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*

Gregorio Weber and Ezra Daniel†

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.

cence of ANS and the quenching of the protein fluorescence. The polarization studies may be expected to yield information about electronic energy transfer among ligand molecules. The quenching of the protein fluorescence and simultaneous appearance of sensitized fluorescence of the ligand is related to energy transfer from the tryptophan residues in BSA to the bound ANS molecules. Both sets of data are potentially capable of revealing the distances, and in the polarization case also the mutual orientation, of the electronic oscillators participating in the transfer. In both types of phenomena the property measured is a weighted average dependent upon the distribution of the ligand molecules among the binding sites. It should thus be possible to detect cooperative features in the binding process by either type of observation.

Materials and Methods

The preparation of solutions and the measurement of concentrations were the same as described in the previous paper (Daniel and Weber, 1966). Fluorescent polarizations were measured with a photoelectric polarization photometer (Weber, 1956). The dye was excited by the 365-mμ group of lines isolated from a mercury arc lamp by means of a 7-37 Corning filter. The fluorescent filter was a Corning glass 3-71, in conjunction with a 2-mm 2 M $NaNO_2$ liquid filter. Fluorescent intensity was measured with a recording fluorophotometer as mentioned in the previous paper.

Theory

Polarization of the Fluorescence Emitted as Function of the Average Number of Ligand Molecules Bound, $\bar{n}$. If the species PX_i, in which i ligand molecules are bound to one protein molecule, contributes a fraction ϕ_i to the total fluorescent intensity, and to each such species an average characteristic emission anisotropy A_i[2] independent of $\bar{n}$ may be assigned, the observed emission anisotropy $\bar{A}$ is given by

$$\bar{A}(N, \bar{n}) = \sum_{i=1}^{n} \phi_i(\bar{n})A_i \qquad (1)$$

Assuming a random distribution of the ligand among N sites of equal binding affinity, the fractions f_r of the protein present in the forms PX_r ($0 \leqslant r \leqslant N$) are given by the successive terms of the normal distribution

$$f_r = \binom{N}{r}\left(\frac{\bar{n}}{N}\right)^r \left(1 - \frac{\bar{n}}{N}\right)^{N-r} \qquad (2)$$

On the assumption of equal fluorescence efficiency of all sites ϕ_r is given by the expression

$$\phi_r = \frac{rf_r}{\sum_{i=0}^{N} rf_r} = \frac{r}{\bar{n}}f_r \qquad (3)$$

From (2) and (3)

$$\phi_r = \frac{r}{\bar{n}}f_i = \binom{N}{r}\frac{r}{N}\left(\frac{\bar{n}}{N}\right)^{r-1}\left(1 - \frac{\bar{n}}{N}\right)^{N-r} \qquad (4)$$

but

$$\binom{N}{r}\frac{r}{N} = \binom{N-1}{r-1}; \left(1 - \frac{\bar{n}}{N}\right)^{N-r} = \left(1 - \frac{\bar{n}}{N}\right)^{(N-1)-(r-1)} \qquad (5)$$

so that

$$\phi_r = \binom{N-1}{r-1}\left(\frac{\bar{n}}{N}\right)^{r-1}\left(1 - \frac{\bar{n}}{N}\right)^{(N-1)-(r-1)} \qquad (6)$$

Equation 6 shows that under the assumptions discussed the fractional fluorescences ϕ_r due to the N species PX_r ($1 \leqslant r \leqslant N$) are given by the successive terms of the binomial distribution for $(N - 1)$ sites calculated for an *a priori* probability of occupation equal to $\bar{n}/N$. Using the values of ϕ_r calculated from eq 6, developing the terms in $(1 - \bar{n}/N)^r$ in powers of $\bar{n}/N$, adding the coefficients of each power, and using eq 1 to express $\bar{A}(N)$, we have

For two sites

$$\bar{A}(2) = A_1 - \frac{\bar{n}}{2}(A_1 - A_2) \qquad (7a)$$

For three sites

$$\bar{A}(3) = A_1 - 2\left(\frac{\bar{n}}{3}\right)(A_1 - A_2) + \left(\frac{\bar{n}}{3}\right)^2(A_1 - 2A_2 + A_3) \qquad (7b)$$

For four sites

$$\bar{A}(4) = A_1 - 3\left(\frac{\bar{n}}{4}\right)(A_1 - A_2) + 3\left(\frac{\bar{n}}{4}\right)^2 \times (A_1 - 2A_2 + A_3) - \left(\frac{\bar{n}}{4}\right)^3(A_1 - 3A_2 + 3A_3 - A_4) \qquad (7c)$$

