Integrals in Eq 24. By the substitution $t = \tan\theta$, and subsequent splitting into partial fractions

$$\epsilon_r \int_{-\pi/2}^{+\pi/2} \frac{\cos^2\eta}{1+\epsilon_r\cos^2\eta}d\eta = \int_{-\infty}^{+\infty} \frac{1}{1+t^2} - \frac{1}{1+\epsilon_r+t^2}dt$$

$$\epsilon_r \int_{-\pi/2}^{+\pi/2} \frac{\cos^4\eta}{1+\epsilon_r\cos^2\eta}d\eta = \int_{-\infty}^{+\infty} \frac{1}{1+\epsilon_r+t^2} + \frac{\epsilon_r}{(1+t^2)^2} - \frac{1}{1+t^2}dt$$

$$\epsilon_r \int_{-\pi/2}^{+\pi/2} \frac{\cos^3\eta\sin\eta}{1+\epsilon_r\cos^2\eta}d\eta = \int_{-\infty}^{+\infty} \frac{t}{1+\epsilon_r+t^2} + \frac{\epsilon_r t}{(1+t^2)} - \frac{t}{1+t^2}dt$$

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Fluorescence Polarization of the Complexes of 1-Anilino-8-naphthalenesulfonate with Bovine Serum Albumin. Evidence for Preferential Orientation of the Ligand*

Sonia R. Anderson† and Gregorio Weber

ABSTRACT: The rotational relaxation time $\langle\rho\rangle$ of the complexes of 1-anilino-8-naphthalenesulfonate with bovine serum albumin has been calculated from independent measurements of fluorescence polarization and lifetime for different values of $\bar{n}$, the average number of 1-anilino-8-naphthalenesulfonate molecules bound per molecule of bovine serum albumin. It increases from 105 ± 3 nsec at $\bar{n} = 1$ to 128 ± 3 nsec at $\bar{n} = 5$ when the wavelength of excitation is 366 nm, but remains constant at 105 nsec for all values of $\bar{n}$ when excitation is at 436 nm. Depolarization by energy transfer in both the bovine serum albumin–1-anilino-8-naphthalenesulfonate adsorbates and concentrated solutions of 1-anilino-8-naphthalenesulfonate in propylene glycol is wavelength dependent, transfer failing in both cases upon excitation at 436 nm. Energy transfer is therefore the origin of the apparent increase in $\langle\rho\rangle$ with $\bar{n}$. Analysis of the results according to the preceding paper shows that a model of preferential orientation of the 1-anilino-8-naphthalenesulfonate molecules in planes parallel to the equator of a prolate ellipsoid of axial ratio 4 accounts for the observations.

The polycyclic anion 1-anilino-8-naphthalenesulfonate has a very low fluorescence yield when free in aqueous solutions, but becomes highly fluorescent upon binding to bovine serum albumin (Weber and Laurence, 1954). The favorable overlap integral between the absorption and emission spectra of 1-anilino-8-naphthalenesulfonate results in electronic energy transfer among 1-anilino-8-naphthalenesulfonate residues bound to the same bovine serum albumin molecule (Weber and Young, 1964). In order to visualize a model depicting the average distance between binding sites and the mutual orientations of the bound 1-anilino-8-naphthalenesulfonate molecules, Weber and Daniel (1966) examined the fluorescence polarizations at different values of $\bar{n}$.[1] The resulting model is a system of "equivalent oscillators" in which the average distance between binding

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[1] Abbreviations used in this work: n, the average number of moles of ligand bound per mole of protein.

