on bovine serum albumin.[10] In the latter case the interpretation of the results in terms of molecular swelling is unquestionably valid. Indeed, it may also be true that in β-lactoglobulin the curvature beyond $Z = +30$ is at least partially a result of dissociation into subunits, as has been suggested.[11] Such dissociation has been reported at somewhat lower ionic strengths and may well persist, in the very acid region, at ionic strength 0.15.

Polyglutamic Acid.—Wada first showed that the potentiometric titration data of polyglutamic acid clearly reveal regions in which helical and coiled molecules, respectively, are being titrated.[19] At low degrees of neutralization the helical form is the predominant species and the appropriate model is therefore that of a cylinder. Wada found that his data in this region give values consistently lower than those calculated from Hill's equation (eq. 6). In an earlier paper we report the results of more precise potentiometric titration experiments on this substance. In Fig. 5 is shown the comparison of the computer results for a rod with the experiments in the region of helix. The agreement is seen to be good, except for the 0.005 M NaCl solution, where the ionic strength may be too low.

Polyampholyte (2-Dimethylaminoethyl Methacrylate–Methacrylic Acid).—Although this synthetic polyampholyte is linear rather than globular, it is instructive to compare its titration behavior to that of proteins. The qualitative features of the titration curves of linear polyampholytes have been fully discussed from a theoretical point of view.[20] The expected behavior is similar to that found in proteins. The experimental results shown in Fig. 6 bear this out. One striking similarity is that the intercept of the pH − log (α/1 − α) *vs.* Z plot varies with ionic strength. Thus, there is a

(19) A. Wada, *J. Mol. Phys.*, **3**, 409 (1960).
(20) S. A. Rice and F. E. Harris, *J. Chem. Phys.*, **24**, 326, 336 (1956).

neutral salt effect on isoionic solutions of the synthetic polyampholyte—behavior which mimicks one of the most important features of protein titration curves, but which is not observed for simple polyions such as polymethacrylic acid.

In contrast to the agreement found between computer calculations of the electrostatic potential and experimental values for simple linear polyions and for proteins, we find that the experimental results for the polyampholyte do not agree with calculations based on any model. In Fig. 6 the results of calculations based on the rod model are shown as dotted lines. The radius of the polymer was taken to be 10 Å., the value obtained from a molecular model of the polymer.

Since Ehrlich and Doty have shown that this polymer is unusually highly coiled in solution,[18] it is perhaps not surprising that calculations based on a rod model should turn out to be inaccurate. Furthermore, the greater length of the side chains bearing the positive charges compared to those terminating in carboxyl groups may produce peculiarities in the ionization caused by the effective local dielectric constant. In any case, it is impossible at the moment to present a more complete discussion of the question.

Ion Binding

The Slope of the Titration Curve.—The appearance of curvature, at high charge, in logarithmic plots of the titration curve is discussed above and it is found that this could result from failure of the Debye–Hückel approximation. The absolute magnitude of the slope, rather than its variation with charge, must now be considered.

Deviations of the observed slopes from the predictions of eq. 4 are usually interpreted in terms of binding of ions other than hydrogen. However, the calculation of the expected slope really involves the computation of the potential at the distance of closest approach of the salt ions (computation of Ψ_a) and calculation of the surface potential Ψ_b from the continuity condition (eq. 9). The quantity Ψ_a depends on the choice of the quantity a, and Ψ_b on the difference $a - b$, which is, in Linderstrøm-Lang's theory, the radius of a salt ion. The difficulty is that $a - b$ can retain this physical meaning, in an exact sense, only if the protons being titrated (say the carboxyl protons) are on the same surface as the positive charges that give the protein its net charge in the acidic region of pH under consideration. In general, the nature of protein side chains makes it unlikely that this is the case, and it is reasonable to allow an ambiguity of ±1 Å. in the value of $a - b$. An ambiguity of this magnitude corresponds to a rather large change in the ion binding as it is conventionally calculated. It is our feeling that, since the titration curves can be fit by values of B (eq. 10) corresponding to $0 < a - b < 2.5$ Å., it is unnecessary to invoke ion binding to explain deviations of the experimental slopes from values calculated with the aid of assumptions that are evidently too rigid. Of course, if the protein molecule is not exactly spherical, even the quantities a and b do not have a rigorous physical meaning.

