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Limitations Inherent in the ΔpH Method of Determining Binding Isotherms of Bovine Serum Albumin*

James M. Cassel† and Jacinto Steinhardt

ABSTRACT: Binding isotherms, determined by the ΔpH method of Scatchard and Black, of a large variety of aliphatic and aromatic anions, containing up to 14 carbon atoms, have been compared with isotherms obtained with the same substances by equilibrium dialysis and in two instances by measurement of potentials across permselective membranes. With symmetric nondeformable ions, *e.g.*, aromatic anions, the ΔpH method gives results in good agreement with those determined by dialysis. With aliphatic long-chain anions, the amount of binding and the binding constants are systematically underestimated by factors which increase with the affinity. In these comparisons, due regard has been taken of (a) the effects of the necessary differences in the amounts of competing electrolyte present when each of the three methods is used, and (b) need to correct for the lack of buffering capacity in very dilute solutions of unbuffered protein. Two methods for evaluation of an empirical electrostatic factor, ω, from the ΔpH binding measurements in 0.001 M NaCl are discussed. Explanation of the much lower ω so computed from that calculated by application of Debye–Hückel theory is sought in terms of applicability of the Linderström-Lang model to noncovalently bound long-chain ions with hydrophobic tails, and of differences between the effective charge Z of the macroion and the charge computed stoichiometrically. It is shown that as a practical matter the ΔpH method cannot be applied to any ligand of high affinity ($K > 10^5$) because of the large effect in this method of small errors in determining concentration or protein molecular weight.

The binding of small ligands to proteins has most often been measured by equilibrium dialysis, a laborious and time-consuming method which precludes any possibility of measuring amounts bound soon after mixing. If the ligand of interest is ionic in nature, *i.e.*, carries a charge, the free-ligand concentration (and hence the binding) may be estimated by electromotive force measurements with electrodes specific for the ligand (Scatchard *et al.*, 1950) or by permselective membrane techniques (Carr, 1955; Scatchard *et al.*, 1957, 1959; Baker and Saroff, 1965). These methods, where applicable, have been shown to give very reliable results. However, electrodes specifically reversible to ligands such as organic anions are rare and have been reported for only a relatively small number of simple ions, and for no highly asymmetric ones. Relatively short-time binding information may be obtained by other methods (Klotz, 1953; Steinhardt and Reynolds, 1969) such as depolarization of fluorescence (Laurence, 1952; Weber, 1952) and gel filtration (Hummel and Dreyer, 1962; Fairclough and Fruton, 1966), but they have not been used for routine and rapid analysis of a large number of protein–ligand systems.

As a part of an extensive study of the interaction of a number of proteins with a series of highly purified ligands, including long-chain anionic detergents, we have examined (a) the reliability, accuracy, and limits of applicability of the ΔpH method for measuring ion binding to bovine serum albumin; and (b) the utility and reliability of the permselective electromotive force method with large asymmetric anions (*i.e.*, octyl sulfate and dodecyl sulfate).

In 1941, Steinhardt showed that changes in position on the pH coordinate of the titration curves of wool fibers obtained with a series of strong acids reflected differences in the tendency of the protein to combine with the respective anions. In 1949, Scatchard and Black demonstrated that the binding of small inorganic anions to serum albumin could be computed from the observed shift in pH produced by addition of ligand as a neutral salt to a system buffered solely by protein. The binding of K^+ to lactoglobulin (Basch and Timasheff, 1967) and of K^+ and Na^+ to α_s-casein (Ho and Waugh, 1965) has been measured by this technique (hereafter referred to as the ΔpH method). It has also been applied in a study of the interaction of several inorganic salts with myosin (Ghosh and Mihalyi, 1952).

In addition to the long-chain ligands, we have examined binding of a number of aromatic anions by the ΔpH method. For all the ligands reported here, we include for comparison binding measurements on the same substances by equilibrium dialysis. Since Scatchard and Black (1949) achieved less satis-

* From the Department of Chemistry, Georgetown University, Washington, D. C. 20007, and the National Bureau of Standards, Washington, D. C. 20234. *Received January 28, 1969.* This work was supported by Grant GM 12085 from the U. S. Public Health Service to J. S. It includes material from the Ph.D. dissertation of J. M. C.

† Polymers Division, Institute for Materials Research, National Bureau of Standards, Washington, D. C.

factory results with protein solutions initially outside the isoionic region, we have restricted our investigation to this region.

