Lapuk, V. K., Chistyakova, L. A., and Kravchenko, N. A. (1968), *Anal. Biochem. 24*, 80.
Leaver, I. H., and Ramsay, G. C. (1969), *Photochem. Photobiol. 9*, 531.
Luse, R. A., and McLaren, D. (1963), *Photochem. Photobiol. 2*, 343.
Marshall, G. R. (1967), *Advan. Exp. Med. Biol. 2*, 48.
Milligan, B., Holt, L. A., and Caldwell, J. B. (1971), *J. Appl. Polym. Sci.* (in press).
O'Donnell, I. J., and Thompson, E. O. P. (1964), *Aust. J. Biol. Sci. 17*, 963.
Opienska-Blauth, J., Charezinski, M., and Berbec, H. (1963), *Anal. Biochem. 6*, 69.
Savige, W. E. (1971), *Aust. J. Chem. 24*, 1285.
Teale, F. W. J. (1959), *Biochim. Biophys. Acta 35*, 543.
Uphaus, R., Grossweiner, L., Katz, J., and Kopple, K. (1959), *Science 129*, 641.
Weil, L. (1965), *Arch. Biochem. Biophys. 110*, 57.
Weil, L., James, S., and Buchert, A. R. (1953), *Arch. Biochem. Biophys. 46*, 266.
Yoshida, Z., and Kato, M. (1954), *J. Amer. Chem. Soc. 76*, 311.

Electrostatic Methods for Measuring the Binding of Ionic Ligands to Proteins*

Clarke J. Halfman and Jacinto Steinhardt

ABSTRACT: The ΔpH method of Scatchard and Black has been used successfully to measure the binding of small ions to proteins. However, the method has been shown to fail in measuring quantitatively the binding of large asymmetric organic ions to bovine serum albumin. The value of w, the electrostatic interaction parameter, for long-chain ligands, differs from its value for smaller or more symmetrical inorganic ions, and is subject to large uncertainties. We now present two methods for determining the interactions of ionic ligands with proteins based on the electrostatic effect of the bound ligand on the hydrogen ion equilibria of the protein. Unlike the ΔpH method, neither depends upon a known value for w nor upon a known relationship between the equivalents of ligand bound and the measured prototropic response. Dependence upon an uncertain theoretical relationship, or determination of an empirical stoichiometric relationship between the measured response and the equivalents of ligand bound, is avoided by obtaining data at two or more protein concentrations. One of the methods uses ΔpH as the measured response to the association of ligand with protein. The response measured in the other method is the equivalents of acid or base which must be added to maintain a constant pH as binding occurs. Values of molal ratios bound and free-ligand concentrations result. The results obtained with both methods agree with those obtained with the familiar standard thermodynamic methods.

Hydrogen ion equilibria of the ionizable groups of proteins are influenced by the net charge of the protein. A theoretical relationship (Tanford, 1961) describing this is

$$\mathrm{pH} = \mathrm{p}K_a - \log (h/n - h) - 0.87wZ \qquad (1)$$

where h is the number of protonated acid groups out of a total of n with dissociation constant K_a; Z is the net charge of the protein; and w is an electrostatic interaction factor which according to the Debye–Huckel theory is given by

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

in dilute solutions, where e is the protonic charge, D is the solvent dielectric constant, k is Boltzmann's constant, T is the absolute temperature, a and b are the distance of closest approach and the radius of the central ion, respectively, and κ has its usual meaning in the Debye theory. The validity of eq 1 and its implications have been repeatedly established from its successful use in analyzing the titration curves of many proteins (Steinhardt and Reynolds, 1969; Steinhardt and Beychok, 1964).

A modification of the equation has also been used to measure the binding of inorganic ions to proteins (Scatchard and Black, 1949). Upon binding charged ligand the net charge of the protein changes by ΔZ, where ΔZ is the charge of the ligand. No change occurs in $\mathrm{p}K_a$ and, if the protein is not too dilute, only a negligible change occurs in $h/n - h$ except at strongly acidic or basic pH. Thus a pH change will be observed which is related to the binding, *i.e.*

$$\Delta Z = \bar{\nu} = \Delta\mathrm{pH}/0.87w \qquad (2)$$

where $\bar{\nu}$ is the mole ratio of bound ligand.