For five sites

$$\bar{A}(5) = A_1 - 4\left(\frac{\bar{n}}{5}\right)(A_1 - A_2) + 6\left(\frac{\bar{n}}{5}\right)^2(A_1 - 2A_2 +$$

[2] The use of the emission anisotropy $A = (1/p - 1/3)^{-1}$ rather than the polarization p itself is discussed under "Polarization of the ANS Fluorescence."

$$A_3) - 4\left(\frac{\bar{n}}{5}\right)^3 (A_1 - 3A_2 + 3A_3 - A_4) +$$

$$\left(\frac{\bar{n}}{5}\right)^4 (A_1 - 4A_2 + 6A_3 - 4A_4 + A_5) \quad (7d)$$

or in general for N sites

$$\bar{A}(N) = A_1 + \sum_{r=1}^{N-1} (-1)^r \binom{N-1}{r} \left(\frac{\bar{n}}{N}\right)^r \times \left[\binom{r}{0} A_1 - \binom{r}{1} A_2 + \ldots \pm \binom{r}{r} A_{r+1}\right] \quad (8)$$

Equation 8 gives the mean anisotropy $\bar{A}$ for the normal distribution as a power series in the relative occupancy $(\bar{n}/N)$, the coefficients $A_1 \ldots A_N$ of the series being the N characteristic values of the emission anisotropy.

In a plot of $\bar{A}$ *vs.* $\bar{n}$ the initial slope, given by the coefficient of $\bar{n}$ in the above expansion, equals $(A_1 - A_2)[(N-1)/N]$. Due to the symmetry properties of the binomial distribution the terminal slope observed when $\bar{n} \to N$ also equals $[(N-1)/N](A_{N-1} - A_N)$. In cooperative binding involving the first two molecules bound ϕ_2 will increase with $\bar{n}$ more rapidly than expected from the normal distribution and the initial slope will be steeper than in this latter case. If the cooperative phenomena involve the binding of the last two molecules similarly the terminal slope will be steeper than that expected for a normal distribution.

Calculation of Electronic Energy Transfer from Depolarization Data (First-Order Approximation). It is clearly difficult to infer the extent of energy transfer from depolarization data if more than two ligand molecules are bound by the protein. In this case a rigorous treatment would demand the consideration of transfer chains involving the three or more fluorescent molecules attached. Fortunately such multiple transfers will not contribute to the depolarization as much as the first transfer, and as a first approximation they may be neglected. Our first-order approximation hypothesis will, therefore, assume that the depolarization results from single transfers involving only a pair of ligand molecules, the excited molecule, and another one. Under conditions of random distribution all N sites in the protein are occupied with equal frequency so that all pairs of sites have the same *a priori* probability of being filled. If the transition probability of transfer between a pair of sites in the protein is $\bar{\nu}(\theta,r)$, a function of the distance and mutual orientation of the bound molecules, and the transition probability of emission is λ, the probability of transfer in the species PX_2 is given by

$$\bar{\nu}/\lambda + \bar{\nu} \quad (9)$$

and the probability of emission by

$$\lambda/\lambda + \bar{\nu}$$

Similarly in the species PX_3 the corresponding quantities are

$$2\bar{\nu}/\lambda + 2\bar{\nu} \quad \text{and} \quad \lambda/\lambda + 2\bar{\nu}$$

and in general for the species PX_r these probabilities are, respectively

$$(r-1)\bar{\nu}/\lambda + (r-1)\bar{\nu} \quad \text{and} \quad \lambda/\lambda + (r-1)\bar{\nu} \quad (10)$$

The radiation emitted after one transfer will have a characteristic mean anistropy A_T determined by the average mutual orientation of the two emission oscillators involved in a transfer. The values of $A_2 \ldots A_N$ as a function of $\bar{\nu}$ and A_T are clearly given by the relations

$$A_2 = A_1 \frac{\lambda}{\lambda + \bar{\nu}} + A_T \frac{\nu}{\lambda + \bar{\nu}} = A_1 - (A_1 - A_T) \frac{\bar{\nu}}{\lambda + \bar{\nu}}$$

$$A_N = A_1 - (A_1 - A_T) \frac{(N-1)\bar{\nu}}{\lambda + (N-1)\bar{\nu}} \quad (11)$$

