Cooperative Effects in Binding by Bovine Serum Albumin. I. The Binding of 1-Anilino-8-naphthalenesulfonate. Fluorimetric Titrations*

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ABSTRACT: The binding of anilinonaphthalenesulfonate by bovine serum albumin at various values of pH, temperature, and ionic strength has been studied by a fluorometric technique. Five moles of anilinonaphthalenesulfonate was bound/mole of serum albumin over the pH range 10–5.0. The equilibrium concentrations of free ligand, free protein, and complex were determined for values of the probability of binding from 0.05 to 0.95. The more complete titration curves obtained contained some 20 binary units of information. Abrupt changes in the apparent reaction order, similar to those demonstrated in the equilibrium of reduced diphosphopyridine nucleotide (DPNH) and beef muscle lactate dehydrogenase, were observed at pH 7, and a continuous increase in the reaction order with probability of binding was seen at pH 5. The results of pH 7, and probably also those at pH 5.0, are not expressible by an equation of Adair's type. We believe that, as in the case of lactate dehydrogenase, the results indicate the presence in the binding system of relaxation effects that are slow in comparison with the rate of dissociation of the ligand.

The binding properties of bovine serum albumin,[1] which make this protein functionally distinct from many others, have received a great deal of attention (Foster, 1960). In a series of studies, Klotz (1947) showed that the binding of aromatic anions by serum albumin could be characterized by the number N of anion molecules bound by the protein and an average or statistical dissociation constant $\bar{K}$. Laurence's studies (1952) showed that the changes in intensity and polarization of the emitted radiation that take place upon combination of a small fluorescent molecule with the protein may be used to advantage to study binding equilibria because of the sensitivity and potential accuracy of photometric determinations. In the studies to be presented we shall use fluorescence methods to determine the equilibrium in solution between 1-anilinonaphthalene-8-sulfonate, free and bound to bovine serum albumin.

In our studies we shall not attempt to characterize the binding by a unique value of K. Instead we will describe the process of binding by means of the *titration curve*. This curve gives the probability of binding p[2] (Weber, 1965) as a function of log [X], the logarithm of the free ligand concentration [X] over a set of values of p evenly distributed between $p = 0$ and $p = 1$ and in number ideally approaching the value $1/(2\delta p)$, where δp is the standard deviation of an independent measurement of p. If the probability of binding is independent of protein concentration the set of $1/2\delta p$ values of p and the corresponding values of free ligand concentration describe completely the binding process under fixed conditions of temperature and solvent composition. In this paper we shall give such complete descriptions of the equilibrium of the dye 1-anilino-8-naphthalenesulfonate and bovine serum albumin for several values of the variables over the pH range 5–10, NaCl concentration up to 3 M, and temperatures of 7, 25, and 39°.

Materials and Methods

Crystallized BSA was a product of Armour. ANS was prepared as described by Weber and Young (1964a). Buffer reagents and other chemicals were analytical grade.

BSA concentrations were determined by measuring light absorption at 280 mμ and using $E^{1\%}_{1cm}$ 6.60. In converting these to molar values, a molecular weight of 66,000 was assumed for BSA. ANS concentrations were calculated using the value 4.95×10^3 for the molar absorption coefficient at 350 mμ (Weber and Young, 1964a).

Titrations were made fluorometrically with the ap-

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[1] Abbreviations used: BSA, bovine serum albumin; ANS, 1-anilino-8-naphthalenesulfonate; DPNH, reduced diphosphopyridine nucleotide.

[2] The probability of binding p equals the degree of saturation $p = \bar{n}/N$ if the total ANS concentration X_0 is at least as large as the total protein concentration P_0 (Weber and Anderson, 1965).