Experimental Section[1]

Materials. Solutions of crystalline bovine serum albumin (Nutritional Biochemicals Inc.) were deionized by passage through a mixed-bed ion-exchange column (Bio-Rad AG 501-X8, 20–50 mesh). A small amount of resin (Dowex 50-X8) in the acid form constituted the base of the column as a trap for any resin decomposition products (Dintzis, 1952). The deionizing procedure is known to remove most of the fatty acid present in the original material (analysis has shown an average of 1 equiv/mole of deionized bovine serum albumin (J. Gallagher, 1968, personal communication)). The pH of 0.1% bovine serum albumin solutions prepared in this manner varied between 5.25 and 5.30. Protein stock solutions were refrigerated and used within 2 weeks.

Highly purified sodium salts of alkyl sulfates and -sulfonates were used (Mann Research Laboratories; see Reynolds *et al.*, 1967). Highly pure octyl and dodecyl sulfate received as a gift from the Chemicals Division, DuPont, were found to give results similar to those determined with the Mann products. All aromatic salts were Eastman Organic Chemicals.

Soluble-absorbing components of the membranes (Visking) used in equilibrium dialysis were removed by boiling in and rinsing with distilled water prior to storing for several days at 2° in large volumes of distilled water.

Methods. pH measurements were made in a water-jacketed reaction vessel. The latter was prepared from the female portion of a 45–50 standard taper joint so that a glass-mixing volume of up to 15 ml was surrounded by circulating water at 25°. Phthalate buffer was used to standardize the pH meter (Radiometer with expanded scale) prior to each pH measurement. The calomel electrode solution and salt bridge contained 1 M KCl, the liquid junction being made through a sealed-in porous pin. The usual procedure was to transfer an aliquot of deionized bovine serum albumin solution to the reaction vessel, provide a gentle flow of purified dry N_2 across the solution surface and slight stirring of the solution with a magnetic stirring bar, and record the pH before and after addition of an aliquot of solution containing ligand as neutral salt. The latter solutions were prepared with deionized water. All points on graphs of ΔpH isotherms represent averages of at least two measurements.

Aromatic ligands received as acids were converted into their sodium salts with NaOH. Concentrations of solutions of nonaromatic salts were determined by weighing and diluting stock solutions volumetrically. Concentrations of aromatic ligand solutions were determined spectroscopically.

The procedure followed in the equilibrium dialysis measurements has been described previously (Cassel *et al.*, 1969).

The cell used in the permselective membrane measurement is a replica of one used by Scatchard *et al.* (1959) who have described its operation. In our work, the anion-selective membrane (A-104B, a quarternary ammonium type made by American Machine and Foundry) was preequilibrated with a solution of ligand.

Potentials across permselective membranes were determined by a pH meter with expanded scale. The solution on one side of the membrane contained protein and ligand and the other side contained ligand alone at approximately equal free ligand concentration. Free ligand concentrations on the protein side of the membrane were calculated from calibration plots of EF/RT *vs.* log C_2 obtained by varying C_2 at constant C_1 (C_2 and C_1 refer to concentrations on opposite sides of the membrane, E is the measured electromotive force, F the Faraday, and T the absolute temperature). The slope of this calibration curve indicated that almost 100% selectivity for anions prevailed. With hexylsulfonate, the slope was 10–15% less than theoretical (2.303 RT/F) independently of the concentration taken for C_1.

Results and Discussion

The ΔpH method of Scatchard and Black rests on the Linderström-Lang titration equation

$$\mathrm{pH} = \mathrm{p}K_{\mathrm{int}} + \log \frac{\bar{r}}{n - r} - 0.87w\bar{z} \qquad (1)$$

where K_{int} is the intrinsic dissociation constant of a group attached to an uncharged protein molecule when it is not subject to either statistical effects (numerous identical groups) or to electrical interactions, n is the number of proton binding sites having the same K_{int} of which $\bar{r}$ do not contain protons, w is an electrostatic interaction term, and $\bar{z}$ is the average net charge of the protein. If $\bar{r}/(n - \bar{r})$ is not changed and the protein is initially at the isoionic point ($\bar{z} = 0$), addition of ligand causes a change in pH, ΔpH, given by

$$\mathrm{pH} = 0.87w\bar{z} = 0.87w\bar{\nu} \qquad (2)$$

where $\bar{\nu}$ is defined as the moles of ligand bound per mole of protein. In the usual Linderström-Lang treatment, w is evaluated according to the Debye–Hückel theory by means of the equation

$$w = \frac{\epsilon^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right) \qquad (3)$$

where the protein molecule is assumed spherical with radius b. D is the dielectric constant, kT the Boltzman energy term, and κ is an ionic strength dependent function, inversely proportional to the thickness of the Debye–Hückel ionic atmosphere. The term a expresses the distance of closest approach of the center of the macroion to any of the surrounding smaller ions.