Substituting the values of eq 11 into eq 1, we have

$$A(\bar{n}) = A_1 - (A_1 - A_T) \sum_{r=2}^{r=N} \phi_r \frac{(r-1)\bar{\nu}}{\lambda + (r-1)\bar{\nu}} \quad (12)$$

which gives the mean anisotropy to be expected if values for A_1, A_T, $\bar{\nu}/\lambda$, and the fractional fluorescences ϕ_r are introduced. In a plot of $\bar{A}$ *vs.* $\bar{n}$ covering the whole range of values of $\bar{n}$, the values of A_1 and A_N may be considered experimental values fixed within the precision characteristic of the method used. If these two values are given, $\bar{\nu}/\lambda$ and A_T are no longer independent of each other, since according to eq 11

$$\frac{A_1 - A_N}{A_N - A_T} = (N-1)\frac{\bar{\nu}}{\lambda} \quad (13)$$

Thus, using in eq 12 the values of $\phi_r(\bar{n})$ characteristics of the normal distribution and the experimental values of A_1 and A_N a single arbitrary parameter (either $\bar{\nu}/\lambda$ or A_T) is left to choice. By varying this single parameter the normal distribution with fixed value of A_1 and A_N that fits best the experimental data may easily be found.

This best fitting random distribution defines an equivalent system of oscillators in which the dependence of $\bar{A}$ upon $\bar{n}$ approaches the experimental values. While the parameters $\bar{\nu}/\lambda$ and A_T define *actual* physical properties of the equivalent system, attributing these to the protein itself requires qualification. The characteristic distance $\bar{\nu}_e$ and the characteristics angle θ_e of the oscillators of emission in the equivalent system are obtained from the values of A_T and $\bar{\nu}/\lambda$ by use of the equations (*e.g.*, Weber, 1966).

$$A_T = A_1 \frac{3\cos^2\theta_e - 1}{2} \quad (14a)$$

$$\frac{\bar{\nu}}{\lambda} = \overline{\nu^2(\theta)}\left(\frac{R}{r_e}\right)^6 \qquad (14b)$$

where $\nu(\theta)$ describes the angular dependence and R is the characteristic distance of transfer. If A_T is positive $\overline{\nu^2(\theta)}$ is comprised between 0.33 and 1. It may be set equal to 1, or better substituted by $\overline{\cos^2\theta_e}$ without introducing an appreciable error in ν_e calculated from the experimental $\bar{\nu}/\lambda$.

Our procedure to define the system of equivalent oscillators will consist of varying systematically the parameter A_T in eq 12; A_1 and A_N, obtained from the experimental data are used together with A_T to determine $\bar{\nu}/\lambda$ by eq 13. If the ϕ_r values are calculated by eq 6 for each experimental value of $\bar{n}$ and these are M in number, the mean standard deviation σ of an experimental value of the emission anisotropy $A_{\text{expt}}(\bar{n})$ from the calculated one $A(\bar{n})$ of eq 12 is given by

$$\sigma = \left\{\sum^{M} [A(\bar{n}) - A_{\text{expt}}(\bar{n})]^2/M - 3\right\}^{1/2} \qquad (15)$$

The use of $M - 3$ in the last equation rather than $M - 1$ is due to the loss of two degrees of freedom by fixing A_1 and A_N.

Quenching of BSA Fluorescence as a Function of $\bar{n}$. The fluorescence of tryptophan in the albumin is quenched by transfer of the excited state of ANS, as shown by the appearance of sensitized fluorescence (Daniel and Weber, 1966). Calculation of the probability of transfer should lead here to an estimate of the average distance between the tryptophan residues and the five binding sites for ANS. The theory is simpler than for the transfer among ANS residues and follows similar lines.

If the quantum yield of fluorescence of BSA measured at 376 mμ, a region where tyrosine emission is negligible, is called q_0, then $Q(\bar{n}) = q(\bar{n})/q_0$ is the relative yield at the same wavelength when on average $\bar{n}$ ANS molecules are adsorbed.

$$Q(\bar{n}) = \sum_{r=0}^{N} f_r(q_r/q_0) \qquad (16)$$

where f_r is the fraction of the BSA population with r bound ANS molecules and q_r/q_0 is the relative fluorescence yield of such molecules. If all binding sites are equally likely to be occupied for all r, then $q_r/q_0 = \lambda/(\lambda + r\bar{\mu})$ where $\bar{\mu}$ is the average rate of transfer from excited tryptophan to ANS. If the values of f_r are those corresponding to the random distribution

$$Q(\bar{n}) = \sum_{r=0}^{N} \binom{N}{r}\left(\frac{\bar{n}}{N}\right)^r\left(1 - \frac{\bar{n}}{N}\right)^{N-r}(1 + r\epsilon)^{-1} \qquad (17)$$

where $\epsilon = \bar{\mu}/\lambda$.