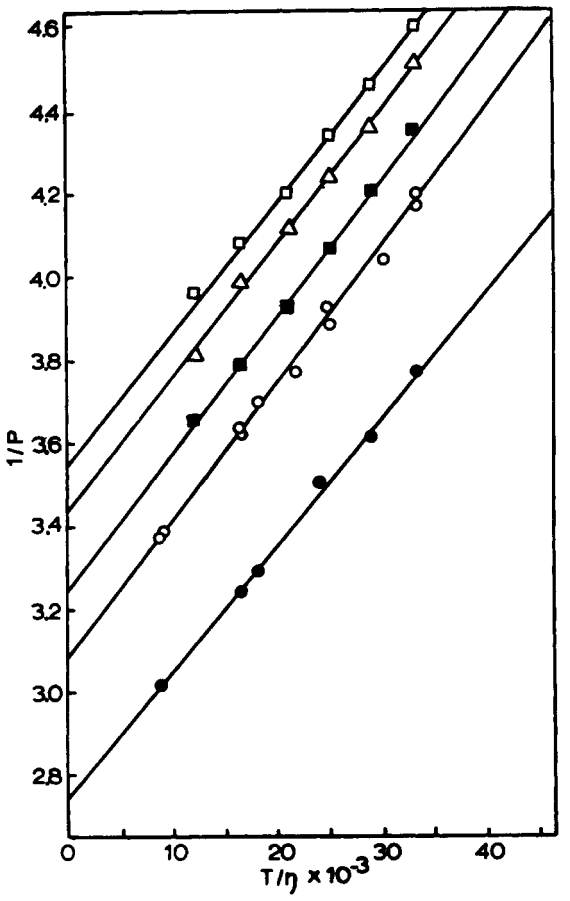

FIGURE 1: Plots of reciprocal of polarization, $1/p$, against T/η (degrees Kelvin per centipoise) for 1-anilino-8-naphthalenesulfonate–bovine serum albumin complexes. The wavelength of the exciting light was 366 nm. Experimental conditions: 0.10 M potassium phosphate, pH 7.0, 25.0°. The 1-anilino-8-naphthalenesulfonate and bovine serum albumin concentrations used are described in Table I. $\bar{n} = 1, \bullet$; $\bar{n} = 2, \circ$; $\bar{n} = 3, \blacksquare$; $\bar{n} = 4, \triangle$; and $\bar{n} = 4.74, \square$.

sites is 21 Å and the average angle between emission oscillators is 33°. Insight into the distribution of ligand among protein molecules was also gained from the experiments: the polarizations observed at pH 7 approach the trend predicted for the random distribution of 1-anilino-8-naphthalenesulfonate among albumin molecules bearing five identical, noninteracting binding sites.

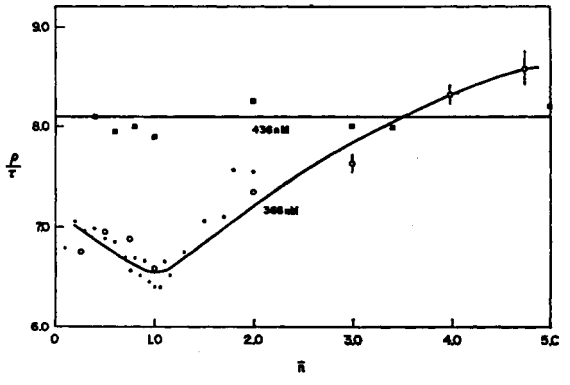

FIGURE 2: The variation of $\langle\rho\rangle/\tau$ with $\bar{n}$ observed upon excitation at 366 and 436 nm (○, □, sucrose was added to solutions at 25°; ●, the temperature was varied).

In the previous paper (Weber and Anderson, 1969), we have shown that additional information can be obtained from fluorescence polarization data. The dependence of the observed rotational relaxation time upon the probability of transfer may reflect the degree of elongation of the protein molecule and the orientation of the ligands with respect to its axial and equatorial planes. We shall expand the model proposed by Weber and Daniel (1966) with new observations which are internally consistent and in excellent agreement with the known hydrodynamic properties of the bovine serum albumin molecules.

Experimental Procedures

Crystallized bovine serum albumin was purchased from Armour Pharmaceutical Company and the magnesium salt of 1-anilino-8-naphthalenesulfonate was prepared by Weber and Young (1964). All other reagents were analytical grade. Bovine serum albumin concentrations were determined spectrophotometrically, using an extinction coefficient of 4.35×10^4 M^{-1} cm^{-1} at 280 nm. 1-Anilino-8-naphthalenesulfonate concentrations were calculated using the value of 4.95×10^3 M^{-1} cm^{-1} for the absorption coefficient at 350 nm.