The reliability of the ΔpH method for measuring the binding of organic ions to proteins has recently been examined (Cassel and Steinhardt, 1966). It appeared that eq 2 was not valid for the binding of large asymmetrical organic ions since results obtained with the ΔpH method did not agree with results obtained by more definitive techniques such as equilibrium dialysis and measurements of potentials across permselective membranes. Moreover, it was obvious that for ions of high affinity at the protein concentration employed (0.1% bovine

* From the Department of Chemistry, Georgetown University, Washington, D. C. 20007. This work was supported by National Science Foundation Grant GB 13391.

serum albumin) the ratio of bound to total ligand was not much less than unity, thus necessitating impracticably high accuracy in measurements of pH, ligand, and protein concentrations (involving protein molecular weight) to obtain from the total ligand and ΔpH the very low concentration of free ligand.

The present investigation provides a means for eliminating the need for a known relationship between $\bar{v}$ and ΔpH by acquiring data at two or more protein concentrations, as described previously for binding-induced physical property changes in general (Halfman, 1970). Results are obtained from solutions sufficiently dilute to provide that the fraction of bound to total lignad is significantly less than one. The ΔpH method, as modified here, nevertheless requires protein concentrations high enough so that the change in pH from binding charged ligand does not depend on protein concentration.

A relationship between $\bar{v}$ and Δh (the equivalents of hydrogen ion which must be added to the protein to maintain constant pH) may be derived from eq 1. This relationship is not concentration dependent. We may write for uncombined protein

$$pH_0 = pK_a - \log (h_0/n - h_0) - 0.87wZ_0$$

As Z_0 changes to Z the pH is maintained constant by the addition of Δh equivalents of acid; then

$$pH_0 = pK_a - \log (h/n - h) - 0.87wZ$$

therefore

$$0.87w(Z - Z_0) = 0.87w\Delta Z = \log (h_0/n - h_0) - \log (h/n - h)$$

Since $\Delta Z = \Delta h - \bar{v}$, for negatively charged ligand, it follows that

$$\bar{v} = \Delta h + \frac{1}{0.87w} \times [\log (h/n - h) - \log (h_0/n - h_0)]$$

If the binding study is performed at a pH at which the ionization of only one class of groups having the same intrinsic pK need be considered, then, $\Delta h = h - h_0$, and

$$\bar{v} = \Delta h + \frac{1}{0.87w} \log \left[\frac{h_0 + \Delta h}{n - (h_0 + \Delta h)} \frac{n - h_0}{h_0} \right] \quad (3)$$

Equation 3 shows that upon binding $\bar{v}$ equivalents of negatively charged ligand at constant pH, the protein will acquire Δh equivalents of hydrogen ion. In an unbuffered solution $\bar{v}$ may be determined from the equivalents of hydrogen ion which must be added to maintain the original pH.

However, eq 3 should not be relied upon for determining $\bar{v}$, since the assumptions involved in its derivation may not be applicable to the binding of large organic ligands to proteins. The equation itself serves only to illustrate the basic principle by which a measurable response related to the binding is provided. Results are computed from plots of Δh against ratio of ligand to protein acquired at at least two protein concentrations.[1] It is unnecessary to evaluate or know the value of w since, as will be shown, $\bar{v}$ is computed from an equation (eq 4) which does not contain w. Application of this "Δh method" is not theoretically limited to measurements with protein concentration above a certain minimum as is the ΔpH method.

Materials and Methods

Distilled, deionized water was used for the preparation of all aqueous solutions. The resin used in the deionizing column was Bio-Rad AG-501-X-8. Inorganic compounds were of reagent grade and used without further purification. Proteins were obtained from Nutritional Biochemicals Corp.; bovine serum albumin (hereafter simply called albumin) was a lyophilized, crystallized preparation (lot 9385); β-lactoglobulin was lot 8438. Protein solutions of desired concentration were prepared from 1 to 2% stock solutions which had been deionized and concentrations determined from $OD^{1\%}_{1\,cm} = 6.67$ (Sterman, 1955) and a molecular weight of 69,000 for albumin, and $OD^{1\%}_{1\,cm} = 9.4$ (Nozaki *et al.*, 1959) and a molecular weight of 37,500 (Townend, 1961) for β-lactoglobulin. The alkyl sulfates were specially prepared and purified products from Mann Research Laboratories, Inc.