The validity of applying eq 2 to anion binding rests on the following. (1) The protein is sufficiently concentrated so that $\bar{r}/n - \bar{r}$ remains essentially constant as the pH changes by the amount ΔpH. This condition is only marginally met by the 0.1% bovine serum albumin solutions used and corrections must therefore be calculated. (2) Only one anion binds. Supporting electrolyte which may be required to suppress Donnan inequalities in the vicinity of the protein ions and to avoid depletion of ionic strength by almost stoichiometric binding (so that $1/\kappa$ does not become absurdly large) requires calculation

[1] "Certain commercial items are identified in this paper in order to specify adequately the experimental procedures. In no case does such identification imply recommendation or endorsement by the National Bureau of Standards."

TABLE I: State of Carboxyl and Imidazole Dissociation in Isoionic 0.1 and 1.0% Bovine Serum Albumin at 25°.

Carboxyl Dissociation[a]					
$(1-\alpha)C$ RCOOH	$\rightleftarrows$	αC $RCOO^-$	+	αC H^+	
[bovine serum albumin]		$[RCOO^-]$		[RCOOH]	$\frac{[RCOO^-]}{RCOOH]} = \frac{\alpha}{1-\alpha}$	α
1.0% (1.5×10^{-4} M)		86.25×10^{-4}		3.75×10^{-4}	22.98	0.958
0.1% (1.5×10^{-5} M)		89.60×10^{-5}		4.05×10^{-6}	221.22	0.9955
Imidazole Dissociation[b]					
$(1-\alpha)C$ RN^+H	$\rightleftarrows$	αC RN	+	αC H^+		
[bovine serum albumin]		[RN]		$[RN^+H]$	$\frac{[RN]}{[RN^+H]} = \frac{\alpha}{1-\alpha}$	α
1% (1.5×10^{-4} M)		1.20×10^{-4}		1.38×10^{-3}	0.087	0.080
0.1% (1.5×10^{-5} M)		3.46×10^{-5}		1.15×10^{-4}	0.300	0.231

[a] Based on 60 carboxyls, pK = 4.3 (Vijai and Foster, 1967). [b] Based on 10 imidazoles, pK = 6.5 (Decker and Foster, 1967).

and allowance for competitive effects. (3) Interaction between macroions is assumed negligible. (4) the Linderström-Lang equation is applicable and w is constant with respect to z, *i.e.*, protein conformation does not change with binding. It is not necessary to assume that the physical model that leads to the Linderström-Lang equation (sphere with homogeneous surface charge) applies to the binding of anions, but only that it applies to the binding of hydrogen ions, which has been amply demonstrated (Tanford, 1962, 1963; Steinhardt and Beychok, 1964). (5) The pH is only affected by the interaction of the ionic portion of the bound ligand with the protein, *i.e.*, the protein is not affected by the nonionic portion of the ligand in such a way as to affect the ionization constants which govern the pH. It is considered that this condition is met in the very dilute solution used here.

To take care of the first condition listed above, we have established the dissociation state of isoionic 0.1% bovine serum albumin by specifying that 10 of the 17 histidines of bovine serum albumin dissociate with a pK = 6.5 (Decker and Foster, 1967) and that 60 of the 100 carboxyls are available for titration in the native state, having a pK of 4.3 (Vijai and Foster, 1967). In a 1% bovine serum albumin solution of 0.1 M in NaCl, there is 0.8 unprotonated imidazole (Decker and Foster, 1967) per molecule. For 1.0% bovine serum albumin isoionic solutions *in water* we have used protonated to nonprotonated ratios of 2.5/57.5 and 9.2/0.8 for carboxyl and imidazole, respectively. Using the Ostwald dilution relationship that a tenfold change in concentration of dissociating acids should give a corresponding change in the relation $\alpha^2/1-\alpha$ where α is the fraction of dissociated groups, we have computed the state of ionization in isoionic 0.1% bovine serum albumin and tabulated these results in Table I. Since a change in pH accompanying the binding of an anion (or cation) must be reflected in an identical per cent change of both carboxyl and imidazole $\alpha/(1-\alpha)$ terms, a relation can be obtained between ΔpH_{exptl}, the concurrent changes in the $\alpha/(1-\alpha)$ terms, and a positive ΔpH correction to be added to ΔpH_{exptl}. Table II is a tabulation of such calculations.