The Effect of Neutral Salts on the Isoionic pH.—Whether or not the Debye–Hückel approximation is used, the smeared charge model predicts that there should be practically no pH change when a neutral salt is added to an isoionic protein solution.[13] Actually, however, such a change is often observed. Scatchard and Black suggest that this pH change is caused by a change in the charge on the macro-ion produced by binding of the salt ions, and present an equation relating the binding to the pH shift[13]

$$\Delta\text{pH} = -0.868w(\Delta Z) \qquad (11)$$

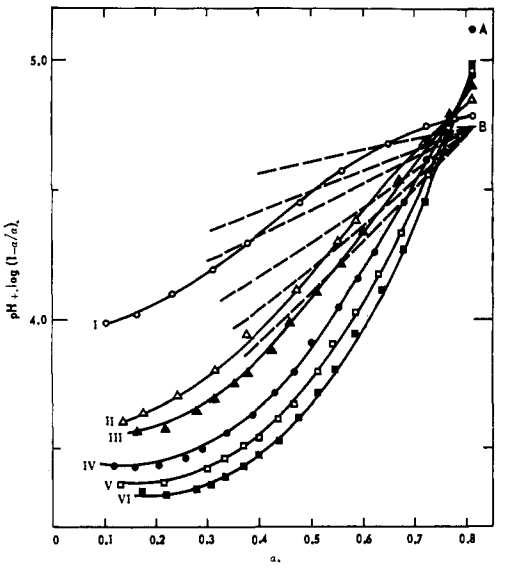

Fig. 6.—Theoretical and experimental titration curves for the carboxyl groups of a copolymer of methacrylic acid and 2-dimethylaminoethyl methacrylate. The titration was performed at 25° using HCl. Point A is the isoionic point, and point B the pK_0^0 of methacrylic acid. Solid curves are drawn to represent best the corresponding experimental points taken at following ionic strengths:

Exptl. curve	I	II	III	IV	V	VI
KCl concn., mole/l.	0.500	0.100	0.050	0.020	0.010	0.005

Dashed curves are theoretical curves at ionic strengths corresponding to the experiments with $a = b = 12.0$ Å.

where w has the same meaning as in eq. 4 and ΔZ is the change in fundamental charge produced by the binding.

The amount of ion binding deduced from eq. 11 is found, in several cases, to be in reasonable agreement with values obtained from measurements of ion activities (see below). However, we note again that it is a major result of the theory of Tanford and Kirkwood that the existence of specific configurations of discrete charges on the molecular surface can lead to exactly such changes in isoionic pH with addition of neutral salts.[14a,b] In general, the effect of the charges on the ionization constant of a given group will not vanish at zero *net* charge. Thus, the pK_0 of eq. 1 and 2, *i.e.*, the $Z = 0$ intercept of the logarithmic titration curve, is not the true intrinsic ionization constant of a carboxyl group, but the apparent ionization constant at zero net charge. The true ionization constant pK_0^0 is given by

$$pK_0^0 = pK_0 - 0.434\frac{(\epsilon)\Delta\Psi_0}{kT} \qquad (12)$$

where $\Delta\Psi_0$ represents the sum of the electrostatic effect of all other charged groups on the carboxyl group at zero net charge. The magnitude and sign of $\Delta\Psi_0$ depends on the detailed distribution of charged groups with respect to the carboxyl groups.

Addition of neutral salt to the isoionic solution shields the carboxyl groups from the influence of the other charges, and it is to be anticipated, therefore, that $\Delta\Psi_0$ will diminish in magnitude with increasing salt concentration so that pK_0 eventually approaches pK_0^0. Thus, the pH of the isoionic solution is expected to change when salt is added, even in the absence of specific ion binding.