The automatic titrating and pH-measuring apparatus (Radiometer) consisted of a type TTTlc titrator, automatic syringe drives (type SBUls), and a type SBR2c Titrigraph recorder. Metrohm and Sargeant combination glass electrodes were used. The syringes delivered 0.5 ml. The titration vessel, containing 8 ml of protein solution, was held at a constant temperature of 25 ± 0.05° by circulating water through the jacket. In the ΔpH experiments the equipment was operated as a titrator with ligand added from a single syringe. The pH-Stat mode was used for the Δh experiments, with acid added from one syringe connected to the equipment in the usual manner while a second syringe, coupled to the chart drive of the recorder, delivered ligand to the protein solution in the titration vessel at a constant rate determined by the chart speed. The x axis of the recorder could thus be calibrated in number of equivalents of ligand added.

Permselective membrane-emf measurements were performed as described previously (Cassel and Steinhardt, 1969) with a cell constructed according to the design of Scatchard (Scatchard *et al.*, 1959). A membrane (A-60, from American Machine and Foundry), selectively permeable to anions, provided the junction between the sample and reference compartments. Each compartment was connected by 1 M KCl liquid junctions to calomel electrodes connected to a Radiometer pH meter. The meter was read on the pH scale, thus directly indicating the log of the ratio of the anion concentrations on each side of the membrane. With 10^{-2} M octyl sulfate in the reference compartment, concentrations of octyl sulfate could be determined with an accuracy of approximately ±5% down to somewhat less than 10^{-5} M.

The equilibrium dialysis procedure has been described previously (Ray *et al.*, 1966), as has the methylene blue assay for unbound detergents (Mukurjee, 1956).

Results

The ΔpH results presented here, unlike those of earlier investigators (Scatchard and Black, 1949; Cassel and Stein-

[1] The actual relationship between ligand bound and the measured response (ΔpH or Δh) may be determined empirically at a high enough protein concentration to assure stoichiometric binding. Such a stoichiometric plot give the empirial value of w. However this procedure, which is not always possible, is unnecessary.

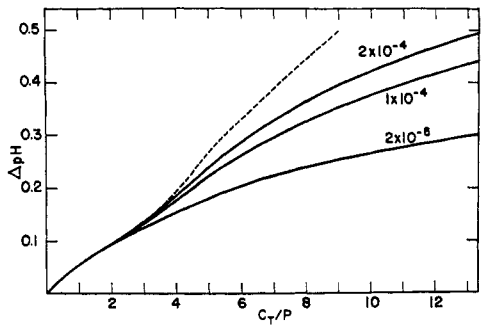

FIGURE 1: Titration of albumin with octyl sulfate in 0.001 M NaCl. The initial pH was 5.35. The solid lines are the recorded titration curves at the protein concentrations indicated. The dashed line is the stoichiometric curve computed from eq 4.

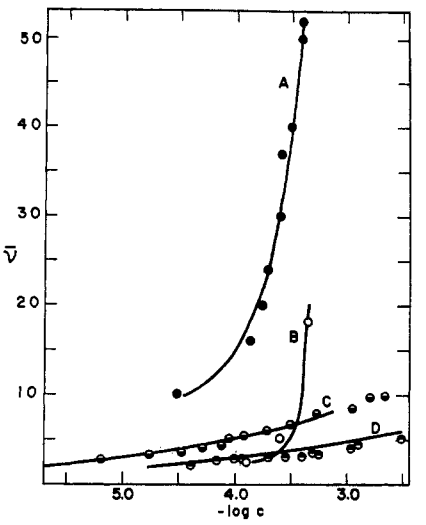

FIGURE 2: Comparison of binding data for several ligand-protein systems obtained by different techniques. (A) Dodecyl sulfate–albumin; the solid line curve represents results obtained by the Δh technique, at a pH of 5.6 in 0.033 M NaCl, from the data of Figure 5; (●) equilibrium dialysis results from Reynolds *et al.* (1967), in pH 5.6 phosphate buffer (μ = 0.033) at 2°. (B) Dodecyl sulfate–β-lactoglobulin; the solid-line curve represents results obtained by the Δh technique at a pH of 6 in 0.01 M NaCl; (○) equilibrium dialysis results at pH 6.0 in 0.01 M sodium phosphate buffer. (C) Octyl sulfate–albumin; the solid line curve represents results obtained by the Δh technique at a pH of 5.6 and by the ΔpH method at an initial pH of 5.35, both in 0.001 M NaCl; (◒) results obtained by the permselective membrane-emf technique for the isoionic protein. (D) Hexyl sulfate–albumin; the solid line curve represents results obtained by the ΔpH method at a pH_0 of 5.35 in 0.001 M NaCl; (◓) results obtained by the permselective emf technique for the isoionic protein (Cassel, 1968).