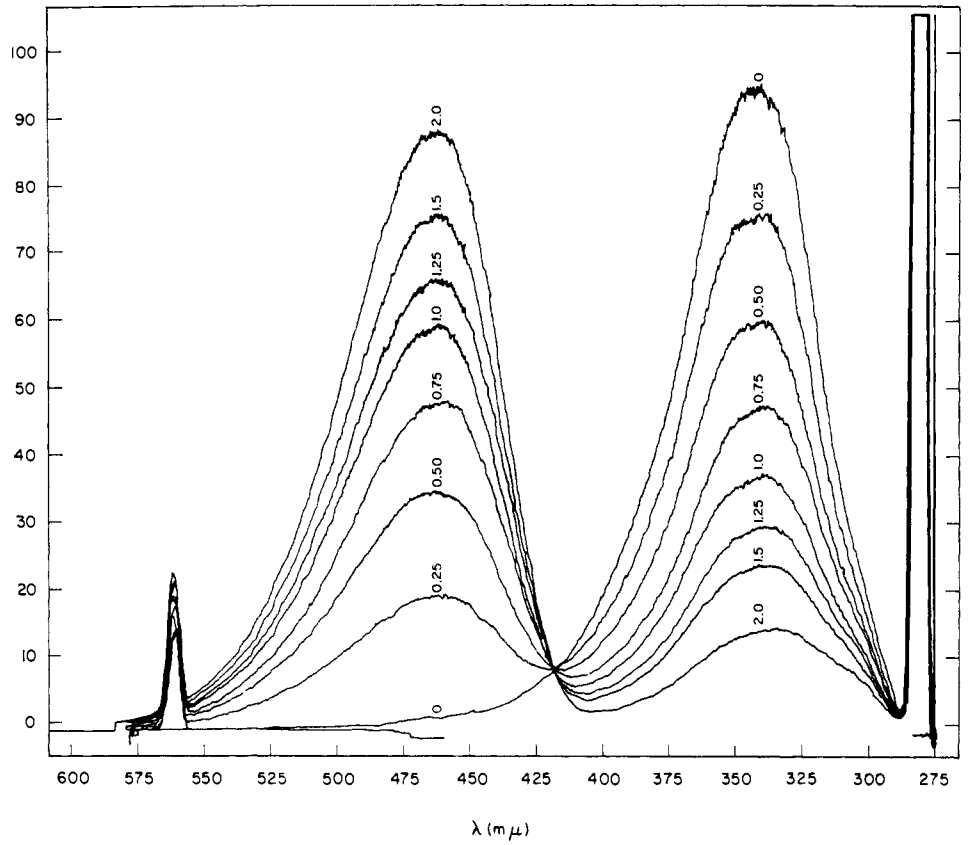

FIGURE 1: Fluorescence spectra of ANS–BSA at various values of $\bar{n}$. BSA concentration 10 mg/ml, in 0.1 M phosphate buffer, pH 7.0. Band widths of excitation and emission 3.0 mμ. An isoemissive point is observed at 416 mμ.

paratus described by Anderson and Weber (1965); the excitation wavelength, 360 mμ, was selected by a monochromator from the emission of a Xenon light source. The fluorescence filter was a Corning glass filter 3-72 in conjunction with a 2-mm, 2 M $NaNO_2$ liquid filter. Fluorescence spectra were obtained with the aid of a recording spectrofluorophotometer (Weber and Young, 1964b).

Titration Procedure

A convenient way to determine the titration curve is to use what may be termed the "dilution method." The principle of the method may be stated thus: A protein–ligand solution for which the ratio $[X_0]/[P_0]$ of total ligand to total protein concentrations equals or slightly exceeds the maximum binding capacity, and initially sufficiently concentrated to ensure virtually stoichiometric binding, is used to cover the whole range of saturations from 0 to 1. Upon dilution, $\bar{n}$ the number of moles of ligand bound/mole of protein takes on successively all the values from $\bar{n} \simeq N$ to $\bar{n} \ll 1$, where N is the maximum number of moles of ligand that can be bound by a mole of protein. In this way, the method gives a cross section of the surface representing the probability of binding p (X_0 and P_0) (Weber, 1965) and, provided concentration-dependent protein interactions may be neglected, yields an unambiguous characterization of this surface.

Anticipating our results, we may point out that ANS is bound firmly by the BSA molecule. This makes it necessary to reach BSA concentrations as low as 10^{-3} M, in order to satisfactorily cover the titration region where $\bar{n} \ll 1$. Originally, it was intended to carry out the titration in the automatic titrator described by Anderson and Weber (1965). However, we found that under the conditions of continuous illumination and vigorous stirring required for automatic titration, the most dilute ANS–BSA solutions (*ca.* 10^{-8} M) lose part of their fluorescence as the experiment proceeds, mainly through photooxidation and adsorption to the walls of the cuvet. It was, therefore, decided to restrict the role of the automatic titrator to the determination of the maximum fluorescence enhancement upon binding. In this case, in which case all the ANS is bound to BSA present in high concentration, the fluorescence readings show no trace of instability. In the determination of the fluorescence signal from solutions in which ANS is only partly bound, we resorted to a manual dilution method in which each solution is individually prepared, measured, and then discarded. This procedure (see also Anderson and Weber, 1965), although time consuming and less elegant than continuous titration,

was found to be reliable and by its use we were able to obtain binding curves repeatable within the limits discussed under Precision and Errors. Because of the exacting experimental requirements mentioned, we think it profitable to describe in detail the procedure followed.

Measurement of $\bar{n}$. If F_c is the fluorescence of a given solution of ANS–BSA and F_b the fluorescence, under the same experimental settings, of an equal concentration of ANS in "excess" BSA,[3] such that all the ANS is bound, then $\bar{n} = (F_c/F_b)(X_0/P_0)$. Implied in this relation are two assumptions which are found to be valid for the system ANS–BSA: (i) The quantum yield of free ANS is negligibly small compared with that of bound ANS; and (ii) the quantum yield of absorbed ANS is at least to a good approximation independent of $\bar{n}$, or is a very slowly varying function of $\bar{n}$.