The exact value of pK_0^0 of protein carboxyl groups is not known, but it is probably close to 4.6.[21] The electrostatic approach outlined above then leads to the

(21) See ref. 8, p. 536.

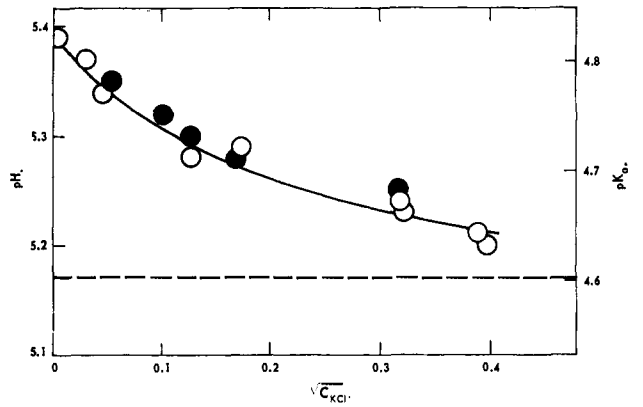

Fig. 7.—The effect of KCl on the isoionic pH and on the pK_0 of β-lactoglobulin. Open (filled) circles represent experiments in which the protein was on the basic (acidic) side of the isoionic point before being subjected to the ion-exchange column. Dashed line represents the supposed pK_0^0 of carboxyl groups in proteins.

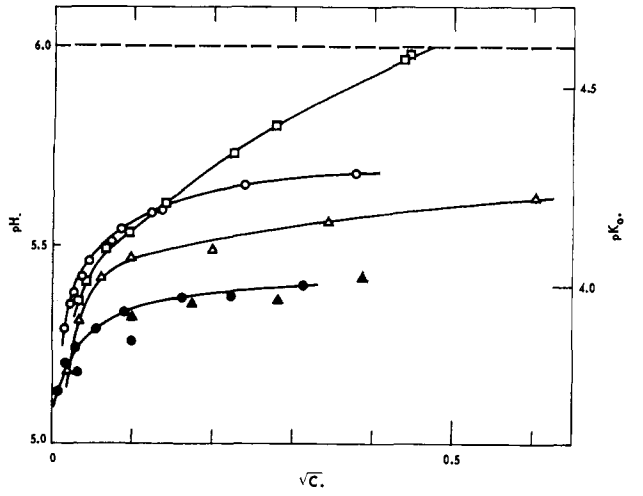

Fig. 8.—The effect of various neutral salts on the isoionic pH and on the pK_0 of bovine serum albumin; data of ref. 22 on effect of salts on isoionic pH: □, NaO_2CCCl_3; ○, NaSCN; △, NaI; ●, NaCl. Data of ref. 10 on the effect of added salt on pK_0; ▲, KCl. Dashed line represents the supposed pK_0^0 of carboxyl groups in proteins.

prediction that, if only carboxyl groups are involved, the value of pK_0 should approach 4.6 at high ionic strength. Since the sign of $\Delta\Psi_0$ may be positive or negative, the value of pK_0 at lower ionic strengths may be either greater or less than 4.6.

In the case of β-lactoglobulin, pK_0 is found to be larger than pK_0^0 (4.6), having the value 4.75 at ionic strength 0.01 and 4.69 at ionic strength 0.15.[11] Using these two values to establish the necessary scale shift, the dependence of isoionic pH on salt concentration (found by Nozaki, *et al.*) may be shown instead as pK_0 *vs.* (square root of) salt concentration. Such a plot is given in Fig. 7 where it is clearly seen that pK_0 decreases toward 4.6, the value of pK_0^0, with increasing concentration of salt.