Fluorescence polarizations were measured with a photoelectric polarization photometer using the isolated lines of a high-pressure mercury arc for excitation (Weber, 1956). The 313-nm line was isolated by means of a combination of a 313-nm interference filter and Corning glass CS 7-54 filter; the 365-nm group of lines, by Corning glass filters CS 7-37 and CS 4-71; the 404-nm line, by a 404-nm interference filter; and the 436-nm line, by a 436-nm interference filter and Corning glass 7-59 filter. In all cases, the fluorescence was observed through a 2-mm layer of 2 M $NaNO_2$ solution and either Corning glass filter CS 3-72 (with excitation at 313, 365, or 404 nm) or Corning filter CS 3-70 (with excitation at 436 nm). The instrument designed by Weber and Bablouzian (1966) was used in the determination of fluorescence polarization spectra. To avoid depolarization by radiative transfer, quartz cuvets of light paths ranging from 1 mm to 1 cm were used. Varying amounts of sucrose were added to the solutions in order to adjust the viscosities to values obtained by interpolation from the tables of Bingham and Jackson (1918).

Direct measurements of fluorescence decay time were made with the cross-correlation phase fluorometer designed by Spencer and Weber (1968). Direct resolution of 0.1 nsec is possible with this instrument.

Sedimentation constants were determined using the Spinco Model E ultracentrifuge.

Results

Dependence of the Relaxation Time upon the Number of 1-Anilino-8-naphthalenesulfonate Molecules Bound. The dependence of the fluorescence polarization, p, upon the rotational relaxation time, ρ, and the lifetime of the

TABLE I: Summary of Physical Constants of Bovine Serum Albumin–1-Anilino-8-naphthalenesulfonate Complexes.[a]

1-Anilino-8-naphthalenesulfonate (M × 10⁴)	$\bar{n}$	$s_{20,w}$ (S)	366-nm Excitation			436-nm Excitation		
			$(\langle\rho\rangle/\tau)_{25°}$	τ (nsec)	$\langle\rho\rangle_{25°}$[b] (nsec)	$(\langle\rho\rangle/\tau)_{25°}$	τ (nsec)	$\langle\rho\rangle_{25°}$[b] (nsec)
0	0	4.13						
1.5	1.0	4.16	6.6	15.9	105	7.9	13.0	103
3.0	2.0	4.13	7.2	15.8	114	8.2	13.4	108
4.5	3.0	4.16	7.7	15.5	119	8.0	13.4	107
6.0	3.98	4.15	8.2	15.4	126	8.0	13.1	105
7.5	4.74	4.13	8.5	15.0	128	8.2	12.6	103

[a] Experimental conditions: 0.10 M phosphate (pH 7.0) and 1.5×10^{-4} M bovine serum albumin. [b] Coefficient of variation is 3%.

excited state, τ, is given by the Perrin equation, where p_0 is the limiting polarization observed in rigid media.

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$$

Since ρ is directly proportional to η/T (the ratio of the viscosity of the solvent to the temperature in degrees Kelvin), a plot of $1/p$ against T/η gives a straight line with intercept at $1/p_0$ when $T/\eta \to 0$. The relaxation time at a specified temperature and viscosity is easily calculated

$$\langle\rho\rangle = \frac{3\tau\left(\frac{1}{p_0} - \frac{1}{3}\right)}{\left(\frac{1}{p} - \frac{1}{p_0}\right)}$$

The values of $\langle\rho\rangle$ presented here were calculated from the fluorescence polarization data and the lifetimes of the excited state determined directly by use of a phase fluorometer (Spencer and Weber, 1968).

