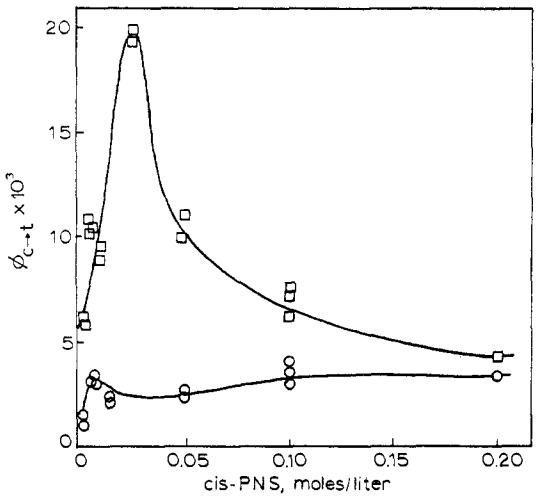

Figure 3. Variation of $\phi_{c\to t}$ with concentration of *cis*-4-nitrostilbene: O, magnesium etioporphyrin; □, zinc etioporphyrin.

the highest concentration studied, $\phi_{c\to t}$ is 0.003. The differences between behavior of zinc and magnesium etioporphyrin are somewhat surprising, particularly since magnesium etioporphyrin is more readily quenched by nitro compounds than is zinc.

The foregoing results indicate that the porphyrin singlet is not the precursor to the isomerizable species in the zinc porphyrin–PNS system. Therefore, the isomerization probably proceeds *via* the porphyrin triplet. Reaction 1 and other reactions leading to triplet states from the singlet complex can be excluded. The fact that isomerization decreases with quenching of the porphyrin singlet *suggests* that reaction 2 must not be very important. Radical anions of the stilbenes are known to undergo very efficient isomerization and participate in chain processes.[40] In preliminary experiments we find that considerable *cis* to *trans* isomerization accompanies the chemical (potassium–glyme) generation of radical anions from *cis*-PNS. Although isomerization of PNS might be somewhat inefficient due to electron localization on the nitro group, some increase in isomerization with concentration would be expected if singlet quenching produced increasing concentrations of free ions.

Though the porphyrin triplet must be the precursor of the isomerizable PNS species, it is unlikely that electronic energy transfer is involved in the system. Triplet energies of the metalloporphyrin are lower than that of *trans*-PNS and probably much lower than that for *cis*. The *trans*-rich stationary states obtained in the isomerization strongly suggest a thermodynamic equilibration. *p*-Benzoquinone was found to quench strongly the isomerization even at low concentrations. For example, addition of 0.001 *M* *p*-benzoquinone (which produces no measurable fluorescence quenching) half-quenches the isomerization of 0.1 *M* *cis*-PNS with zinc etio. Our results suggest that either a complex originating from the triplet state with considerable charge separation or a radical anion of PNS is responsible for the isomerization. If the isomerization precursor is the radical anion of PNS, our results indicate that, at least in a nonpolar solvent such as benzene, the porphyrin triplet is more effective than the singlet in donating electrons even though its energy content is considerably lower.

Acknowledgments. We thank the Petroleum Research Fund, administered by the American Chemical Society (Grant 782-G1,2), and the U. S. Public Health Service (Grant No. GM 15,238-01) for support of this work.

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The Effect of Added Neutral Salt on the Isoionic pH of Proteins and Synthetic Polyampholytes[1]

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Abstract: The method of determining the degree of ion binding on proteins from the pH shift of isoionic solution observed when a neutral salt is added is critically examined by comparing the ionization constants of carboxyl group on several proteins and synthetic polyampholytes. It is concluded that the pH shift of isoionic solution cannot always be considered to give the degree of ion binding.

It is often reported that some ions are bound by protein molecules in a neutral electrolyte solution. Several methods of determining the degree of ion binding have been proposed.[2,3] In particular, the method presented almost two decades ago[4] has been rather commonly used in assessing ion binding;[2,3] that is, the degree of ion binding is estimated from the small change in pH observed when a neutral salt is added to an iso-

(1) This investigation was carried out, in part, in the laboratory of Professor A. Holtzer, Department of Chemistry, Washington University, St. Louis, Mo., where it was supported by Research Grant RG-5488 from the Division of General Medical Sciences, U. S. Public Health Service.