In Figure 1, the binding isotherms of four aromatic anionic ligands as determined by both dialysis and ΔpH measurement are shown. C_{eq}, determined directly in the dialysis measurement, is computed in the ΔpH experiment as the difference between total ligand, C_T, and ligand calculated to be bound ($\bar{\nu}C_p$, where C_p is protein concentration). Except for the binding data at low $\bar{\nu}$ values for the rather high-affinity dinitro-1-naphtholate, the two methods give results which are in very satisfactory agreement. Equally good agreement between the two methods was found with 2,5-dichlorobenzenesulfonate, 2-naphthalenesulfonate, 2-anthraquinonesulfonate, and 2,4,-6-trinitrocresolate, despite the presence in the dialysis experiment of buffer ions and their absence in the ΔpH measurements. The association constant, K, thus does not depend strongly upon ionic strength.

The binding of alkyl sulfates and -sulfonates as determined

TABLE II: Correction of ΔpH_{exptl} for Variable $\alpha/(1-\alpha)$ Due to $\alpha/(1-\alpha)$ Factor.[a]

ΔpH_{exptl}	$\Delta\alpha/(1-\alpha)$ (%)	$(\alpha/(1-\alpha))_I$[b] Imidazole	Carboxyl	ΔpH[c]
1.70	15	0.255	169	0.142
1.10	14	0.258	171	0.131
0.95	13.5	0.2595	172	0.127
0.73	12.5	0.2625	174	0.116
0.45	10	0.270	179	0.092
0.17	5	0.285	189	0.044
0.08	2.5	0.2925	194	0.022
0	0	0.300	199	0

[a] Calculated for 0.1% bovine serum albumin solution. [b] $\alpha/(1-\alpha)$ ratio at pH_{exptl} of system. [c] A positive correction to be added to ΔpH_{exptl} is computed by log $(X/0.300)(y)/(199)$, where X and y are $(\alpha/(1-\alpha)_I$ values for imidazole and carboxyl at pH of system.

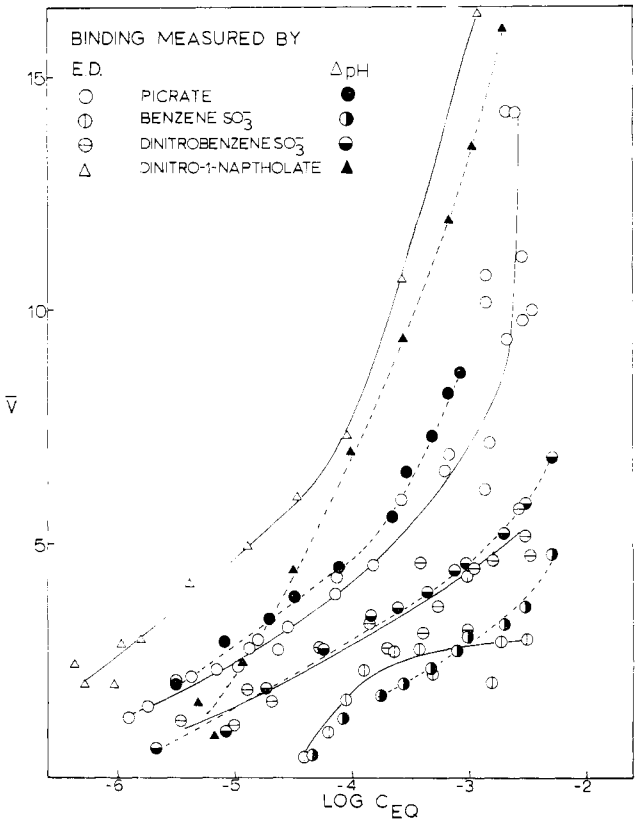

FIGURE 1: The binding of aromatic anionic ligands to bovine serum albumin at 25° as calculated from dialysis and ΔpH measurements. Ordinate is $\bar{r}$, the molal binding ratio. Absissa is log C_{Eq}, the equilibrium concentration of ligand. Equilibrium dialysis measurements were made at 0.033 μ (phosphate, pH 5.6).