Weber and Young (1964) and Weber and Daniel (1966) found that the fluorescence polarization of the 1-anilino-8-naphthalenesulfonate–bovine serum albumin complexes observed upon excitation at 366 nm decreases monotonically with increasing $\bar{n}$. Any of three factors could contribute to this phenomenon: changes in the rotational relaxation time of the bovine serum albumin molecule, variation in the lifetime of the excited state, and energy transfer among bound 1-anilino-8-naphthalenesulfonate residues. The values of $\langle\rho\rangle/\tau$ determined by Weber and Daniel (1966) varied from 6.6 ± 0.2 when $\bar{n} \leq 1$ to 7.7 when $\bar{n} = 2$. However, they did not consider the significance of the variation and concluded that energy transfer alone was responsible for the change in polarization. Absolute values were not assigned to $\langle\rho\rangle$ since direct measurements of τ were not available. However, the constancy of the fluorescence yield was invoked to rule out variation in τ.

We have extended the measurements of $\langle\rho\rangle/\tau$ and p to cover the range of $\bar{n}$ from 1 to 5. The results indicate that not only is the change in $\langle\rho\rangle/\tau$ at $\bar{n} = 2$ significant, but that the increase continues beyond $\bar{n} = 2$ (Figures 1 and 2). Direct measurements of τ carried out with the cross-correlation phase fluorometer give values decreasing from 16 to 15 nsecs when $\bar{n}$ varies from 1 to 5. Thus $\langle\rho\rangle$ increases from 105 ± 3 nsec at $\bar{n} = 1$ to 128 ± 3 nsec at $\bar{n} = 5$ (Table I).

If the 23% increase in $\langle\rho\rangle$ reflects a gross change in the hydrodynamic properties of the bovine serum albumin molecule, there must be related changes in the frictional coefficient for translation. Careful determinations of

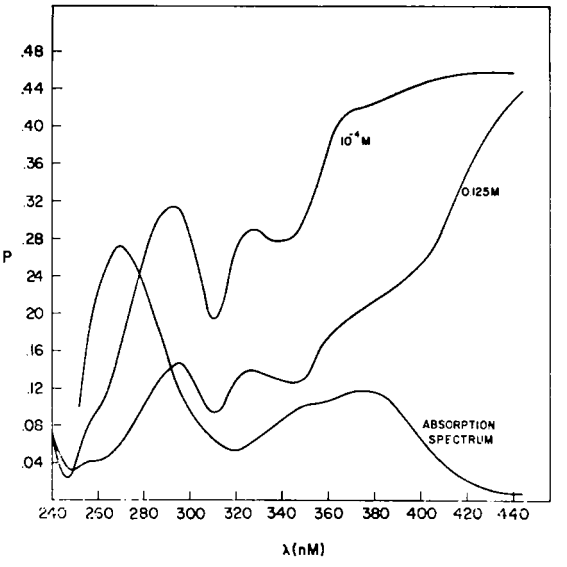

FIGURE 3: Fluorescence polarization spectra of dilute (10^{-4} M) and concentrated (0.125 M) solutions of 1-anilino-8-naphthalenesulfonate in propylene glycol at −55°. The absorption spectrum at 25° is superimposed to demonstrate the correlation of the electronic transitions with the different polarization values. (This figure has been reproduced from Weber, 1968.)

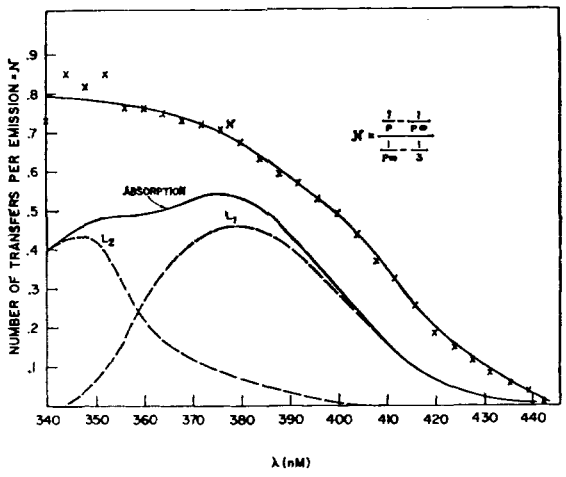

FIGURE 4: A plot of N, the average number of *effective* transfers per emission in the concentrated solution of 1-anilino-8-naphthalenesulfonate, against the wavelength of the exciting light (x-x). The absorption spectrum (——) and the positions of the two overlapping absorption bands estimated from the polarization spectra of dilute solutions (- - -) are superimposed. The experimental conditions are given under Figure 3.