A. A "stock" solution of ANS–BSA in buffer[4] is prepared with $P_0 = 1.0 \times 10^{-4}$ M and $X_0 = 6.0 \times 10^{-4}$ M. This ratio of ANS:BSA, slightly higher than N, was chosen to enable us to cover the whole range of values of $\bar{n}$.

B. To determine the maximum enhancement F_b, the automatic titrator is used. A 0.5-ml syringe is filled with the "stock solution" of ANS–BSA and the contents are mechanically injected into a square cuvet of 4-cm^2 section containing 10 ml of a concentrated solution of BSA ($\simeq$1%) so as to ensure that practically all the ANS is bound. The mixture is stirred magnetically during the process and the fluorescence is continuously recorded. If the rate of flow of solution out of the syringe is known, the record then gives a continuous plot of F_b *vs.* ANS concentration.

C. To determine F_c, 10 ml of buffer from a container kept at a temperature near that at which the experiment is to be done is pipetted into the cuvet, and water-saturated nitrogen gas is bubbled through plastic tubing into the solution. After a time which is judged sufficient for temperature equilibration and deoxygenation, the nitrogen tubing is withdrawn from the solution and left to hang above the surface of the liquid to maintain an atmosphere of nitrogen. The exciting light is screened off and a given volume of ANS–BSA "stock solution" is delivered with a microsyringe into the buffer contained in the cuvet. The contents are mixed gently with a polyethylene rod, the light screen is removed, and the photocurrent produced is graphically recorded. The reading is F_c. When the dilutions are very large, the signal usually decreases very slowly with time. The "initial" value, observed during the first minute of recording, is used for every value of $\bar{n}$.

Presentation of the Binding Data. From the known total concentrations of ANS and BSA and the measured value of $\bar{n}$, the concentration of free ANS, [X] is calculated making use of the relation

$$[X] = X_0 - \bar{n}P_0$$

where X_0 = total concentration of ANS and P_0 = total concentration of protein in the particular solution to which $\bar{n}$ applies. Log [X] is calculated and plotted *vs.* $\bar{n}$ to give the titration curve.

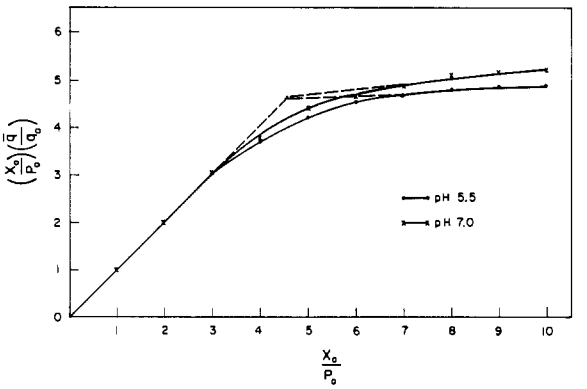

FIGURE 2: Stoichiometric plot of relative fluorescent efficiency of ANS–BSA mixtures (explanation in text). X_0 = molarity of total ANS; P_0, molarity of total protein; buffer = 0.1 M phosphate.

Results

Fluorescence Spectra of ANS–BSA Complexes. The fluorescence spectra of ANS–BSA complexes, excited at a wavelength of 280 mμ, at which both BSA and ANS absorb, are shown in Figure 1. In this experiment the BSA concentrations were in all cases 10 mg/ml so that virtually all the ANS added was bound to the protein. It is seen that as $\bar{n}$ increases, ANS fluorescence (λ_{max} 469 mμ) increases and concurrently the protein fluorescence (λ_{max} 343 mμ) decreases. Inspection of the spectra indicates that the fluorescence of tyrosine is relatively less quenched than that of tryptophan. It is to be noted that the ANS spectra do not change in maximum of emission or band width (±1 mμ) over the range of values of $\bar{n}$ recorded ($\bar{n}$ = 0.25–2.0). The constancy of the emission spectrum of adsorbed ANS was found to apply, within the same limits of error, up to values of $\bar{n}$ at least as large as 4.5. The spectra of Figure 1 show an isoemissive point at 416 mμ. As described in a previous paper (Anderson and Weber, 1965) the existence of this feature shows that the quantum yield of fluorescence of the absorbed molecules is the same over the range of values of $\bar{n}$ represented. The possible variation of the fluorescent yield at larger values of $\bar{n}$ is discussed below.

Stoichiometry and Fluorescence Yield of Bound ANS. The value of 5 moles of ANS bound/mole of protein, determined as originally done by Weber and Young (1964a) was found to be maintained over the pH range studied. The criticism may be leveled to such determina-

[3] By "excess" BSA is meant a concentration large enough to ensure complete binding of all the ANS present.