In a plot of Q *vs.* $\bar{n}$ approaching $\bar{n} = 0$ and $\bar{n} = N$, the initial slope equals

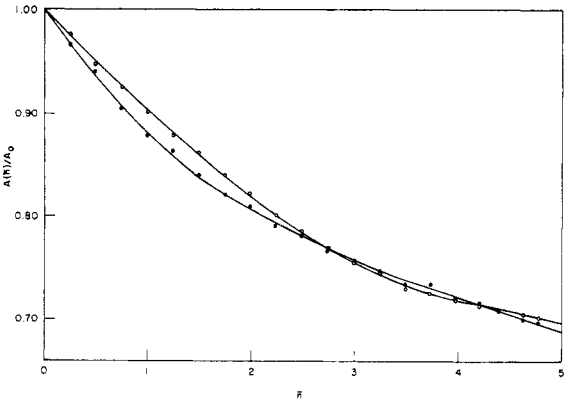

FIGURE 1: Plot of the emission anisotropy $A_{\bar{n}}$, normalized by dividing by $A_{\bar{n}\to 0}$, as a function of $\bar{n}$. BSA concentration throughout equals 10 mg/ml; ○ buffer, 0.1 M phosphate, pH 7.0, 20°. ● buffer, 0.1 M acetate, pH 5.0, 20°.

$$S_i = [dQ(\bar{n})/d\bar{n}]_{n\to 0} = -\epsilon/(1 + \epsilon)$$

and the final slope

$$S_f = [dQ(\bar{n})/d\bar{n}]_{n\to 0} = \frac{-\epsilon}{(1 + N\epsilon)[1 + (N - 1)\epsilon]} \qquad (18)$$

From the last two equations

$$S_f = \frac{S_i(1 - S_i)}{[1 + (N - 1)S_i][1 + (N - 2)S_i]} \qquad (19)$$

The initial and final slopes must be related as indicated by eq 19 if the random transfer theory is at all applicable to our case.

Results

Polarization of the ANS Fluorescence. In Table I we present the results of our measurements on the polarization of the fluorescence of ANS adsorbed on BSA, as a function of $\bar{n}$, where $\bar{n}$ is the number of moles of ANS bound/mole of protein. The results are expressed in terms of the emission anisotropy $A = (1/p - 1/3)^{-1}$ (Jablonski, 1960) which possesses the property of additivity (Weber, 1952), and is, therefore, to be preferred for our purposes over the polarization. The BSA concentration in these experiments was kept constant throughout and equal to *ca.* 10 mg/ml. A 10-fold dilution did not have a perceptible effect in the experimental results, thereby eliminating the protein concentration as an important variable in the polarization measurements. For the sake of comparison, the values of the anisotropies at pH 7.0 and 5.0, expressed as fractions of the limiting values at $\bar{n} \to 0$, are shown in Figure 1. An immediate observation is apparent, *viz.*, the values of A or p decrease with increasing $\bar{n}$. The question to be answered, therefore, is what does the decrease in p reflect?

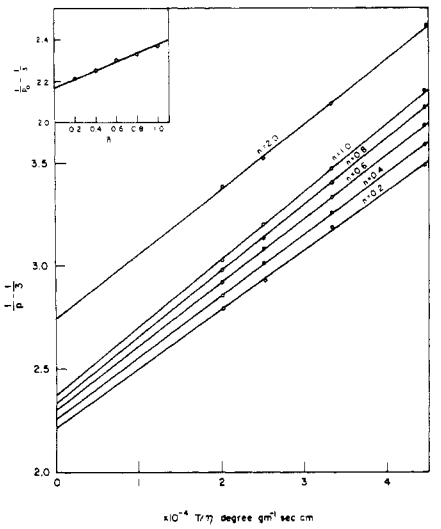

FIGURE 2: Plot of $1/p - {}^1/_3$ *vs.* T/η for ANS–BSA for various values of $\bar{n}$. T/η was varied by changing the temperature. BSA concentration throughout 1 mg/ml. Inset, plot of the intercepts $1/p_0 - {}^1/_3$ *vs.* $\bar{n}$.