$s_{20,w}$, however, reveal constancy within 1% (Table I). Apparently a true hydrodynamic change is not involved.

Is the increase in $\langle\rho\rangle$ related to the increasing probability of transfer? The calculations in the preceding paper (Weber and Anderson, 1969) show that if the bovine serum albumin molecule preserves its size and shape at all degrees of binding, it would be reasonable to attribute the variation in $\langle\rho\rangle$ with $\bar{n}$ to preferential orientation of the ligand. However, it is possible to go beyond this and to directly correlate the increase in $\langle\rho\rangle$ with transfer.

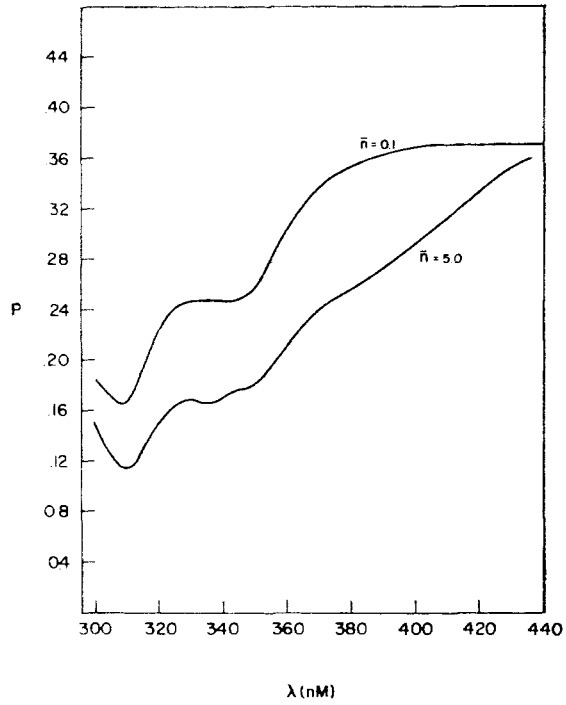

FIGURE 5: Fluorescence polarization spectra of 1-anilino-8-naphthalenesulfonate–bovine serum albumin complexes when $\bar{n}$ = 0.1 and when $\bar{n}$ = 5. Conditions: 0.10 M potassium phosphate, pH 7.0, 5°.

TABLE II: Fluorescence Spectra of 0.16 M 1-Anilino-8-naphthalenesulfonate Dissolved in Propylene Glycol.[a]

Wavelength of Exciting Light (nm)	$\lambda_{-1/2}$ (nm)	λ_{max} (nm)	$\lambda_{+1/2}$ (nm)
270	446	480	525
290	447	480	525
320	446	480	523
340	446	480	523
360	447	480	523
400	446	480	523
420	447	482	525
440	450	484	528

[a] Thin layers 1 mm in depth were examined. Band widths of excitation and emission: 3 mn. Note: the spectra are not corrected for grating transmission and photomultiplier response.

Dependence of Energy Transfer upon the Wavelength of the Exciting Light. Fluorescence polarization spectra of thin layers of indole solutions in propylene glycol have shown that the concentration depolarization disappears upon excitation at the long-wavelength edge of the absorption band (Weber, 1960). In our search for a way to correlate the apparent increase in relaxation time with transfer among preferentially bound 1-anilino-8-naphthalenesulfonate molecules, we have obtained fluorescence polarization excitation spectra of 1-anilino-8-naphthalenesulfonate dissolved in propylene glycol at −55° (Figure 3). Propylene glycol forms a rigid glass at this temperature. Light of varying wavelength, with a band width of 10–20 Å over most of the spectrum, was used for excitation. The over-all polarization of the emission at wavelengths greater than 500 mμ was recorded.