In the case of bovine serum albumin, pK_0 is apparently less than pK_0^0 indicating that, at zero net charge, the influence of the surrounding charges is opposite to that in β-lactoglobulin. In albumin, the electrostatic picture requires that pK_0 increase with ionic strength. Unfortunately, in this case, the literature does not provide a firm experimental test of this prediction; the only precise study of the effect of neutral salts on the isoionic pH[13,22] was performed without a parallel study of the

(22) G. Scatchard, J. S. Coleman, and A. L. Shen, *J. Am. Chem. Soc.*, **79**, 12 (1957).

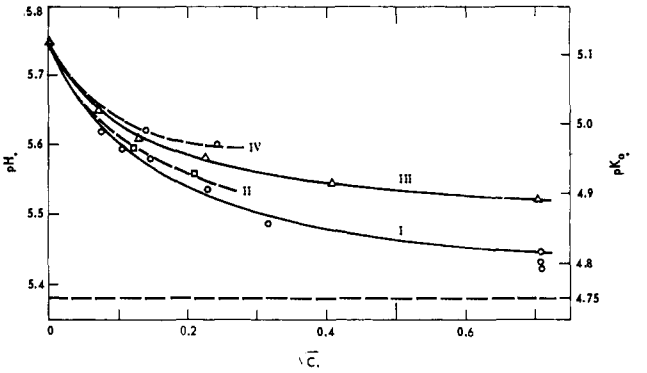

Fig. 9.—The effect of various neutral salts on the isoionic pH and on the pK_0 of the copolymer of methacrylic acid and 2-dimethylaminoethyl methacrylate: I, KCl; II, KBr; III, KI; IV, KSCN. The dashed line represents the pK_0^0 of the carboxyl groups of methacrylic acid.

titration, and the precise titration study[10] did not include measurements of isoionic pH *vs.* salt concentration. To make the comparison, we simply assume that the pK_0 observed by Tanford, *et al.*, in a titration experiment corresponds to the isoionic pH measured by Scatchard, *et al.*, at the same salt concentration. Using this method of computation, we show the data of Scatchard, *et al.*,[22] in Fig. 8 as pK_0 *vs.* square root of salt concentration. It is apparent that pK_0 increases, as expected, although the limit at high ionic strength is not clear.

Unfortunately, the data for conalbumin cannot be used in this context since the isoionic pH is in a region where carboxyls are not the only groups being titrated.

For the synthetic linear polyampholyte, a similar, neutral salt effect is observed. The data for several different salts are shown in Fig. 9 as pK_0 *vs.* square root of salt concentration. In this case, we expect pK_0^0 to have the value characteristic of polymethacrylic acid: 4.75. Although small differences exist among experiments with different salts, the observed behavior is qualitatively as anticipated. The direction of the change indicates that at zero net charge the undissociated carboxyl groups are more influenced by the negatively charged carboxylate groups already present on the molecule than by the equal number of positively charged, amino groups. This could result if the polymerization is not quite random, methacrylate tending to add to methacrylate. This conclusion is in agreement with a study of the process of polymerization used in synthesizing this substance, where it was found that the positive and negative monomer units tend to occur in groups of two or three rather than in strict alternation, or at random.[18]

Small differences (about 0.1 pH unit) observed in Fig. 9 for different salts are equally apparent in measurements on simpler polymers such as polymethacrylic acid. This effect is shown in Fig. 10; a maximum difference of about 0.1 pH unit appears in pK_0 when different salts are employed at the same concentration. This difference probably arises because the definition even of pK_0^0 is not that of a true, thermodynamic equilibrium constant, and variations with different salts would exist even in the potentiometric titration of monobasic carboxylic acids. The definition of pK_0^0 in use here is

$$K_0^0 = \frac{a_{H^+}\alpha}{(1-\alpha)} = \frac{a_{H^+}[RCOO^-]}{[RCOOH]} = K_a\left(\frac{\gamma_{RCOOH}}{\gamma_{RCOO^-}}\right)$$

where K_a is the true, thermodynamic equilibrium constant, the bracket denotes concentrations, and the γ's activity coefficients. The quantity pK_0^0 would

thus include, particularly, the residual effects of the salt ions in the solution on the single carboxylate anion.