[4] In the experiments at pH 10 and 2, the "stock solution" was ANS–BSA in distilled water, and this was diluted in buffer of the required pH only prior to reading.

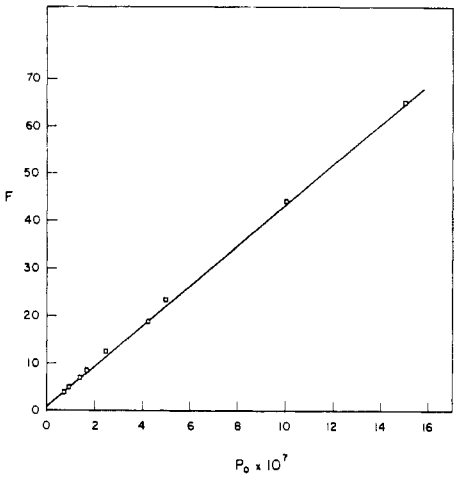

FIGURE 3: Independence of $\bar{n}$ from protein concentration P_0. Plot of P_0 *vs.* fluorescence for a solution of protein with $\bar{n} = 1.1$ diluted with the equilibrium concentration of free ligand.

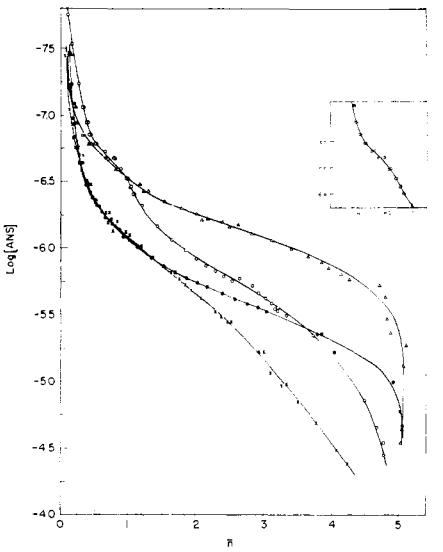

FIGURE 4: Binding curves of ANS by BSA at 25°. O—O, buffer, 0.1 M phosphate, pH 7.0. ×—×, buffer, 0.1 M phosphate, pH 10.0. △—△, buffer, 0.1 M phosphate, pH 5.0. ●—●, buffer, 0.1 M acetate, pH 5.0. Inset: portion of curve at 0.1 M phosphate, pH 7.0.

tions that they tacitly assume the fluorescent yield at any value of $\bar{n}$ to be the same as that found for very small $\bar{n}$. The approximate constancy of the quantum yield with number bound was verified by direct determinations at varying degrees of saturation: P_0 was held constant at 1.5×10^{-4} M and X_0 was varied in ten equal steps from 1.5×10^{-4} to 1.5×10^{-3} M. In the spectrofluorimeter used (Weber and Young, 1964b) excitation and observation take place through the front face of the cuvet so that there is no upper limit to the absorbancy of the solutions that may be examined. The emission spectrum of the solutions was recorded for each value of X_0 together with that of an internal standard (quinine sulfate in 0.1 M sulfuric acid) having the same optical density at the exciting wavelength (345 mμ). At this wavelength the absorption coefficients of ANS and ANS–BSA complexes are virtually the same. Table I shows that the fluorescent yield remains the same at least up to $\bar{n} = 3$, and that at $\bar{n} = 5$ it cannot be significantly <0.90 at pH 7, or 0.86 at pH 5. The maximum number N of moles bound can best be derived from a stoichiometric plot: A plot of $(X_0/P_0)\varphi(m)$ *vs.* X_0/P_0, where m is a parameter that changes with binding, and $\varphi(m)$ a function of m such that $\varphi(m) = 1$ for complete binding and $\varphi(m) = 0$ for no binding, may be termed a stoichiometric plot since it ideally reaches the value N, when all N binding sites are occupied. When the parameter m is the average quantum yield $\bar{q}$

$$\varphi(\bar{q}) = (\bar{q} - q_1)/(q_b - q_1)$$

where q_1 is the fluorescent yield of free ligand and q_b the fluorescent yield of bound ligand. Under our conditions of excitation and observation q_b/q_1 was nearly 180, so that we can set $\varphi(\bar{q}) = \bar{q}/q_b$ without appreciable error.

In Figure 2 we have plotted the data of Table I using $(\bar{q}/q_0)(X_0/P_0)$ instead of $(\bar{q}/q_b)(X_0/P_0)$, where q_0 is the yield for $\bar{n} = 1$ *vs.* X_0/P_0. A departure from the initial slope may thus be due, either to incomplete binding, or reduced yield, or both, but it is apparent from the plot that the number of molecules bound *that make a significant contribution to the fluorescent yield* cannot be appreciably different from five.