(2) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press, New York, N. Y., 1958.

(3) C. Tanford, "Physical Chemistry of Macromolecules," John Wiley & Sons, Inc., New York, N. Y., 1961.

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

ionic solution of the protein. If a simple smeared charge model is adopted as a picture of the protein, it is predicted that there should be no pH change in the solution when a neutral salt is added, since the isoionic protein has practically zero net charge. In practice, however, a small but measurable change of pH is generally observed. It seemed reasonable at one time to retain the smeared charge model, the pH change then being ascribed to a change in the net charge on the protein produced by specific binding of an excess of one of the ions of the salt. This approach leads to an equation relating the amount of bound ion to the pH shift[4]

$$\Delta\text{pH} = -0.868w\Delta\text{pH} \qquad (1)$$

where

$$w = \frac{e^2}{2DkT}\left[\frac{b}{1} - \frac{\kappa}{1 + \kappa a}\right] \qquad (2)$$

and ΔZ is the change in the valence produced by ion binding, b the radius of the sphere, a the distance of the closest approach of small ions to the macroion, and κ the Debye–Hückel reciprocal length of the ionic atmosphere.

It has been reported that the degree of ion binding obtained from this method is consistent with the results of other methods, that is, degree of ion binding obtained from measurements of ionic activities or membrane equilibrium,[5–7] and also from comparison of the experimental potentiometric titration curves with Linderstrom–Lang's equation.[8,9] However, the applicability of this interpretation to *all* cases has been questioned by Nagasawa and Holtzer.[10] They pointed out that such small pH changes can occur without real ion binding since the protein molecule does not have a uniformly smeared charge but has a discrete charge assembly. For the purpose, they quoted a theoretical study of Tanford and Kirkwood[11,12] who computed the electrostatic potentials of spherical particles having various fixed conformations of discrete distributions of positively and negatively charged groups, and showed that the effect of charges on the ionization constant of a group does not always vanish even at zero net charge, but that addition of a neutral salt to isoionic solution would shield dissociating groups from the influence of other charges so that the pH of the isoionic solution is expected to change when a neutral salt is added, even in the absence of specific ion binding. Concerning the deviation of observed potentiometric titration curves from Linderstrom–Lang's theory, which is also explained by postulating ion binding, Nagasawa and Holtzer pointed out that this deviation might as easily stem from the Debye–Hückel approximation used in Linderstrom–Lang's theory as from the presence of ion binding. This analysis thus reveals a serious ambiguity in the method of interpretation of isoionic pH changes.

(5) G. Scatchard, J. S. Coleman, and A. L. Shen, *J. Am. Chem. Soc.*, 79, 12 (1957).
(6) G. Scatchard, I. H. Scheinberg, and S. H. Armstrong, *ibid.*, 72, 535, 541 (1950).
(7) G. Scatchard, Y. V. Wu, and A. L. Shen, *ibid.*, 81, 6095 (1959).
(8) Y. Nozaki, L. G. Bunville, and C. Tanford, *ibid.*, 81, 5523 (1959).
(9) C. Tanford, "Electrochemistry in Biology and Medicine," T. Shedlovsky, Ed., John Wiley & Sons, Inc., New York, N. Y., 1955, p 248.
(10) M. Nagasawa and A. Holtzer, *J. Am. Chem. Soc.*, 86, 531 (1964).
(11) C. Tanford and J. G. Kirkwood, *ibid.*, 79, 5333 (1957).
(12) C. Tanford, *ibid.*, 79, 5340 (1957).

One of the conclusions reached by Nagasawa and Holtzer was that as the concentration of an added salt increases, the apparent dissociation constants of ionizable groups on a protein molecule must approach their intrinsic dissociation constants, and the pH shift (sometimes positive and sometimes negative) of isoionic solutions can be reasonably interpreted from these changes in their apparent ionization constants. To show that all ionization constants of ionizable groups actually approach their intrinsic values as the concentration of added salt increases, however, they quoted the results of studies of various proteins,[4,5,8] some of which, it seemed, might not be sufficiently reliable for the purpose.