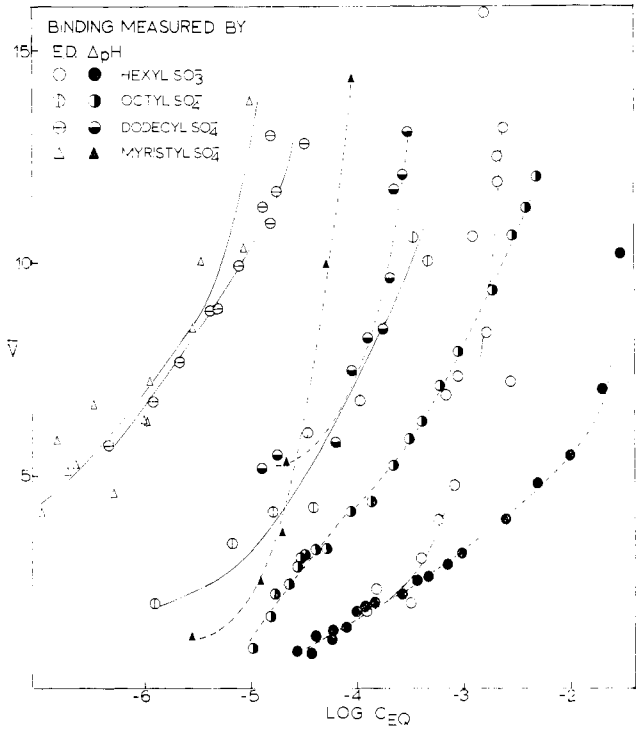

FIGURE 2: The binding of aliphatic anionic ligands to bovine serum albumin at 25° as calculated from dialysis and ΔpH measurements. Dialysis measurements same conditions as Figure 1.

by the same methods is given in Figure 2, and binding measurements on two of the ligands by the permselective membrane technique are shown in Figure 3. With each of the *long-chain* ligands the ΔpH method significantly underestimates the binding, the discrepancy being more marked for the higher affinity ligands. The permselective membrane measurements on octyl sulfate are in excellent agreement with the dialysis results. Those on hexylsulfonate agree with both ΔpH and dialysis at the low $\bar{\nu}$ range, but tend to fall midway between them at higher $\bar{\nu}$. Attempts to employ the permselective membrane methods with dodecyl sulfate were not successful.

Reciprocal plots ($1/\bar{\nu}$ *vs.* $1/C_{eq}$) of the calculated binding data of Figures 1 and 2 were used to obtain by extrapolation to $1/C_{eq} = 0$ values of the number of identical sites, and from the slope of the straight lines, $1/nK$, where K is the association constant for the ligand. In Figure 4, the log of K as determined by ΔpH measurements for a given ligand is plotted against its counterpart determined by equilibrium dialysis. Ligands for which the two methods give concordant results scatter along the solid line which represents a 1:1 relationship, while those in which K is significantly underestimated by the ΔpH method are displaced toward the lower right side of the figure.

The equilibrium dialysis measurements of some of the aliphatic ligands show that a very large fraction (>0.95) of the ligands in these systems is taken up by the protein. This results in the depletion of free electrolyte and values for $1/\kappa$ (the "ionic atmosphere thickness") which are large compared with the average distance between macroions. The applicability of the Debye–Hückel theory on which the Linderström-Lang equation rests is thus called into question for these cases. A neutral inorganic salt, the ions of which have been shown to have relatively small affinities for bovine serum albumin, was therefore added (0.001 *m* NaCl). Only one Cl^- is bound at all tightly (K = 2400), the others being bound with extremely low energies (K = 100) (Scatchard *et al.*, 1957). Therefore, while Cl^- might fill, on the average, 0.7 ligand site of the set of 5 to 9 available to long-chain ionic ligands (Reynolds *et al.*, 1967), competition for the other sites should be negligible.

Figure 5 shows the binding of myristyl sulfate as computed by ΔpH measurements in 0.001 M NaCl solutions of bovine serum albumin. The calculated binding isotherm still drastically underestimates the state of association of this ligand even after conditions for validity of the theory have been met.

An explanation for the discrepancies exhibited in Figure 5 may be sought in part in the interaction factor w. The factor used was computed from the Debye–Hückel theory for a macroion of radius 30 Å and a ligand of 2.5 Å, assuming that the protein could be treated as an impenetrable conducting sphere (with charge distributed uniformly on its surface) and with distance of closest approach calculated as the sum of the radii of the macroion and the support electrolyte ions.

Tanford *et al.* (1955) have shown that the empirically determined w from titration data on this protein is substantially smaller, *e.g.*, 20% smaller at μ = 0.01. An empirical value of w may also be computed from our ΔpH measurements by at least two procedures. The first involves knowledge of n and K, the number of and the association constant, respectively, for the high-energy myristyl sulfate binding sites. These are