Independence of Degree of Dissociation and Protein Concentration. The principle of the demonstration (Anderson and Weber, 1965) is the following: A solution of ligand–protein complex, diluted with ligand at

TABLE I: Relative Fluorescent Efficiencies of ANS–BSA Solutions as a Function of Their Molar Ratio X_0/P_0.[a]

X_0/P_0	$\bar{q}$(pH 7.0)	$\bar{q}$(pH 5.5)
1	1.00	1.00
2	1.00	0.99
3	1.02	0.99
4	0.94	0.91
5	0.89	0.84
6	0.77	0.75
7	0.70	0.67
8	0.63	0.60
9	0.57	0.54
10	0.52	0.49

[a] All solutions were 1.5×10^{-4} M in albumin.

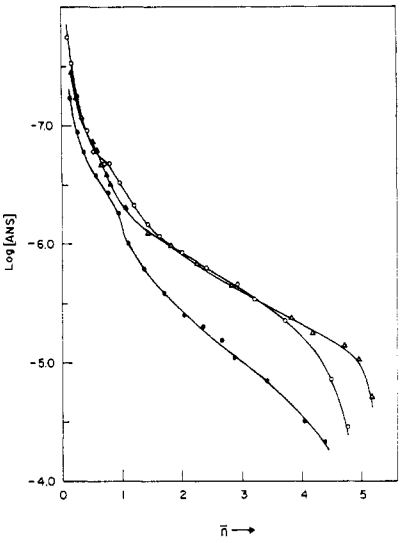

FIGURE 5: Binding curves of ANS by BSA; pH 7.0 at 25°. O—O, buffer, 0.1 M phosphate, for comparison. ●—●, buffer, 0.1 M phosphate +0.5 M NaCl. △—△, buffer, 0.1 M phosphate + 3.0 M NaCl.

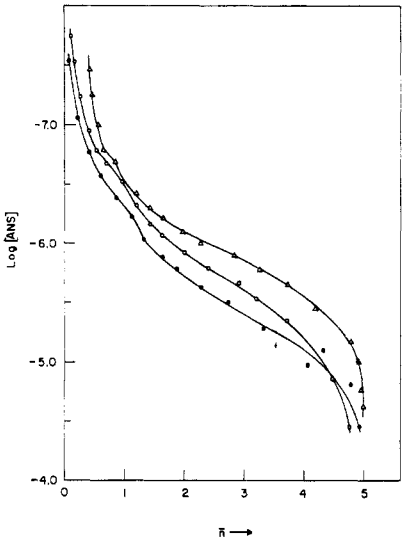

FIGURE 6: Binding curves of ANS by BSA at pH 7, 0.1 M phosphate buffer at different temperatures. O—O, 25°. △—△, 7°. ●—●, 37°.

concentration [X] equal to that of free ligand in the original solution, will maintain the same value of $\bar{n}$, if the degree of dissociation is independent of protein concentration. The fluorescence F in arbitrary units equals in fact

$$F = q_1[X] + \bar{q}[PX] = q_1[X] + \bar{q}\bar{n}P_0$$

According to the last equation a plot of total protein P_0 *vs.* fluorescence under the conditions described should be linear with slope $\bar{q}\bar{n}$ and intercept $q_1[X]$. Figure 3 shows data obtained for $\bar{n} = 1.1$. Similar linear plots were obtained for $\bar{n} = 3$, using the same protein concentrations, so that over this range of values of $\bar{n}$ and $[P_0]$, these two are truly independent variables.

Binding Curves. Figures 4–6 show the general behavior. The features which present the greater interest are summarized in Table II. Binding of the simple type (Weber, 1965), is one in which independently of N, the titration curve is characterized entirely by conforming to the relation $(\bar{n}/N) = [X]/([X] + K)$, where K is the unique dissociation constant of the system. The "span" of a binding curve is given by the number of log units of [X] covered in the range $\bar{n}/N = 0.1$ to $\bar{n}/N = 0.9$. For simple binding the span is 1.908 log units of [X]. It is seen that the titration at pH 5 shows a span of 1 log unit, so that cooperative binding is present. On the other hand, at pH 7 the span is almost normal, but the curve differs from that of the simple type due to the existence of a clear inflexion at $\bar{n} = 1$ (see inset of Figure 4).