hardt, 1969), were analyzed without evaluating w, by making use of plots of equivalents of total ligand added (C_t/P) *vs.* ΔpH, at several protein concentrations. Such data were acquired by titrating albumin with octyl sulfate at three concentrations (Figure 1). Each value of ΔpH corresponds to a certain value of $\bar{\nu}$, which is not necessarily the theoretical value predicted by eq 2; for each value of $\bar{\nu}$ there is a corresponding concentration of free ligand (c). Since the value of ΔpH and c corresponding to a certain value of $\bar{\nu}$ should be independent of protein concentration, we may equate two expressions for c

$$c = P_i[(C_t/P)_i - \bar{\nu}] = P_j[(C_t/P)_j - \bar{\nu}]$$

where $(C_t/P)_i$ and $(C_t/P)_j$ are the total equivalents of ligand added at protein concentrations P_i and P_j, respectively, to attain a certain value of the measured response corresponding to a certain value of $\bar{\nu}$. From this equality it immediately follows (Halfman, 1970) that

$$\bar{\nu} = \frac{(C_t/P)_i - \frac{P_j}{P_i}(C_t/P)_j}{1 - P_j/P_i} \qquad (4)$$

Equation 4 may be used to compute binding results from data acquired at two or more protein concentrations even when the relationship between the measured response and $\bar{\nu}$ is unknown. Results computed from the application of eq 4 to the data of Figure 1 for the binding of octyl sulfate and results obtained in a like manner for the binding of hexyl sulfate are in agreement with results obtained by the permselective membrane-emf technique (Figure 2). Neither an empirically determined nor a theoretically computed relationship between $\bar{\nu}$ and ΔpH is used with this procedure. The experimental relationship between $\bar{\nu}$ and ΔpH (Figure 1, dashed curve) is, indeed, not linear and $0.87w$, calculated from the initial and final slope, is 0.055, compared to a value of 0.078 calculated from the Debye–Hückel theory at this ionic strength. Empirical values of w obtained in binding studies of proteins with long-chain ligands are substantially lower than those given by the Debye–Hückel theory (Cassel and Steinhardt, 1969).

To use eq 4 it is necessary to employ protein concentrations low enough to assure that the fraction of bound to total ligand is significantly less than one. For this reason the ΔpH method is limited to the study of ligands of relatively low affinity, since the response to binding, ΔpH, will become markedly dependent on protein concentration at low concentrations. The lower practical limit depends on the initial pH (see Appendix). A plot of the dependence of relative error, $\bar{\nu}_{obsd}/\bar{\nu}_{true}$ (where $\bar{\nu}_{obsd}$ would be obtained from ΔpH according to eq 2 and $\bar{\nu}_{true}$ is obtained from eq 5), on protein concentration is given in Figure 3. An error of less than 10% is obtained only when the protein concentrations are above 2×10^{-5} M at pH 5, 1×10^{-5} M at pH 6, and 10^{-6} M at pH 7. At pH 5.6 solutions of albumin more concentrated than about 0.1% are required.

Since the pH is maintained at a constant value in the Δh method, described by eq 3, low protein concentrations may be employed without introducing errors due to changes in ionization of the protein. Figure 4 is a plot of eq 3 with n_{COOH} = 50, pK_{COOH} = 4.3 (Vijai and Foster, 1967), and w = 0.045 (the theoretical value at this ionic strength) at an ionic strength of 0.033 and a pH of 5.6. Also included in Figure 4 is $\bar{\nu}$ *vs.* Δh, computed from the data of Figure 5 by eq 4, for the binding of dodecyl sulfate to albumin. Disagreement between the theoretical and experimental plot as in the ΔpH response shows that eq 3, used by itself, does not describe the influence of charged long-chain ligand on the hydrogen ion equilibria of the protein sufficiently well to be used to measure the binding. However, eq 3 illustrates how the electrostatic charge of the bound ligand provides a measurable response to the binding, *i.e.*, the equivalents of hydrogen ion necessary to maintain a constant pH.