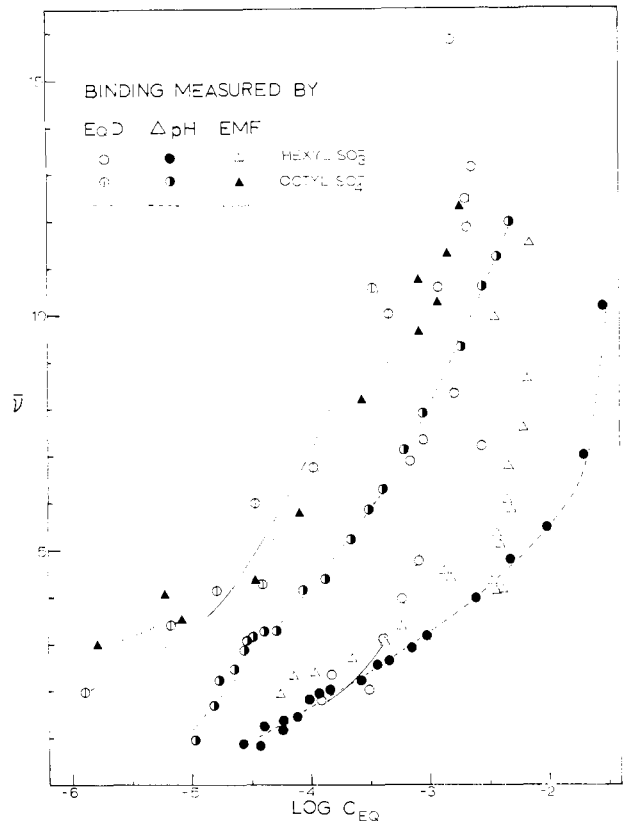

FIGURE 3: The binding of hexylsulfonate and octyl sulfate to bovine serum albumin at 25° as calculated from dialysis, ΔpH, and permselective membrane electromotive force.

obtained by analysis of the equilibrium dialysis data, using the relation

$$\bar{\nu} = \sum \bar{\nu}_i = \sum \frac{n_i K_i C_{eq}}{1 + K_i C_{eq}} \tag{4}$$

and restricting the experiment to concentrations of ligand (myristyl sulfate) sufficiently dilute that the binding of ligand may be interpreted in terms of one set of binding sites, so that

$$\bar{\nu} \cong \bar{\nu}_1 = \frac{n_1 K_1 C_{eq}}{1 + K_1 C_{eq}} \tag{5}$$

From eq 2

$$\frac{1.15\Delta pH}{w} = \bar{\nu} \text{ and } C_T - C_p\left(\frac{1.15\Delta pH}{w}\right) = C_{eq}$$

giving

$$\frac{1.15\Delta pH}{w} = \frac{nK\left[C_T - C_p\left(\frac{1.15\Delta pH}{w}\right)\right]}{1 + K\left[C_T - C_p\left(\frac{1.15\Delta pH}{w}\right)\right]} \tag{6}$$

This relationship contains only one unknown, if we employ values for n and K determined by the equilibrium dialysis

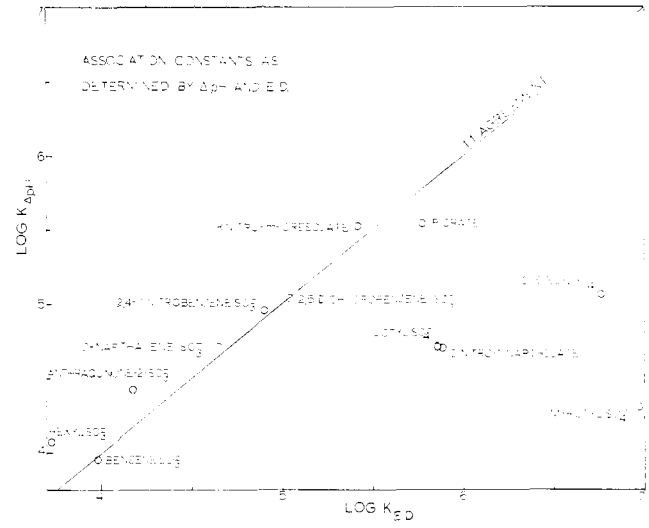

FIGURE 4: Log–log plot of association constants determined by ΔpH *vs.* those determined by equilibrium dialysis. Supporting electrolyte absent from all ΔpH measurements. Solid line represents 1:1 agreement between methods.

measurements.[2] Values of w calculated in this manner for each of the ΔpH measurements of Figure 5 range between 0.045 and 0.058 and average 0.049, hardly over half the w computed by the Debye–Hückel theory for the ideal system.

A more satisfactory method for extracting an empirical w from the ΔpH data of Figure 5, which does not require predetermined values for n and K, depends upon the fact that when the ligand has a high affinity, and is present to the extent of only a few equivalents per mole of protein, practically all the ligand exists in the bound state. Thus, when the molar content of total ligand does not exceed one-half the number of sites ($n/2$) times the mole concentration of protein, then $1/C_{eq} \geq K$ and the ratio of bound to total ligand is $\geq nKC_p/(2 + KC_p n)$. As long as nK exceeds $20/C_p$ or 1.3×10^6 (0.1% protein), the ratio of bound to total ligand is over 0.9. At lower values of $\bar{\nu}$, such as $0.2n$, or at higher protein concentration, this ratio approximates unity closely. As a limit, therefore, at the lowest values of $\bar{\nu}$

$$\Delta pH \cong -0.87 w C_T/C_p \tag{7}$$

The slope of a plot of ΔpH against C_T/C_p will then give -0.87 w from which w itself may be obtained. Such plots are quickly and easily obtained with automatic titrators using ordinary glass electrodes and titrating with ligand instead of with base. The ΔpH effect (rising pH as anionic ligand is bound) controls the operation of the titrator.