The shape and span of the titration curves are also reflected in the plot of log $\bar{n}/(N - \bar{n})$ *vs.* log [X] (Hill's plot) shown in Figure 7. The numbers at different points of the curve give the values of the slope in the

TABLE II

Buffer, M, pH, °C	$-\text{Log}\ [\text{ANS}]_{\bar{n}=2.5}$	Span[a]	Remarks
Phosphate, 0.1, 7.0, 25	5.78	1.95	Normal span
Phosphate, 0.1, 7.0, 25	5.80	1.77	Normal span
Acetate, 0.1, 5.0, 25	5.64	1.19	Reduced span
Acetate, 0.1, 5.0, 25	5.63		
Phosphate, 0.1, 5.0, 25	6.17	1.00	Reduced span
Phosphate, 0.1, 10.1, 25	5.44	2.20	Extended span
Phosphate, 0.1, 2.0, 25	5.07	...[b]	
Phosphate, 0.1, + NaCl, 0.5, 7.0, 25	5.23	2.40	Extended span
Phosphate, 0.1, + NaCl, 0.0, 7.0, 25	5.75	1.69	
Phosphate, 0.1, 7.0, 39	5.57	1.61	
Phosphate, 0.1, 7.0, 39	5.57		
Phosphate, 0.1, 7.0, 7	5.97	1.80	

[a] By span is meant the number of log units covered in the range $\bar{n} = 0.5$–4.5. [b] At pH 2.0 more than five sites are available on BSA for ANS binding.

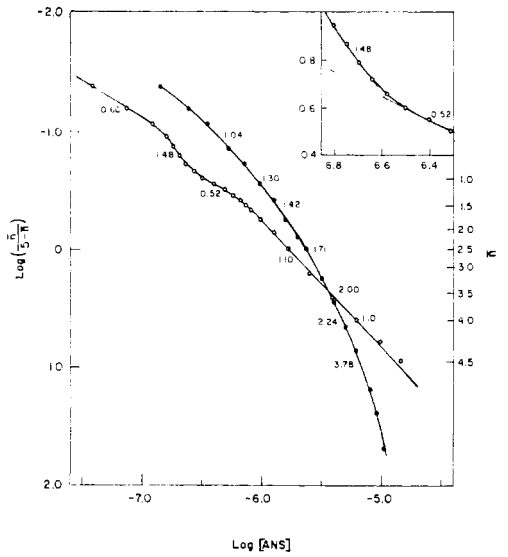

FIGURE 7: Hill's plot of the binding data of Figure 4. ●—●, 0.1 M phosphate, pH 5.0. O—O, 0.1 M phosphate, pH 7.0.

Hill's plot or what is equivalent, the apparent order j of the binding reaction. Simple binding is characterized by a straight line of unit slope. The binding of ANS by BSA at any pH and temperature studied clearly departs from such simple behavior. From the figure, it is noticeable that changes in the order of the reaction with $\bar{n}$ occur in both cases shown. At pH 5 it increases steadily from $j < 1$ to j appreciably higher than 3. Moreover no decrease in j with $\bar{n}$ was noticed up to the highest values of n reliably determined which were at least as high as $\bar{n} = 4.8$. At pH 7 the apparent order of the reaction increased first to a value well above 1 (at $\bar{n} = 0.8$), then decreased to a value well below it (at $\bar{n} = 1.2$). This behavior resembles closely the one described by Anderson and Weber (1965) in beef muscle lactate dehydrogenase and some of its hybrids.

Discussion

It is clear from the results presented that binding of ANS by BSA was never of the simple type. Inspection of Table II shows that since the span of the titration curve in most cases was normal or even shorter, heterogeneity of the binding albumin population need not be considered.

In attempting to describe the binding curves similar problems arise here as already considered in previous work on the binding of M hybrids of lactate dehydrogenase (Anderson and Weber, 1965). In particular, fitting of the curve at pH 7 by a set of five arbitrary equilibrium constants by the use of Adair's equation does not seem feasible. This is shown by application of the test derived by Weber and Anderson (Weber and Anderson, 1965). In a system that obeys Adair's equation the maximum rates of increase or decrease of the slope of the Hill plots with $\bar{n}$ are given by

$$\frac{N-s}{N-\bar{n}} \leqslant \frac{\Delta s}{\Delta \bar{n}} \leqslant \frac{N-s}{N-\bar{n}}\left(\frac{N}{s}+\frac{N}{\bar{n}}-2\right)$$

Using the values of j (the experimental slope), we obtain from the data shown in Figure 7 for BSA at pH 7

$$\Delta s/\Delta n = -2.4; \qquad \frac{N-s}{N-\bar{n}} = -1.0$$

so that fitting by Adair's equation is not possible within experimental errors.

The curve at pH 5 shows continuous increase in j with N, and at $\bar{n}$ values of 4 and 4.5, j equals, respectively, 2.24 and 3.78. Application of the test above shows that the maximum possible rate of increase compatible with Adair's equation at $\bar{n} = 4.25$ and average slope 3.02 equals $\Delta s/\Delta \bar{n} = +2$ while the observed rate of increase is 1.56/0.5 = 3.12. However, as discussed in the Appendix (see Precision and Errors) the uncertainty in the calculated slope, δj, is here *ca.* 0.5. Therefore, the observed difference with the limiting value of +2 is barely significant.

The failure of Adair's equation to fit the experimental results excludes any explanation of these in terms of tautomerism of protein forms in equilibrium, if the equilibrium between any pair of tautomers is to be described by a simple first-order constant (Weber and Anderson, 1965).