To answer this question more clearly, therefore, it seemed necessary to measure the ionization constant of an ionizable group on several proteins as functions of ionic strength and to compare them with each other. Moreover, it seemed pertinent for this purpose to compare the results on proteins with those for a series of polyampholytes with the same species of positively and negatively charged groups in varying composition ratios. In the present work, we therefore determined the ionization constant of the carboxylic acid groups of β-lactoglobulin, bovine serum albumin, and human serum albumin as well as of a series of synthetic polyampholytes which have only carboxyl and dimethylamino groups. It is concluded that the pH shift of isoionic solution observed when a neutral salt is added *cannot always* be considered to give the degree of ion binding.

However, it is to be noted that we do not deny that *some* ions may bind to proteins, but merely point out that there is another effect that can alter the experimental findings in a similar way, making it impossible, in general, to assess this binding.

Experimental Section

Samples. The protein samples were crystallized bovine serum albumin (Lot B70411), crystallized human serum albumin (Lot 23), and three times crystallized β-lactoglobulin (Lot 35) of Pentex Inc., Kankakee, Ill. About 1 g of each protein was dissolved into 100 ml of deionized distilled water, and the solution was centrifuged at 15,000 rpm for 45 min to remove the particulate matter. The solution was deionized by passing through a mixed-bed ion-exchange resin, Amberlite MB-1. It was found that isoionic β-lactoglobulin is not very soluble but is salted in by addition of a slight amount of NaCl, while both isoionic serum albumins are completely soluble. The potentiometric titration of the protein solution was carried out in 0.500 *M* NaCl solution using 0.500 *M* HCl and NaOH solutions delivered with a microburet. An example of the potentiometric titration curves is shown in Figure 1. The number of carboxylic acid groups and amino groups in a molecule of each protein and, hence, the degree of dissociation of carboxylic acid groups at the isoionic point was determined from the potentiometric titration data of the protein by using the method of Tanford.[9]

The amount of H^+ bound on the protein molecule is given as the difference between the added amount of HCl and the amount of HCl remaining free in the solution; the latter can be calculated from pH values assuming that the activity coefficient of hydrogen ion is not affected by the presence of charged protein. If we denote the number of moles of H^+ bound by x and the number of moles of free H^+ by y, there must be the following relationship between x, y, and the molar concentrations of COO^- and COOH of the protein at the isoionic point (A and B in Figure 1)

$$\frac{y[1 - (x + B)/(A + B)]}{V[(x + B)/(A + B)]} = \frac{(H^+)(COO^-)}{(COOH)} = K \qquad (3)$$

where K is the ionization constant for ionization of COOH and V is the volume of the solution. This expression can be rewritten as

$$x = A - KV[(x + B)/y] \qquad (4)$$

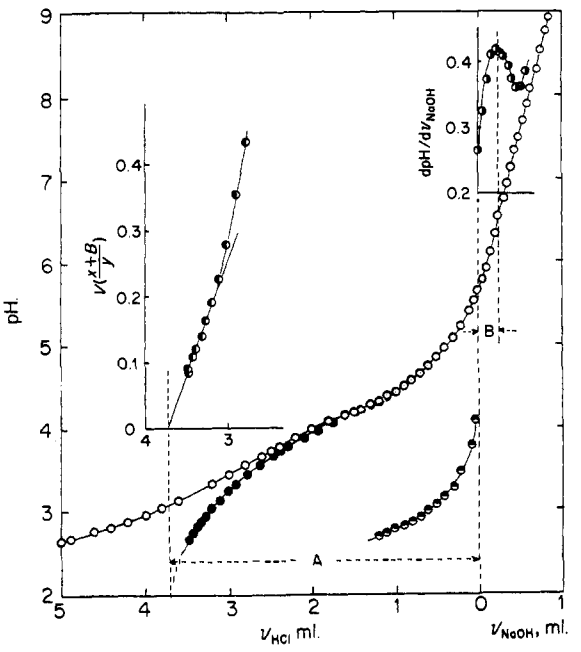

Figure 1. An example of potentiometric titration curves of human serum albumin. Concentration of protein is about 0.5%. Both concentrations of HCl and NaOH solutions are 0.500 *M*. Ionic strength is 0.500 *M* NaCl. The volume of the initial sample solution is 30 ml. The open circles denote the plot of pH *vs.* volume of 0.05 *M* HCl added, while the filled circles denote the plot of pH *vs.* amount of H^+ (expressed as volume of 0.05 *M* HCl) bound to protein. The upper-filled circles denote the plot of pH *vs.* volume of 0.05 *M* HCl added in a blank experiment. The left-filled circles are plotted according to eq 4. $A + B$ gives the total amount of COOH of the protein, while A gives the amount of COO^- at the isoionic point.