Figure 6 displays the myristyl sulfate ΔpH data plotted in this manner; a straight line results. The value for w is 0.043, in fairly good agreement with the value calculated algebraically above. Similar plots have resulted with data obtained with other ligands.

An inherent limitation in the ΔpH procedure is very clearly

[2] An alternative procedure is to assume only the value of n_1 and then by sets of simultaneous equations determine values of w for arbitrarily paired ΔpH measurements, taking the average of such w values as the empirical w.

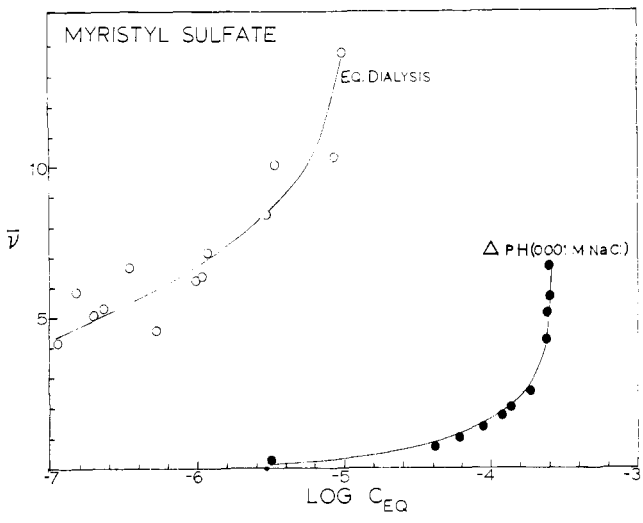

FIGURE 5: Apparent binding of myristyl sulfate to bovine serum albumin at 25° as computed from ΔpH measurements in 0.001 M NaCl solutions and from dialysis measurements. Dialysis conditions same as Figure 1.

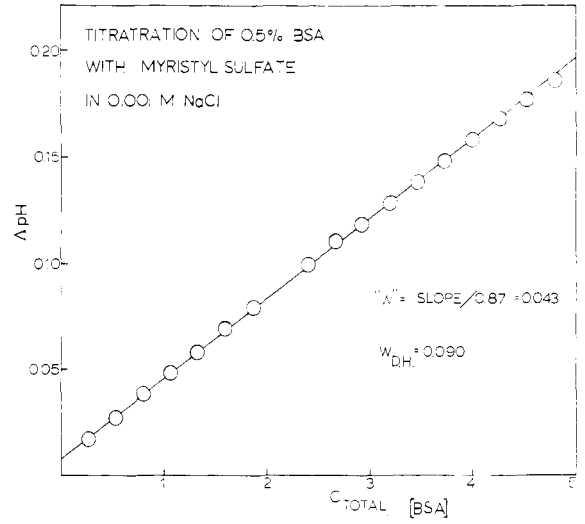

FIGURE 6: Titration of 0.5% bovine serum albumin with myristyl sulfate in 0.001 M NaCl.

demonstrated by these measurements. In this method unlike most others for measuring binding, the free ligand concentration is not determined directly but is calculated as the difference between total and bound ligand concentrations. When K is of the order of 10^6, as it is for these high-affinity ligands, an almost unattainable accuracy in both C_T and C_p is required for estimation of the difference in total and bound ligand concentrations since the difference, the free ligand, is such a small fraction of them. The estimation of C_p requires not only a very precise method of measuring protein concentration, but also a commensurately accurate protein *molecular weight*. The very fact that w can be determined empirically by the method just described illustrates the difficulty of determining K from these data by means of eq 2. The sensitivity to error of the method when applied to ligands of very high affinity, illustrated by the fact that if we employ the empirical value of w determined from the plot in Figure 6, the computed free ligand concentrations based on these ΔpH measurements are all zero or negative (the bound ligand concentration may appear to exceed the total ligand concentration by 3–10%), due to an accumulation of small errors.[3] Thus, the determination of an empirical w does not permit binding of high-affinity long-chain ligands to be measured by the ΔpH procedure. If it could be shown that the values of w obtained as above do not depend upon the anion nor on K, they could be used with ligands of lower affinity. This does not seem to be the case since "normal" values of w adequately describe the data obtained with anions of lower affinity (Figure 1).