By reference to the Perrin (1926) equation

$$A^{-1} = \frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho_h}\right) \quad (20)$$

it is seen that a change in the polarization of the fluorescence, p, may be due to a change in the lifetime of the excited state, τ, in the mean harmonic rotational relaxation time, ρ_h, or in the limiting polarization, p_0. The constancy of τ is suggested by the constancy of the relative quantum yields of the adsorbed ANS, within the limits set in the previous paper. The constancy of ρ_h was in turn suggested by the finding of identical values of the sedimentation constant $s_{20,w}$ at $\bar{n} = 0$, 0.5, and 1.0. In order to unequivocally distinguish between the three possibilities mentioned above, a series of experiments were carried out and their results shown in Figure 2. In this figure $(1/p - {}^1/_3)$ is plotted *vs.* T/η where T is the absolute temperature and η the viscosity of the solvent. $T/\bar{\eta}$ was varied by changing the temperature in several solutions with different values of $\bar{n}$. The values τ/ρ_h obtained from the slope and intercept of the plots and the values of p_0 are tabulated (Table II) to show that changes in τ/ρ_h are of minimal importance and that the changes in p observed are quantitatively accounted for by the changes in the limiting polarizations p_0 attained in the absence of molecular rotations. From the values of ρ_h/τ of Table I and the known value of 120 nsec for ρ_h in neutral BSA solutions a value of τ of *ca.* 16 nsec would be calculated, nearly twice that estimated by Weber and Young (1964) from oscillator strength of the last absorption band and quantum yield. The error in the latter figure arises from an erroneous estimate of the oscillator strength. The last absorption band of ANS ($\lambda_{max} \sim$ 350 mμ) corresponds to two electronic transitions, not one, so that the molar absorption coefficient assumed for the transition is spuriously high leading to a value of τ which is shorter than the actual value. The degenerate character of the longest wavelength absorption band is clearly demonstrated by observations of the fluorescent polarization spectrum of aromatic amines (Weber, 1966) and of ANS itself (Stryer, 1965). More-

TABLE I: Emission Anisotropy of ANS–BSA at Various $\bar{n}$.

	A_n	
$\bar{n}$	pH 7.0	pH 5.0
0.00	0.346_0[a]	0.333_0[a]
0.25	0.337_7	0.321_2
0.50	0.327_7	0.312_9
0.75	0.319_8	0.300_5
1.00	0.311_6	0.292_1
1.25	0.303_6	0.286_9
1.50	0.297_9	0.279_2
1.75	0.290_4	0.272_7
2.00	0.284_5	0.269_0
2.25	0.276_6	0.262_8
2.50	0.271_5	0.259_6
2.75	0.266_4	0.255_0
3.00	0.261_4	0.251_7
3.25	0.257_9	0.248_4
3.50	0.252_8	0.244_1
3.74	0.250_9	0.244_1
3.98	0.248_4	0.239_6
4.21	0.246_5	0.238_1
4.40	0.244_8	0.235_1
4.63	0.243_7	0.232_5
4.78	0.242_7	0.231_6
5.00	0.240_0[a]	0.230_0[a]

[a] Extrapolated.

TABLE II: Polarization of the Fluorescence of ANS–BSA at Various $\bar{n}$.

$\bar{n}$	$(1/p_0 - {}^1/_3)$	$(1/p - {}^1/_3)_{25^0}$	ρ_h/τ[a]
0.2	2.210	3.175	6.87
0.4	2.255	3.255	6.77
0.6	2.295	3.335	6.62
0.8	2.330	3.405	6.50
1.0	2.370	3.470	6.46
2.0	2.740	3.795	7.79

[a] The third column gives the ratio ρ_h/τ (eq 20). The value of ρ_h/τ for $\bar{n} = 2$ differs from the others by twice the standard deviation expected. A significant change in hydrodynamic parameter with $\bar{n}$ is thus possible.

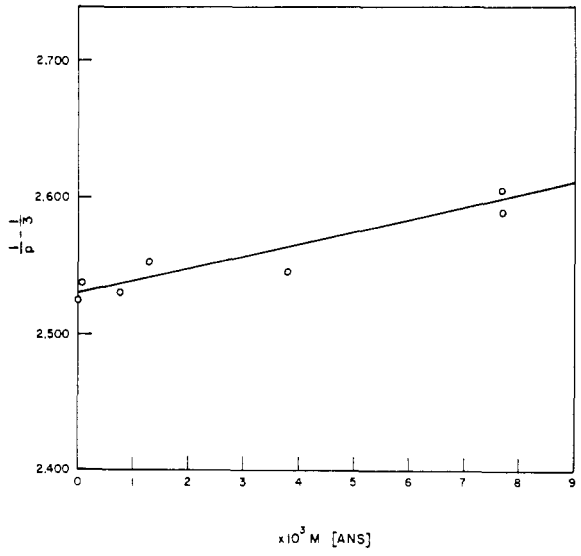

FIGURE 3: ANS in 90% glycerol–water solution. Plot of $1/p - {}^1/_3$ *vs.* concentration of ANS.