since it is believed that K does not change much with addition of HCl at the end of titration if the ionic strength is high. The maximum amount of bound H^+, A, can be obtained by linear extrapolation to zero $KV[(x + B)/y]$ of a plot of x *vs.* $KV[(x + B)/y]$. The degrees of ionization of the carboxyl groups of β-lactoglobulin and bovine serum albumin at their isoionic points, $A/(A + B)$, thus determined are summarized in Table I. The results agree with the data of Tanford, *et al.*[8,13]

Polyampholytes used here are copolymers of methacrylic acid and 2-dimethylaminoethyl methacrylate. Glacial methacrylic acid, which was kindly supplied from Mitsubishi Rayon Co. Ltd., and 2-dimethylaminoethyl methacrylate obtained from Bordon Chem. Co., were distilled at reduced pressure under nitrogen gas immediately before use. The former boiled at 38–38.2° and the latter at 17–18° (2–3 mm). Copolymerization was carried out in ampoules at 60° using absolute methanol as solvent and 2,2-azobis(butyronitrile) as initiator in almost the same way as previously reported by Doty and Ehrlich.[14] The degree of conversion was about 10–20%. The compositions of the polyampholytes obtained are shown in Table I.

Methods of purification of the samples were varied with the different compositions of the samples because of their different solubilities. Samples 1 and 2 were purified by repeated precipitation from methanol solution with acetone. Then, they were again dissolved in water and passed through a mixed-bed ion-exchange column of Amberlite IR 120 and 400 to obtain isoionic solutions of the samples. Samples 4–9 were insoluble in any solvent usually used. Therefore, the samples which were precipitated during the polymerization process were washed with methanol, acetone, and ether successively and dried. Then, they were dissolved in dilute hydrochloric acid solutions. Since they are coagulated if passed through mixed-bed ion-exchange resin columns, the mixture of Amberlite IR 120 and 400 was added to the sample solutions with stirring to remove excess HCl. This process was repeated until Cl^- was not

(13) C. Tanford, S. A. Swanson, and W. S. Shore, *J. Am. Chem. Soc.*, 77, 6414 (1955).
(14) G. Ehrlich and P. Doty, *ibid.*, 76, 3764 (1954).

Table I

Protein no.	Sample names	Isoionic pH	Degree of ionization of COOH at isoionic point
1	Human serum albumin	5.5_0	0.93_9
2	Bovine serum albumin	5.3_0	0.94_0
3	β-Lactoglobulin	5.2_6	0.76_5
4	No. 1 + HCl	4.85[a]	(0.89_6)
Poly-ampholyte no.	**COOH/N (mole ratio)**		
1	18.8/81.1	8.7_6	0.22[c]
2	46.4/53.7	7.5_7	0.92–0.91[c]
3	54.2/45.8	5.5_2	0.80_3
4	57.7/42.3	5.2_7	0.73
5	65.6/34.4	5.1_4	0.52_4
6	70.1/29.9	4.9_5	0.41_7
7	81.5/18.5	4.7_6	0.26
8	85.2/14.8	4.6_8	0.17_4
9	94.6/5.4	4.5_0	0.05_7
10[b]	70.1/29.9	(3.6_6)	(0.42_0)
11[b]	87.9/12.1	(3.5_8)	(0.14_3)
12	No. 9 + HCl	(3.2_6)	(0.02_1)

[a] At 1% solution. [b] These samples contain a slight amount of HCl. The degree of ionization of COOH of these samples as well as of no. 12 and of protein no. 4 are the values measured when these samples are dissolved for measurements. [c] These values are the degree of ionization of amino group.

detected by adding $AgNO_3$ solution. The isoionic solutions thus made were found to remain as milk-white solutions.