Obviously application of the Debye–Hückel theory to protein–proton interactions tends to overestimate the electrostatic work required to remove a proton from these macroions. Reynolds *et al.* (1967) have questioned the validity of extending to protein–detergent ion association the assumption embodied in the usual theoretical derivation of w (see, for example, Tanford, 1963, or Steinhardt and Beychok, 1964), that the charge distribution on the macroion due to associated anions can be treated as continuous and long range, rather than as discrete points having the expected short-range effects only. The ΔpH method, however, depends only upon application of the w term to the dissociation of *hydrogen ion;* this applicability has been repeatedly demonstrated. Nevertheless, it appears that the w term may alter with the size or shape of associated anions, since the "normal" w has been shown to describe the binding isotherm of the more symmetrical aromatic anions (Figure 1).

Long-chain aliphatic ions are thus in a unique class with respect to properties affecting the Scatchard and Black method. It is firmly established that the Linderström-Lang equation, which contains an electrostatic interaction term, is needed to describe H^+ titration curves of protein, and that proteins also obey the Scatchard–Black equation in their binding of small or symmetrical anions. It is as firmly established that the titration curves with detergent anions of native bovine serum albumin do not require electrostatic corrections (Reynolds *et al.*, 1967, among others); in fact, the inclusion of such terms spoils any fit of the data. No paradox is involved. The conducting sphere model on which the Linderström-Lang equation is based may be a good approximation to reality where H^+ is concerned, because the H^+ ion fluctuates over all sites as a result of rapid interchange with its conjugate base, water, in the solvent. It is not certain that anions held strongly at a relatively small number of fixed sites *in the native protein*, and with no mechanism for rapid fluctuation available, will bind in accordance with the same model. This does not affect the validity of the Scatchard–Black method, since only H^+ ion dissociation are involved in it. Evidence to date appears to indicate that anions of high affinity, at least, widely spaced on the large protein surface, interact if at all only very weakly. The abnormally low value of w, however, is not the result of this situation, since the w value relates only to hydrogen ions. It is suggested that the low values are consequences of one or more of the following: (a) the charges are few and fixed in position, (b) they may be above the "surface" of the protein ion, rather than on it, owing to their hydrophobic tails, and (c)

[3] The results are negative because C_T rather than bound C was used in obtaining w.

because of the effect of counter ions the apparent low w actually represent a low (less than stoichiometric) $\bar{z}$.

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The Molecular Weights of the α Chains of Chicken Bone Collagen by High-Speed Sedimentation Equilibrium*

Elton P. Katz, Camille J. Francois, and Melvin J. Glimcher

ABSTRACT: High-speed sedimentation equilibrium studies were made of the $\alpha 1$, $\alpha 2$, and $\alpha 3$ chains of chicken bone collagen obtained by free-flow electrophoresis of a neutral pH, KSCN, and LiCl extract. All three components were found to be molecularly homogeneous. The molecular weights found were: $\alpha 1$, 92, 100; $\alpha 2$, 91, 200; and $\alpha 3$, 101,000. This gives a molecular weight of the chicken bone macromolecule of 284,000.

A model has been proposed, relating the unequal chain lengths for the α components of the chicken bone collagen macromolecule to certain of its distinctive physicochemical properties.

Although the molecular structure, macromolecular aggregation state, and amino acid composition of bone collagens are qualitatively similar to those of the collagens of unmineralized soft tissues (Eastoe, 1956; Piez and Likins, 1960; Glimcher and Krane, 1968), the collagen as it is organized in bone, possesses certain characteristic physicochemical properties which distinguish it from most of the soft tissue collagens (Glimcher and Katz, 1965; Glimcher *et al.*, 1965). For example, in contrast to the collagens of most other tissues, very little of the collagen of demineralized bone, even in young, rapidly growing animals, can be extracted in solutions normally used to extract the undenatured protein from unmineralized soft tissues (Glimcher and Katz, 1965). On the other hand, unlike the insoluble collagen of bovine Achilles tendon, for example, a large fraction of chicken bone collagen can be extracted as gelatin at neutral pH and in the cold by certain salts known to denature the protein (Glimcher and Katz, 1965). Chicken bone gelatin, like the gelatin obtained from the collagen of soft tissues, contains α components (the single-chain units making up the triple-stranded collagen molecules) as well as the higher molecular weight β-dimer and γ-trimer components formed by covalently cross-linking α chains (Glimcher and Katz, 1965; Francois and Glimcher, 1967a). However, the proportion of α chains in the chicken bone gelatin was found to be unusually high as compared with other normal tissue collagens, especially in the first extracts which

* From the Department of Orthopedic Surgery, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts. *Received September 24, 1968.* This study was aided by grants from the National Institutes of Health (AM-06375) and The John A. Hartford Foundation, Inc.