Results were obtained from the Δh response caused by the addition of dodecyl sulfate to albumin at several protein concentrations (Figure 5) by computation from eq 4; they are in

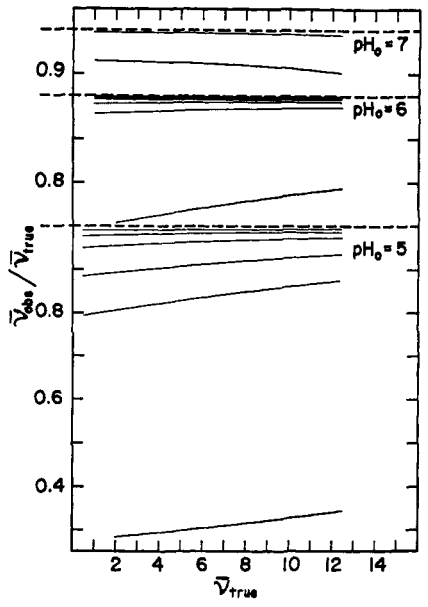

FIGURE 3: Relative error in measuring binding by the ΔpH method as a function of protein concentration and initial pH, determined from $\bar{\nu}_{obsd} = \Delta pH/0.87W$ and $\bar{\nu}_{true}$ from eq 5. At pH_0 of 5.0 and 6.0 the curves correspond to the following protein concentrations, in order of increasing error: 2×10^{-4}, 10^{-4}, 5×10^{-5}, 2×10^{-5}, 1×10^{-5}, and 10^{-6} M. For pH_0 of 7.0 the curves correspond to protein concentrations of 10^{-5} and 10^{-6} M.

agreement with results (Reynolds *et al.*, 1967) obtained by equilibrium dialysis (Figure 2). Results were also obtained in a like manner from the Δh response for the binding of octyl sulfate to albumin; they are in agreement with those obtained by the permselective membrane-emf technique (Figure 2). Results obtained for the binding of dodecyl sulfate to β-lactoglobulin by this Δh method (Figure 2) are in agreement with equilibrium dialysis results.

Discussion

Binding isotherms were obtained from the influence of the presence of charged ligands on the hydrogen ion equilibria of proteins. Binding results calculated from both experimental Δh and ΔpH responses agreed with those obtained from equilibrium dialysis and permselective membrane-emf measurements (Figure 2). However, when electrostatic forces contribute to the binding or when the binding is pH dependent for any other reason, isotherms obtained by the two methods may not agree. Thus in the Δh method and in equilibrium dialysis (performed in the presence of a buffer) the ionization state of titratable groups of the protein is altered as the charge of the protein is changed by binding charged ligand when the pH is held at a constant value; the net change in charge is not equal to the equivalents of bound ligand. On the other hand, in the ΔpH method and in the permselective membrane-emf technique (no buffer present except the protein) the ionization state of the protein is not appreciably altered as the pH changes, except at very dilute protein concentrations; the net change in charge of the protein is equal to $\bar{\nu}$.

The experimental influence of charged ligand on the hydrogen ion equilibria of albumin is not exactly that predicted by the Scatchard-Black relationship. Thus, the binding of octyl sulfate in the absence of buffer does not cause a consistent linear change in pH (Figure 1); a reduced rise in pH occurred

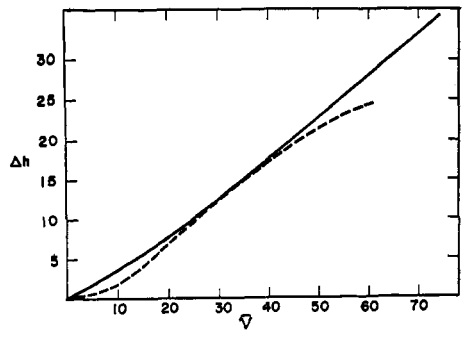

FIGURE 4: Relationship between $\bar{\nu}$ and Δh for anion binding to albumin at pH 5.6 and ionic strength 0.033. The solid line curve is a theoretical plot of eq 3; the parameters used are given in the text. The dashed curve is the experimental relationship computed from the data of Figure 5.