The compositions of copolymers as well as the degrees of ionization of carboxylic acid groups and amino groups at the isoionic point were also determined from the potentiometric titration curves. An example of the potentiometric titration curves for polyampholytes was shown previously.[10]

The molecular weights of the polyampholyte samples were not determined, because it is believed, from analogy with the potentiometric titration behavior of linear polyelectrolytes, that the pH of a polyampholyte solution does not vary with molecular weight except in the case of extremely low molecular weight, and also because this work is not concerned with a quantitative discussion of the pH change. The intrinsic viscosity of sample 3 in 0.1 *M* KCl solution was 0.86, and those of the other samples were not very low either, so all samples were indeed polymeric.

Sodium chloride used was the analytical grade of Fisher Chem. Co., while potassium chloride was recrystallized three times from that of special grade of Katayama Chem. Co. All solutions were prepared with conductivity water, which was made by passing ordinary distilled water through a mixed-bed ion-exchange resin column.

Measurements of pH. The measurements of pH of the protein solutions were carried out at 25 ± 2° with a Radiometer M4 instrument, while the pH's of the polyampholyte solutions were measured at 25 ± 0.1° in an atmosphere of nitrogen gas with Beckman glass electrode and a pH meter of Horiba P type, whose sensitivity was ±0.002 pH. The pH standards used were NBS pH standards or Beckman pH standards. The salt molarities used here were from 0 to 0.5 *M*. Salt concentrations were brought to the desired values by addition of measured volumes of concentrated NaCl or KCl solutions. The concentrations of the sample solutions were about 1% for proteins and from 0.4 to 3% for polyampholytes. The isoionic pH is almost independent of the sample concentration.[4]

The pH of a solution of isoionic human serum albumin (No. 4) was adjusted to be 4.85 with HCl to be compared with the data of Scatchard and Black.[4]

Results

The pH changes observed when NaCl or KCl is added to isoionic solutions of the proteins or polyampholytes are plotted against the square root of the salt concentrations in Figures 2 and 3. In both protein

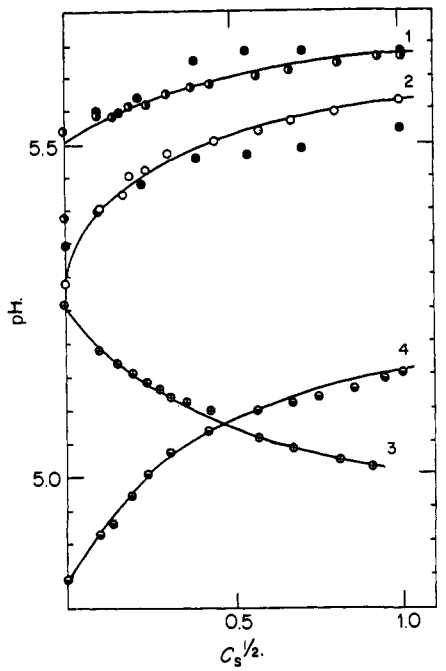

Figure 2. The changes in isoionic pH of proteins with addition of NaCl. The numbers show the sample numbers in Table I. The filled circles show the data obtained for the solutions prepared separately, while other circles show the data obtained by adding a concentrated NaCl solution to the sample solution consecutively with a microburet.