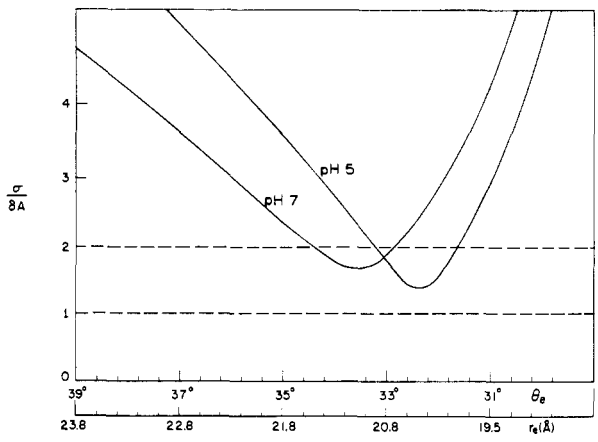

FIGURE 4: Plot of $\sigma/\Delta A$, the average standard deviation between the anisotropies due to specified systems of equivalent oscillators and the experimental data (eq 15). Ordinate, ratio of calculated standard deviation σ to experimental standard deviation ΔA. Abscissa, values of average distance (in A units) and related angle θ between emission oscillators (in degrees), for random distributions having the same anisotropy values as the experimental at $\bar{n} = 0$ and 5.

over, direct measurements of τ (L. Stryer, personal communication) give a value close to 16 nsec.

In order to determine the factors affecting p_0 the system ANS–BSA was compared to the system ANS–glycerol. The polarization of the fluorescence of ANS in glycerol was measured as a function of ANS concentration and the range of concentrations over which no appreciable concentrational depolarization takes place was determined (Figure 3). A solution of ANS in glycerol in this latter range (1.5×10^{-5} M) was used to measure the effect of temperature upon the polarization, and a value of $p_0 = 0.43$ was obtained by extrapolation to $T/\eta \rightarrow 0$. This value is close, though not identical with the value of $(1/p_0 - {}^1/_3)$ extrapolated to $\bar{n} \rightarrow 0$ ($p_0 = 0.40$) shown in the inset of Figure 2. Thus the limiting polarization of ANS is not seriously affected by its adsorption on BSA. The changes in p_0 observed can, therefore, be attributed unequivocally to electronic energy transfer occurring when two or more ANS molecules are adsorbed on the same albumin molecule.

Concentration depolarization of the fluorescence due to energy transfer can actually be observed in glycerol solutions of ANS, as shown by the data of Figure 3. R, the characteristic distance for energy transfer, is calculated by the use of the equation (Weber, 1954; Weber, 1966)

$$R = (2a)^{1/2}\left(\frac{1.68s}{\frac{1}{P_0} - \frac{1}{3}}\right)^{1/6} \qquad (21)$$

where a is the radius of the molecule, and s is the slope of the straight line obtained when $(1/p - {}^1/_3)$ is plotted *vs.* the concentration of the fluorescent species. Using the value of the slope in Figure 3 for s, and setting $2a = 10^{-7}$ cm, we obtain $R = 14$ A for energy transfer in glycerol. The quantum yield of ANS in the glycerol solution used (*ca.* 90% glycerol–water) was ${}^1/_8$ of that of ANS adsorbed upon serum albumin. Therefore, the value of R for the latter must be greater than that in glycerol by a factor of $8^{1/6} = 1.41$, on the likely assumption that the lifetimes of the excited state are proportional to the observed yields. We shall, therefore, assume that $R = 20$ A for ANS–BSA complexes. This value is smaller than $R = 24$ A estimated from spectroscopic data by Weber and Young (1964).