between the binding of 1 and 5 equiv. Likewise, binding of approximately the first 10 equiv of dodecyl sulfate required fewer equivalents of acid than theoretically predicted to maintain a constant pH. The hydrocarbon chain of the ligands may alter the pK_a of ionizable groups in the vicinity of the binding sites, or bound chloride ions may be released as the long-chain anions are bound. An influence of the hydrocarbon chain of organic ligands on the pK_a of ionizable groups is suggested by the observations that the binding of long-chain alcohols to albumin slightly increases the acidity of albumin (Ray *et al.*, 1966) and that the binding of lysolecithin (zwitterionic, no net charge) shifts the titration curve of albumin to lower values of pH (Halfman, 1970).

Although the theoretical (Scatchard and Black) electrostatic influence of charged ligands on the hydrogen ion equilibria of the proteins is not precisely followed, one may nevertheless obtain accurate binding isotherms by measuring the experimental response at several protein concentrations. Results are then computed from eq 4, which it will be noted does not contain w. Although an empirical response can be measured at successively dilute protein concentrations, one must have some understanding of the physical basis of the response so that its concentration dependence can be analyzed. The ΔpH response is an example of a binding induced physical property change which is concentration dependent (Figure 3) and is therefore useful for determining free-ligand concentrations only at protein concentrations greater than *ca.* 10^{-5} M (Figure 3). Application of the Δh method is not limited theoretically by a minimum protein concentration but is limited in practice by trace contaminants which buffer. Attempts to

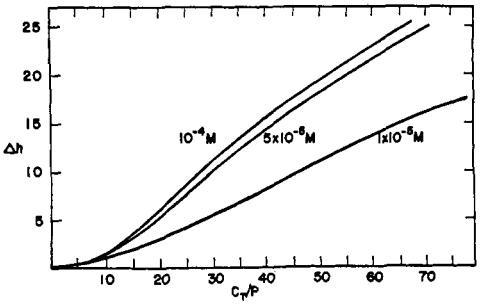

FIGURE 5: Response of the pH stat to the binding of dodecyl sulfate to albumin at a pH of 5.6 in 0.033 M NaCl at the indicated protein concentrations.

use protein concentrations lower than 2×10^{-6} M have therefore met with little success. With albumin–dodecyl sulfate the low response of Δh to the binding (Figure 5) in the low $\bar{v}$ region of the isotherm compounded the difficulties of measuring free-ligand concentrations below approximately 3×10^{-5} M. The isotherms obtained therefore for dodecyl sulfate binding to albumin do not extend reliably to values of $\bar{v}$ below about 10. The simple and convenient methods presented in the present paper will find their widest use in measuring the regions of binding isotherms at values of free-ligand concentration greater than 10^{-5} M.

Appendix

Part of the error at low protein concentrations in the ΔpH method is caused by a significant change in the degree of ionization of each type of ionizable group as the pH changes, so that instead of the simple linear relationship between acquired charge and ΔpH (eq 2), the following will be true

$$\Delta\text{pH} = 0.87w(\bar{v} - \epsilon\Delta h_i) + \log\left[\frac{h_i^0}{n_i - h_i^0}\frac{n - h_i}{h_i}\right] \quad (5)$$

where h_i^0 is the number of protonated species of a class of ionizable groups, i, at the initial pH and h_i is the number of protonated species after a pH change of ΔpH. The error in $\bar{v}_{obsd}$ from assuming the validity of eq 1 compared to $\bar{v}_{true}$ in eq 5 may be estimated on the basis of the following considerations. At pH values below 7 only changes in carboxyl and imidazole protonation contribute to $\Sigma\Delta h_i$. The product, $[h_i^0/(n_i - h_i^0)] \times [(n_i - h_i)/h_i]$, will be the same for whatever groups are considered. At any protein concentration a number of equivalents of base or acid may be added to attain any desired initial pH. At this initial pH the degree of ionization of any class of groups will be independent of protein concentration. However, the equivalents of acid or base necessary to attain this pH will depend on protein concentration. Letting B/P equal the equivalents of acid or base added, plus the sum of all the charged groups on the protein, except for carboxylate (COO^-) and imidazolium (N^+)

$$B/P = ((\text{COO}^-) - (\text{N}^+))_0 - \left(\frac{(\text{H}^+) - (\text{OH}^-)}{P}\right)_0$$