The existence of protein tautomeric forms in equilibrium with different affinities for the ligand has been proposed as an explanation of the cooperative binding of O_2 by hemoglobin (Changeux, 1964) and also of the activation of enzymes by small molecules which are not their substrates (Monod *et al.*, 1965). It appears from the data in this paper as well as from those of Anderson and Weber (1965) that the cooperative binding phenomena observed here cannot result from equilibria of the simple type invoked by these authors, since these would always give rise to titration curves that can be fitted by Adair's equation.

For reasons discussed elsewhere (Weber and Anderson, 1965) we believe that our present results indicate the existence in the protein molecule of relaxation effects upon binding and dissociation of the ligand, with times comparable to or longer than the lifetime of the protein–ligand complexes. The direct demonstration of relaxation effects in the protein molecules with times longer than the reciprocal of the dissociation rate of the ligand could be decisive in excluding mechanisms that invoke simple tautomer equilibria governed by detailed balance. The work of Gibson (1959) and the recent observations of Sundaram and Fincham (1964) indicate that such "slow" relaxation phenomena may be observed in protein molecules.

The binding of ANS by BSA at pH 5 in either acetate or phosphate buffers shows very clear cooperative character. Since albumin is a single peptide chain and the fragments isolated after a brief peptic digestion are

very different in physical properties and amino acid distribution (Weber and Young, 1964b), it does not seem possible to assign to the serum albumin molecule the kind of symmetry present in proteins made up of subunits. Thus, such structural symmetry is not a necessary condition for the observation of cooperative effects in binding of ligands by proteins.

Binding Affinities. The absolute value of log [X] for $\bar{n} = 2.5$ was found to be as high as 6.20 ± 0.03 (at pH 5) and only slightly lower, 5.78 ± 0.02 at pH 7.

At pH 10 the binding of the first mole of ANS has an affinity comparable to that at pH 7, the values of log [X] for equal $\bar{n}$ differing by only *ca.* 0.3–0.4 log unit which corresponds to a factor of two in apparent dissociation constants.

The binding affinity was greatest at pH 7, at least for the first mole bound. At pH 2 (not shown in Figure 4), the number of molecules bound was much larger than 5, but the binding affinity for the first 5 moles was considerably lower than at pH 7, the titration curves being separated on the average by *ca.* 0.8 log unit or a difference of almost one order of magnitude in average dissociation constant. Comparing the two curves at pH 5, it is seen that, at the same molarity, phosphate interferes less with binding of ANS than does acetate. The addition of NaCl (Figure 5) weakens the affinity for ANS, but this effect is reversed at high concentration (3 M), where the curve takes on some cooperative character. The decrease in affinity at acid pH, the comparable affinities for the first mole bound at pH 7 and 10, and the fact that phosphate interferes less with binding than acetate at the isoelectric point indicate very clearly that purely electrostatic factors do not have major importance in the binding phenomena studied. The increase in dissociation constant and number bound at low pH is to be related to the expansion of the molecule below pH 4 (Foster, 1960; Weber and Young, 1964a,b) with a considerable change in the native conformation. As found for other ligands (Klotz and Urquhart, 1949; Kharush, 1950) the binding affinity of BSA for ANS decreases with increasing temperature. Calculation of thermodynamic quantities from the data in Figures 4–6 is possible but because of the complex character of the phenomena their significance and origin would not be clear and thus detailed discussion at this stage would be unprofitable.

Appendix

Precision and Errors

The value of $\bar{n}$, the average number of ANS molecules absorbed/protein molecule, is calculated by the equation

$$\bar{n} = \frac{X_0 - [X]}{P_0} = \frac{X_0}{P_0}\frac{F_c}{F_b} \qquad (1)$$

Therefore, the dependence of $\delta\bar{n}$, the standard deviation in $\bar{n}$, upon δF_c and δF_b, the standard deviation of the photometric measurements, is given by the rule of the propagation of errors as

$$\frac{\delta\bar{n}}{\bar{n}} = \left[\left(\frac{\delta F_c}{F_c}\right)^2 + \left(\frac{\delta F_b}{F_b}\right)^2\right]^{1/2} \simeq \sqrt{2}\frac{\delta F}{\bar{F}} \qquad (2)$$

The ratio $\delta F/\bar{F}$ was in our measurements 0.02 for $\bar{n} > 0.3$. Therefore, we shall take $\delta\bar{n}/\bar{n} = 0.03$. The dependence of the calculated [X] upon $\bar{n}$ is given by

$$\delta[X] = P_0\delta\bar{n}$$

since $[X] = X_0 - \bar{n}P_0$. The error in log [X], δ log [X], is

$$\delta \log [X] = \frac{\delta[X]}{[X]} \simeq \left(\frac{\delta\bar{n}}{\bar{n}}\right)\left(\frac{\bar{n}}{N - \bar{n}}\right) \qquad (3)$$

since X_0/P_0 was approximately equal to N in the experiments. Similarly the error in $\log R \equiv \log \bar{n}/(N - n)$ is given by

$$\delta \log R = \left(\frac{N}{N - \bar{n}}\right)\left(\frac{\delta\bar{n}}{\bar{n}}\right) \qquad (4)$$