With the purpose of determining the system of equivalent oscillators that best represents the results obtained in the BSA–ANS system, Figure 4 shows a plot of the standard deviation σ of eq 15 *vs.* the parameter A_T or ν/λ for the polarization data of Table I. It is seen that both minima define similar values of $\bar{\nu}/\lambda$ and A_T. In the abscissa we have marked the equivalent distances r_e and angles θ_e calculated by means of eq 14. The ordinate units are experimental standard deviations δA in the measurement of A. In our hands, from observations involving 20 pairs of A values, $\delta A = \pm 0.0011$. It will be noticed that the best fits are only within 1.5 and 1.75 experimental standard deviations, respectively. A satisfactory fit should place the minimum within one experimental standard deviation. The difference could result from insufficiency of the first-order approximation in the calculation of the probability of transfer, or less likely because of departures from the random distribution. A more refined analysis seems possible once the causes that determine the peculiarities of the titration curves are known, but the data at hand seem sufficient to give an estimate of the distance among the

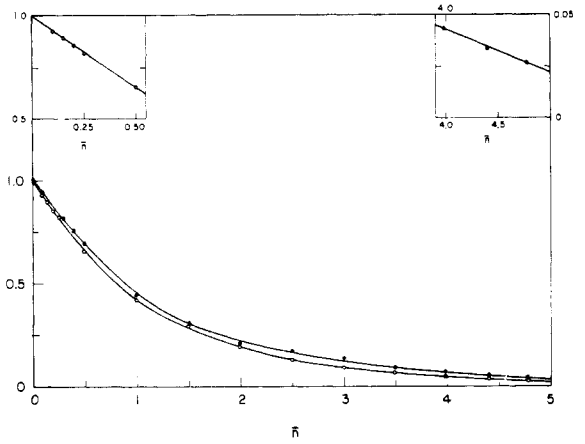

FIGURE 5: Plot of efficiency of protein fluorescence *vs.* $\bar{n}$. BSA concentration constant throughout (10 mg/ml). Excitation at 290 mμ. Fluorescence measured at 375 mμ. Band widths of excitation and fluorescent measurement = 3 mμ. The data have been corrected for absorption of the exciting light by ANS. ○ buffer, 0.1 M phosphate, pH 7.0, 25°; ● buffer, 0.1 M acetate, pH 5.0, 25°. Insets, initial and terminal portions of the curve in 0.1 M phosphate, pH 7.0, 25°, to show the initial and final slopes.

binding sites and the relative orientations of the bound molecules. The value of r_e = 21 A is 14% lower than the value of 24 A estimated by Weber and Young (1964) for the distance between the first pair of molecules bound. It confirms their view that the sites are not randomly distributed but are all found in a fraction of the total area or volume of the protein.

The agreement between our value of r_e and the one obtained by Weber and Young (1964) in spite of the large differences in the A_T values introduced is simply a consequence of the fortunate dependence of $\bar{\nu}/\lambda$ upon the sixth power of the distance, a circumstance that makes r_e relatively independent of changes in θ_e. The value of θ_e of 33° observed requires special comment. For random orientation a value close to 53° would be obtained, so that the experimental data reflect the existence of considerable preferential orientation in the bound molecules. This seems to us one of the more interesting qualitative conclusions that emerges from the present study.

It is worthwhile to stress that we cannot presently estimate to what extent the values of θ_e and r_e calculated for the equivalent system represent the actual physical situation in the protein. It seems that the most important condition to be fulfilled for the equivalent system to approach the real one is that of random occupancy of binding sites. This condition, which gives rise to eq 11, implies that the average values $\bar{\nu}/\lambda$ and A_T are independent of $\bar{n}$, and this may obtain even in cases of cooperative binding when the distribution of the molecular species with different numbers of bound ligand molecules is not normal. On the other hand, if such normal distribution is present, random occupancy is ensured. According to the titration curves of Figure 4 of Daniel and Weber (1966), binding at pH 7 with a span of 1.8 log units must closely approach the random distribution, at least for $\bar{n} > 1$, while the binding at pH 5.0 obviously does not. Yet in both cases very similar values of θ_e and r_e were calculated. We believe that this similarity arises from the fact that random occupancy of the different sites takes place in either case, and therefore that the values of θ_e and r_e given will be found to be close to those obtaining in the protein, if and when such direct information becomes available.

Quenching of Protein Fluorescence. The values of the relative fluorescence yield at 375 mμ are shown in Figure 5 as a function of $\bar{n}$, the average number bound. The results have been corrected for the absorption of both exciting and fluorescent light by the ligand. At pH 7 the initial slope S_i = 0.690. According to eq 19 the final slope ought to have the value 0.0194 in good agreement with the observed 0.021. We conclude that the random transfer hypothesis used to derive eq 19 is a good approximation to our case.