$(COO^-)_0$ and $(N^+)_0$ are determined from

$$(\text{COO}^-)_0 = n/(1 + 10\text{p}K_1 - \text{pH}_0 - 0.87wZ_0)$$

$$(\text{N}^+)_0 = n_2/(1 + 10\text{pH} - \text{p}K_2 + 0.87wZ_0)$$

Z_0 may be estimated from published titration curves. After the pH change occurs by binding charged ligand

$$\Delta h_i = ((\text{N}^+) - (\text{COO}^-)) - ((\text{N}^+) - (\text{COO}^-))_0$$

$$= -\left[B/P + \left(\frac{(\text{H}^+) - (\text{OH}^-)}{P}\right)\right] + \left[B/P + \left(\frac{(\text{H}^+) - (\text{OH}^-)}{P}\right)\right]_0$$

$$= \left(\frac{(\text{H}^+) - (\text{OH}^-)}{P}\right)_0 - \left(\frac{(\text{H}^+) - (\text{OH}^-)}{P}\right) \quad (6)$$

Also since

$$\text{pH}_0 = \text{p}K_1 - \log\left(\frac{n_1 - (\text{COO}^-)}{(\text{COO}^-)}\right)_0 - 0.87wZ_0$$

$$= \text{p}K_2 - \log\left(\frac{(\text{N}^+)}{n_2 - (\text{N}^+)}\right)_0 - 0.87wZ_0$$

$$\text{pH} = \text{p}K_1 - \log\left(\frac{n_1 - (\text{COO}^-)}{(\text{COO}^-)}\right) - 0.87wZ$$

$$= \text{p}K_2 - \log\left(\frac{(\text{N}^+)}{n_2 - (\text{N}^+)}\right) - 0.87wZ$$

therefore

$$\text{pH} - \text{pH}_0 = \Delta\text{pH} = \log\left(\frac{n_1 - (\text{COO}^-)}{(\text{COO}^-)}\right)_0\left(\frac{(\text{COO}^-)}{n_1 - (\text{COO}^-)}\right) - 0.87w(Z - Z_0)$$

$$= \log\left(\frac{(\text{N}^+)}{n_2 - (\text{N}^+)}\right)_0\left(\frac{n_2 - (\text{N}^+)}{(\text{N}^+)}\right) - 0.87wZ(Z - Z_0)$$

$$\left(\frac{(\text{N}^+)}{n_2 - (\text{N}^+)}\right)_0\left(\frac{n_2 - (\text{N}^+)}{(\text{N}^+)}\right) = \left(\frac{n_1 - (\text{COO}^-)}{(\text{COO}^-)}\right)_0\left(\frac{(\text{COO}^-)}{n_1 - (\text{COO}^-)}\right)$$

Since

$$\left(\frac{(\text{N}^+)}{n_2 - (\text{N}^+)}\right)_0 = \frac{(\text{H}^+)}{K_2} \times 10^{-0.87wZ_0}$$

$$\left(\frac{n_1 - (\text{COO}^-)}{(\text{COO}^-)}\right)_0 = \frac{(\text{H}^+)}{K_1} \times 10^{-0.87wZ_0}$$

therefore

$$\left(\frac{(\text{N}^+)}{n_2 - (\text{N}^+)}\right)\Big/\left(\frac{n_1 - (\text{COO}^-)}{(\text{COO}^-)}\right)_0 = \frac{K_1}{K_2}$$

$$\left(\frac{n_1 - (\text{COO}^-)}{(\text{COO}^-)}\right) = \frac{K_1}{K_2} \times \left(\frac{(\text{N}^+)}{n_2 - (\text{N}^+)}\right)$$

Since

$$(\text{N}^+) = (\text{COO}^-) - \frac{B}{P} - \frac{(\text{H}^+) - (\text{OH}^-)}{P}$$

and $\alpha = (\text{COO}^-)/n_1$, a quadratic, eq 7, may be developed in $1 - \alpha$.

$$\left(\frac{K_1}{K_2} - 1\right)(1 - \alpha)^2 + \left[(1 + a) - \left(a - \frac{n_2}{n_1}\right)\frac{K_1}{K_2}\right](1 - \alpha) - a = 0 \quad (7)$$

where

$$a = \frac{1}{n_1}\left(n_1 - \frac{B}{P} - \frac{(H^+) - (OH^-)}{P}\right)$$

After determining $1 - \alpha$, and consequently α, from eq 7 and $\Sigma\Delta h_i$ from eq 6, eq 5 may be solved for $\bar{v}$ from which the influence of protein concentration on the results obtained by the ΔpH method may be estimated (Figure 3).