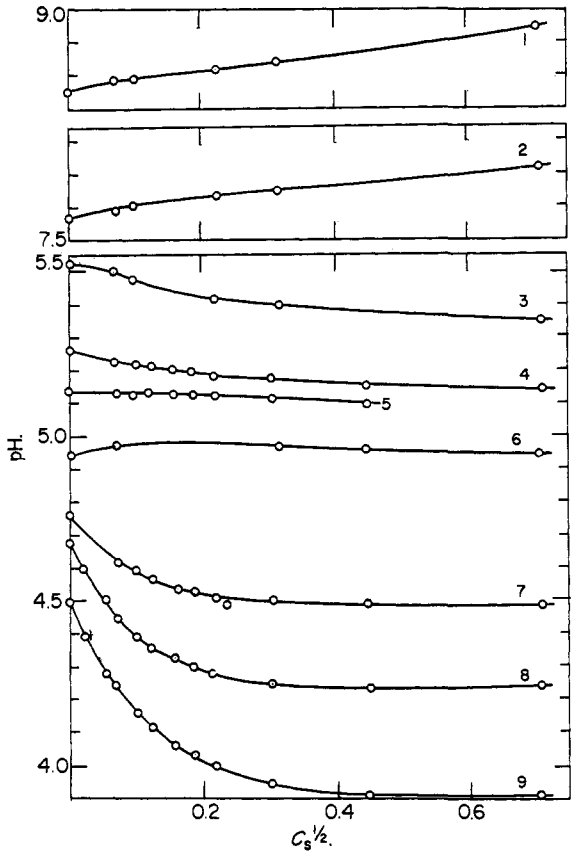

Figure 3. The changes in isoionic pH of polyampholytes with addition of KCl. The numbers show the sample numbers in Table I.

and polyampholyte solutions, the samples having the higher content of carboxylic acid group show the lower

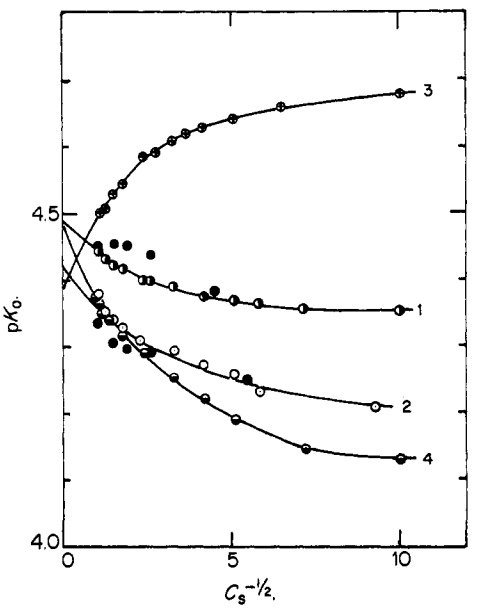

Figure 4. The changes in the apparent ionization constants, pK_0, of COOH of proteins with addition of NaCl. The data correspond to the data in Figure 2.

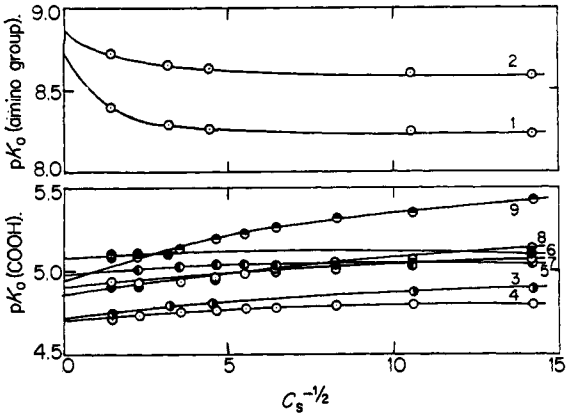

Figure 5. The changes in the apparent ionization constant, pK_0, of COOH or amino group with addition of KCl. These data correspond to the data in Figure 3.

isoionic pH, though in the literature the isoionic pH of bovine serum albumin is found to be lower than that of β-lactoglobulin.[4,5,8] Moreover, the pH of the sample solutions of higher content of amino groups is increased with increase of ionic strength, whereas those of the samples having the higher content of carboxylic acid group are decreased.

If we define the apparent dissociation constant of an ionizable group at the isoionic point by

$$pK_0 = pH_I + \log[(1-\alpha_I)/\alpha_I] \qquad (5)$$

where α_I is the degree of ionization of the group and pH_I the pH at the isoionic point, pK_0 of COOH on the proteins is changed as a function of ionic strength as shown in Figure 4 and pK_0 of COOH and amino group on the polyampholytes are as shown in Figure 5. It is observed that pK_0 of COOH differs with different proteins and polyampholytes but all data converge to their intrinsic values as the ionic strength increases.