Introducing S_i = 0.69 in eq 18 we have $\bar{\mu}/\lambda$ = 0.45. This value depends upon both mutual orientation and distance of the oscillators involved. Following the considerations outlined in the discussion of eq 14b we shall set the orientation factor equal to 1 so that the average tryptophan–ANS distance equals

$$\langle r \rangle = R\epsilon^{1/6} = 0.88R \qquad (22)$$

where R is the characteristic distance for the transfer. R may be calculated from spectroscopic data by the use of Forster's (1947) equation

$$R = \left(\frac{1660}{n^2}\frac{\tau J_{\bar{\nu}}}{\bar{\nu}^2}\right)^{1/6} 10^{-6}\ \text{cm} \qquad (23)$$

in which n is the refractive index of the medium, τ the lifetime of the excitation in tryptophan, $J\bar{\nu}$ the overlap integral of the absorption spectrum of ANS and emission spectra of tryptophan, and $\bar{\nu}$ the mean wavenumber of the transferred quanta. For the system in question $\bar{\nu}$ = 28,000 cm^{-1}, $J\bar{\nu} \sim 1.0 \times 10^{11}$ cm^3/mM^2. τ has not been directly measured in serum albumin, but assuming it to be equal to 6 ± 2 nsec, eq 23 gives R = 29 ± 1.5 A, the uncertainty assigned to R arising from that assumed in τ. With this value of R, $\langle r \rangle \simeq$ 33 A. Of greater interest to us than this value is the qualitative difference observed between the quenching curves of pH 7 and pH 5 depicted in Figure 5. For equal $\bar{n}$, the yield at pH 5 is systematically higher than that at pH 7. Such relation may be expected from the cooperative character displayed by the titration curve at pH 5 since in such case the number of molecules with no ligand attached, and therefore strongly fluorescent, will be greater than expected for the normal distribution.

Discussion

The results shown in this study confirm and extend the earlier finding by Weber and Young (1964) that the polarization of ANS fluorescence decreases with increasing values of $\bar{n}$. These authors attributed the decrease to energy transfer among ANS molecules adsorbed on the same BSA molecule. The experiments described in this paper establish conclusively that energy transfer is the sole factor contributing to the decrease of p with $\bar{n}$.

Granted that the depolarization curves as a function of $\bar{n}$ result from the distribution of species with different A values depending upon the number of molecules of ANS adsorbed, the observed differences between the curves at pH 7.0 and 5.0 should reflect differences in distribution. It will be noticed that both the initial slope $(d\bar{1}/d\bar{n})_{n\to 0}$, and the final slope $(d\bar{1}/d\bar{n})_{\bar{n}\to 5}$ are greater in the pH 5.0 curve. The former shows that as $\bar{n}$ increases the rate of appearance of molecules with two ANS molecules adsorbed is faster at pH 5 than at pH 7.0. The latter shows that as $\bar{n}$ decreases from 5, the rate of appearance of molecules with 3 ANS molecules adsorbed is faster at pH 5.0 than at 7.0. These are precisely the effects to be expected from the titration curves shown in Figure 4 of the paper of Daniel and Weber (1966).

From both the polarization observations and those of the quenching of protein fluorescence it follows that the cooperative character of the binding observed at pH 5.0 is to be traced to a departure of the distribution of the species of protein complexes with different numbers of ligand molecules from the values present in the normal distribution. Although such departure is logically expected to occur, we have here independent evidence of its existence. It is to be noted that the consequences of the cooperative behavior in binding at pH 5, demonstrated by titration at protein concentrations of 10^{-7} M, were manifest in the polarization measurements carried out at protein concentration of 10^{-4} M.

The task of demonstrating by polarization experiments departures from the random distribution of ligand would be far simpler and the conclusions correspondingly more certain, in cases with a smaller number of binding sites. Cases with two binding sites seem particularly favorable, as indicated by eq 7a.

In conclusion we wish to stress two aspects of this work: first, the possibility of obtaining from fluorescence depolarization data information about *both* the orientation and distance among the bound ligand molecules in cases in which random occupancy of binding sites is approached. The application of this method to proteins made up of identical subunits should give results of great interest. The investigations of Gally and Edelman (1965) upon the interaction of ANS with Bence–Jones proteins, and the recent work of Stryer (1965) on ANS–apomyoglobin and ANS–apohemoglobin complexes, have shown that the use of this technique need not be confined to studies on serum albumin. Second, the cooperative effects in binding may reveal themselves in the phenomena of depolarization and fluorescence quenching and these can, in favorable cases, complement and clarify the titration data.

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