It thus appears that the effect of neutral salt on the isoionic pH of polyampholyte may be a feature introduced by electrostatic forces that do not vanish at zero net charge, and therefore that, in this sense, the smeared charge model is inappropriate. If this view is adopted, specific ion binding need not be postulated and the limit approached by pK_0 at high ionic strength is, except for a minor ambiguity, the true intrinsic dissociation constant of the group in question. These qualitative features all appear in the theory of the discrete charge model, and, as shown here, agree with experiment.

Measurement of Activity Coefficients.—The results of measurements of ion activity coefficients are often quoted as further evidence for the existence of ion binding in proteins. The assumption is made that the activity coefficient of the free ion is the same in the presence or absence of protein; measured changes in the ion activity coefficient are then interpreted as a simple result of the removal of small ions from the solution by binding to the protein. The extent of binding deduced in this manner is in good agreement with that calculated from the pH shift.[22-24]

However, although the postulate of site ion binding can explain the titration results, we have seen that a combination of the discrete charge model and use of the nonlinearized Poisson–Boltzmann equation can also do so. To be consistent, we must now raise the question whether the ion activity results can also be explained in this alternative manner.

To produce such an explanation for the case of isoionic protein, it is only necessary to suppose, again, that the large number of charged groups on the protein surface exert a residual electrostatic effect even at zero net charge, and that this alters the activity coefficient of the small ions. Since, in the conventional approach, the ion binding is calculated, as noted above, on the assumption that such changes in activity coefficient are absent, the computed values for binding will be more or less in error, depending on how drastically the isoionic protein affects the activity coefficient of the small ions. Since the observed changes in ion activity are often rather small in isoionic solutions, it may be that, in some cases, the entire change is caused by such a residual electrostatic effect and that interpretation based solely on site binding of ions is incorrect.

In solutions of highly charged proteins, strong, general electrostatic interactions exist between the macromolecule and its counter-ions and these forces must affect the activity of the counter-ions. Consequently, in these cases also, interpretation exclusively in terms of ion binding at specific sites may not be completely valid.

In this latter context, the results of extensive measurements of ion activity in solutions of synthetic, linear polyelectrolytes can provide a helpful comparison. One of the remarkable features of these results is that the observed counter-ion activity in solutions containing added salt is the sum of the activity of the counter-ions in a solution containing polymer only and the activity in a solution containing salt only.[25-27] Such additivity implies that the number of ions bound is roughly independent of the concentration of added salt at a given (substantial) value of the polyion charge,[28] a result which cannot be explained on the basis of site ion binding (which would follow the mass action law), but which *is* consistent with calculations of ion localization based on general electrostatic interaction between the poly-ion and the small ions.[16]

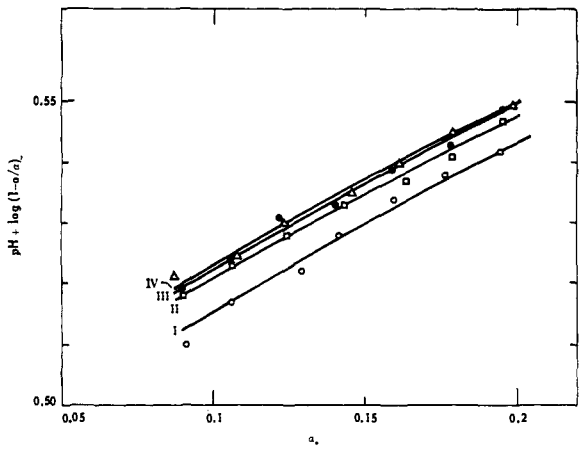

Fig. 10.—The effect of various neutral salts on the potentiometric titration of polymethacrylic acid at 25°. Titration was performed with KOH in presence of 0.500 *M* salt in each case. I (○), KCl; II (□), KBr; III (●), KI; IV (△), KSCN.