The slope j is calculated by means of the equation

$$j = \frac{\log R_1 - \log R_2}{\log [X_1] - \log [X_2]} = \frac{\Delta \log R}{\Delta \log [X]}$$

where R_1 and R_2, and $[X]_1$ and $[X]_2$ are corresponding values of R and [X]. From the last equation the error in j, δj, is given by

$$\delta j = \frac{\delta(\Delta \log R)(\Delta \log [X]) - \delta(\Delta \log [X])(\Delta \log R)}{(\Delta \log [X])^2}$$

We shall use the relations $\delta(\Delta \log R) \simeq \sqrt{2}\,\delta \log R$, $\delta(\Delta \log [X]) = \sqrt{2}\,\delta \log [X]$ because $\Delta \log R$ and $\Delta \log [X]$ are calculated from independent values of [X]. The propagation of errors rule yields, after use of eq 3 and 4 to express $\delta \log R$ and $\delta \log [X]$ as functions of $\bar{n}$ and $\delta\bar{n}$

$$\delta j = \frac{\sqrt{2}}{j\Delta \log R}\frac{N}{N - \bar{n}}\left(1 + \left(j\frac{\bar{n}}{N}\right)^2\right)^{1/2}\frac{\delta\bar{n}}{\bar{n}} \qquad (5)$$

For $\bar{n} = 1, j \simeq 1, j\Delta \log R \simeq 0.2$, and $\delta\bar{n}/\bar{n} = 0.03$

$$\delta j = \pm 0.29$$

The uncertainty in the slope, δj, is expected to be considerably smaller than that predicted by (5) because the experimental j is calculated from a smoothed out plot of log [X] *vs.* $\bar{n}$ and not from individual pairs of values of log [X] and $\log \bar{n}/(N - \bar{n})$, as used to derive (5). If m values of $\bar{n}$ or [X] have been used to calculate the slope the error will be reduced by the factor $1/\sqrt{m - 1}$. In the region $\bar{n} = 1$, if we use $m - 1 = 3$ as the experimental value, we obtain $\delta j = \pm 0.18$. This is in keeping with the experimental reproducibility of the j values

which were better than ± 0.15. Thus the significance of the difference between the values of $\Delta s/\Delta n$ and $(N - s)/N - \bar{n}$ experimentally found in the titration curves at pH 7, at $\bar{n} = 1$, is not in question.

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Cooperative Effects in Binding by Bovine Serum Albumin. II. The Binding of 1-Anilino-8-naphthalenesulfonate. Polarization of the Ligand Fluorescence and Quenching of the Protein Fluorescence*

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ABSTRACT: The decrease in the polarization of the fluorescence of anilinonaphthalenesulfonate adsorbed upon bovine serum albumin with the average number bound is found to be due solely to electronic energy transfer among the ligand molecules. A descriptive theory of this phenomenon is developed using two simplifying assumptions: (1) random distribution of the ligand molecules among the protein binding sites. (2) A single transfer of the excited state is responsible for the depolarization. Under these assumptions, a "system of equivalent oscillators" may be defined which best fits the experimental data. The equivalent system for the albumin–anilinonaphthalenesulfonate case is one in which the average distance between a pair of binding sites is 21 A and the average angle between two emission oscillators is 33°. The polarization data show the existence of cooperative features in the binding o pH 5 by comparison with that at pH 7, a phenomenon already seen in the titration curves. The quenching of tryptophan fluorescence by transfer of the excited state to the anilinonaphthalenesulfonate may be used to reach a similar conclusion. In addition, it leads to an estimate of *ca.* 33 A for the average distance between the partners involved in this transfer.

In the previous paper (Daniel and Weber, 1966), the binding behavior of the dye 1-anilino-8-naphthalenesulfonate, ANS,[1] to bovine serum albumin, BSA, was reported. As a result of that study we concluded that at pH 7.0 the binding corresponded to a normal titration curve with a span of 1.9 log units, and that changes in reaction order were observed in the neighborhood of $\bar{n} = 1$. At pH 5.0 on the other hand, the binding was increasingly cooperative with the number of moles of dye bound and the over-all span of the titration was reduced to *ca.* 1 log unit.

In this paper, two properties of the system ANS–BSA are considered, *viz.*, the polarization of the fluores-

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[1] Abbreviations used: ANS, 1-anilino-8-naphthalenesulfonate; BSA, bovine serum albumin.