Discussion

The changes in isoionic pH with addition of neutral salts can be interpreted if we assume that chloride ion is bound on HSA and BSA as well as on the polyampholyte samples 1 and 2 since pH increases with increase of ionic strength, whereas sodium or potassium ion must be assumed to be bound on other molecules since the pH decreases with increase of ionic strength. However, if this assumption were correct, the pK_0's of all samples would be the same at zero ionic strength, because there is no net charge and no ion binding on the molecules at the isoionic point; at the higher salt concentrations the value should diverge. This conclusion is contradicted by the experimental results (Figures 4 and 5).

As is stated in the introductory section, the wanted analysis of such data includes the assumption that the charges on the molecules are uniformly smeared out. In general, the degree of dissociation α of an ionizable group on proteins or polyelectrolytes is related to the pH of the solution by [10,15]

$$\mathrm{pH} = pK_0^0 - \log[(1-\alpha)/\alpha] = 0.434e\psi/kT \quad (6)$$

or

$$pK_0 = pK_0^0 - 0.434e\psi/kT \quad (7)$$

where pK_0^0 is the intrinsic ionization constant of the group, e the absolute magnitude of the electronic charge, and ψ is the electrostatic potential at the position from which protons are dissociated. If we consider the dissociation of an ionizable group, say COOH, eq 6 must be satisfied with pK_0^0 and α of COOH and also with the value of ψ at the position of COOH. The assumption of smeared charge means that ψ would always be zero if the solution is isoionic, since there is no net charge on the molecule. Actually, however, the charges are discretely situated on the molecule. Even though the net charge of the molecule is zero at the isoionic state, the electrostatic potential at the position of the ionizable group is not always zero because of the interaction between point charges. Therefore, it is natural that pK_0 differs with different samples even at their isoionic points.

If ψ is not zero, the magnitude of the potential always decreases as a neutral salt is added, approaching the intrinsic dissociation constant of the group, *i.e.*, $pK_0 = pK_0^0$ at infinite ionic strength. In the case of a methacrylic acid group, pK_0^0 is believed to be in the neighborhood of 4.75,[15] while in the case of proteins, pK_0^0 of COOH is believed to be about 4.6.[16] This speculation agrees well with the present experimental results.

In the solutions of the polyampholyte samples 1 and 2, in which the amino group content is larger than the acid group content, pK_0 of COOH cannot be estimated since $\alpha_{COOH} = 1$, but, instead, pK_0 of the amino group can be estimated. It is observed in Figure 5 that pK_0 of the amino group asymptotically approaches the intrinsic dissociation constant of dimethylamino groups which is considered to be about 9.

One of the ambiguities in this work may be that samples 4–9 are not completely soluble at their isoionic states but become milk-white suspensions. Even when the molecules are soluble in the isoionic state, the molecules are tightly coiled.[14] For the purpose of quantitative discussion of the pH shift, the shapes of the molecules in isoionic solution would certainly have to be clarified. For the purpose of this work, however, it is our opinion that our ignorance of the precise shape of molecule is not important. Another ambiguity may be that the isoionic pH's of the polyampholyte samples 6–9 are lower than those of the proteins, so that the net charges of the polyampholytes may have some effect on the isoionic pH changes with ionic strength shown in Figure 5. At least, however, even those experimental results are not in contradiction with the present conclusions.

Thus, it is concluded that if the discrete charge model is adopted in place of the smeared charge model, the change in pH with addition of a neutral salt can be interpreted qualitatively without assuming specific ion binding. Moreover, the discrete charge model can explain the change in pK_0 with ionic strength, which the more conventional interpretation cannot. Although the situation in proteins is surely very complicated, it is certain that the pH shift of isoionic solution observed when a neutral salt is added *cannot always* be considered to give the degree of ion binding. However, it should once more be stressed that certain ions are surely bound to proteins; in that case the degree of ion binding may be estimated from the pH shift.

Acknowledgment. We wish to thank Professor Alfred Holtzer for his constant interest in this work and for critically reading this manuscript. We also wish to thank Mr. Y. Kato and Mr. M. Nakayama for their assistance in experiments.

(15) M. Nagasawa, T. Murase, and K. Kondo, *J. Phys. Chem.*, **69**, 4005 (1965).

(16) See ref 2, pp 534–536.