Unfortunately, the heteropolymeric nature of proteins makes it impossible to distinguish unequivocally between site binding and electrostatic binding by experiments analogous to the ones described for synthetic homopolymers. Thus, it might appear at first sight that measurements of the activity coefficient of, say, chloride ions as a function of sodium chloride concentration in solutions containing, say, bovine serum albumin at a given (substantial) charge would constitute a crucial test of the two alternatives. It is unfortunate that, in spite of the extensive measurements that have been made on proteins, we have been able to unearth only one pair of experimental values that could be used in this manner.[29] The two solutions of interest contained essentially the same concentration of bovine serum mercaptalbumin at almost the same charge (36.5 protons bound in one case, 32.8 in the other), but varied in the amount of sodium chloride present, one solution being about 0.01 molal in NaCl and the other about 0.05 molal. In spite of this large difference in salt concentration, the number of chloride ions bound per albumin molecule (11.2 and 11.9, respectively), as deduced from chloride activity measurements, was virtually identical in the two solutions. This finding would appear to argue in favor of the general electrostatic interpretation of binding as in the case of linear polyelectrolytes; however, this view is somewhat superficial and the site-binding idea can explain the data as well, if it is supposed that there are several kinds of sites with different binding constants and that the stronger binding sites are already completely filled in both solutions and that weaker binding sites are not appreciably occupied until still higher concentrations of added salt.[30] Although the latter explanation seems the more *ad hoc*, the variety of amino acids present in proteins lends it credence.

There is no doubt that binding of small ions to specific sites on a protein molecule occurs in many cases. However, it is possible, we feel, to offer an alternative description of titration and ion activity measurements in purely electrostatic terms and, indeed, studies on synthetic polymers have provided a considerable body of experimental evidence supporting the idea of electro-

(23) G. Scatchard, I. H. Scheinberg, and S. H. Armstrong, *J. Am. Chem. Soc.*, **72**, 535, 541 (1950).
(24) G. Scatchard, Y. V. Wu, and A. L. Shen, *ibid.*, **81**, 6095 (1959).
(25) R. A. Mock and C. A. Marshall, *J. Polymer Sci.*, **13**, 203 (1954).
(26) M. Nagasawa, M. Izumi, and I. Kagawa, *ibid.*, **37**, 375 (1959).
(27) Z. Alexandrowiez, *ibid.*, **43**, 337 (1960).
(28) S. A. Rice and M. Nagasawa, "Polyelectrolyte Solutions," Academic Press, Inc., New York, N. Y., 1961, Chapter 8.
(29) These data appear in Table III of ref. 22.
(30) We are grateful to Prof. George Scatchard for pointing this out to us.

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.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND THE ADOLPHUS BUSCH III LABORATORY OF MOLECULAR BIOLOGY, WASHINGTON UNIVERSITY, ST. LOUIS, MO.]

The Helix–Coil Transition in Solutions of Polyglutamic Acid[1]

BY MITSURU NAGASAWA[2] AND ALFRED HOLTZER

RECEIVED JUNE 10, 1963

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.[3] Recently, Wada showed that this transition appears very clearly in titration studies of polyglutamic acid.[4] 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.[5] 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.*[3,4]

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. and 0.0200 *M* NaCl. These solutions were titrated in a nitrogen atmosphere at 25 ± 1°, 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 ± 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 λ, n the solvent refractive index, M_0 the monomer molecular weight (129), and λ_0 is taken as 2130 Å.[6] 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,[6] 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.[6-8] In our experiments the value of b_0 could be obtained with a precision of ±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[9,10]

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

where $\text{p}K_0$ is the negative logarithm of the intrinsic dissociation constant of a carboxylic acid group, α 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.* α curves are shown. As Wada pointed out,[4] region B contains the titration curve of completely helical poly-

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

(2) Department of Synthetic Chemistry, Nagoya University, Chikusa-ku, Nagoya, Japan.

(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).

(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).