References

Cassel, J. M., and Steinhardt, J. (1969), *Biochemistry 8*, 2603.

Halfman, C. J. (1970), Ph.D. Thesis, University of Illinois.

Mukerjee, P. (1956), *Anal. Chem. 28*, 870.

Nozaki, Y., Bunville, L. G., and Tanford, C. (1959), *J. Amer. Chem. Soc. 81*, 5523.

Ray, A., Reynolds, J. A., Polet, H., and Steinhardt, J. (1966), *Biochemistry 5*, 2606.

Reynolds, J., Herbert, S., Polet, H., and Steinhardt, J. (1967), *Biochemistry 6*, 937.

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

Scatchard, G., Wu, Y. U., and Shen, A. L. (1959), *J. Amer. Chem. Soc. 81*, 6104.

Steinhardt, J., and Beychok, S. (1964), *Proteins 2*, 139.

Steinhardt, J., and Reynolds, J. (1969), Multiple Equilibria in Proteins, New York, N. Y., Academic Press, Chapter 5.

Sterman, M. D. (1955), Ph.D. Thesis, Purdue University.

Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, Chapter 7.

Townend, R. (1961), *J. Amer. Chem. Soc. 83*, 1419.

Vijai, K. K., and Foster, J. F. (1967), *Biochemistry 6*, 1152.

Structure–Volume Relationships for Proteins. Comparative Dilatometric Study of Acid–Base Reactions of Lysozyme and Ovalbumin in Water and Denaturing Media*

Sam Katz† and Jane E. Miller

ABSTRACT: The sign, magnitude, and the time dependence of the volume changes produced by the reaction of proteins with acids and bases are determined by the nature of the reactive species, medium, temperature, composition, and structural organization of the protein. Dilatometric analysis was used to determine the magnitude of the volume changes produced by the reaction of lysozyme and ovalbumin with HCl and NaOH in water and denaturing media. The volume increase which resulted from the reaction of lysozyme with hydroxyl ion in 8 M urea was about 85% of that produced by the same process in water. These data indicate that only minor structural changes were produced by this treatment. The volume changes produced by the protonation of lysozyme in 8 M urea were substantially lower than those predicted from model studies, whereas the volume effects in 6 M guanidine hydrochloride were larger than anticipated. From these data, it is apparent that the degree of denaturation in the two media differ substantially, a conclusion which is in accord with other physical measurements. The time dependence and magnitude of the volume isotherms for ovalbumin reacting with H^+ and OH^- both in water and in denaturants differ substantially from lysozyme. Lysozyme reached steady-state values in all media immediately upon mixing, while ovalbumin, reacting with H^+ or OH^- in 8 M urea, exhibited time-dependent volume effects. The initial rapid volume increases are associated with the ionic reactions involved in neutralization, while the slower volume decreases are related to the formation of molecular aggregates. The singular volume effects produced by ovalbumin at elevated pH in 8 M urea are explicable in terms of the titration of tyrosyl residues which are "masked" in the native protein but which are normalized in this medium.

This study was to determine whether proteins would produce volume changes characteristic of their composition and structural organization upon reaction with acids and bases in water and denaturing media. If so, this could provide another technique for determining the presence of certain types of structural organization in proteins. This approach could be used to study such phenomena as the normalizing of "buried" ionic groups, conformational changes, enzyme–inhibitor interactions, etc. The analysis of the volume changes resulting from the reaction of acids and bases with the appropriate prototropic groups in protein is facilitated by the existence of comparable data for organic acids and bases in water (Weber, 1930; Kauzmann *et al.*, 1962) and in denaturing media (Katz and Miller, 1971).

Lysozyme and ovalbumin, proteins which incorporate 30–35% helical structure and some β structure (Jirgenson, 1969), were selected because of their different response to urea and guanidine hydrochloride. Lysozyme, which contains four disulfide bonds, shows little structural alteration upon exposure to 9 M urea (Steiner, 1964; Warren and Gordon, 1970) but

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† To whom to address correspondence.