The polarization spectrum of a dilute (10^{-4} M) solution of 1-anilino-8-naphthalenesulfonate reflects the relative contributions of the electronic transitions in absorption (Figure 3). In fact we can outline the individual positions of the two overlapping absorption bands which compose the long-wavelength absorption peak (Figure 4). Transfer among 1-anilino-8-naphthalenesulfonate molecules in a concentrated (0.125 M) solution results in a proportional decrease in polarization throughout the region 260–380 nm. However, the extent of transfer declines steadily upon excitation at wavelengths greater than 380 nm. The average number of *effective* transfers per emission in the concentrated solution of 1-anilino-8-naphthalenesulfonate can be calculated from its polarization, p, and the corresponding polarization, p_∞, of a dilute solution in which no transfer occurs (Weber, 1966).

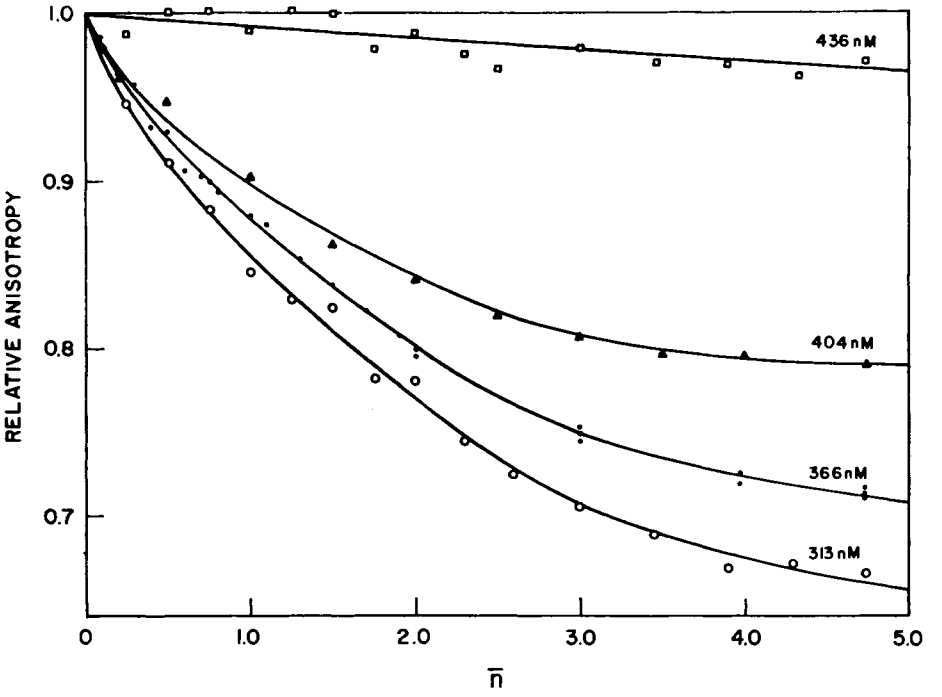

FIGURE 6: Plot of relative emission anisotropy ($A(\bar{n})/A(\bar{n} \rightarrow 0)$) as a function of $\bar{n}$ at different wavelengths of excitation: 436 nm, □: 404 nm, ▲; 366 nm, ●; and 313 nm, ○. The absolute values of $A(\bar{n} \rightarrow 0)$ are as follows: 436 nm, 0.408; 404 nm, 0.407; 366 nm, 0.343; and 313 nm, 0.248. Conditions: 0.10 M potassium phosphate, pH 7.0, 25°; 10 mg/ml of bovine serum albumin.

$$N = \frac{\frac{1}{p} - \frac{1}{p_\infty}}{\frac{1}{p_\infty} - \frac{1}{3}}$$

A plot of N *vs.* wavelength shows that the degree of transfer varies from 0.8 at 340 nm to approximately 0 at 440 nm (Figure 4). There appears to be no correlation between the change in N and the positions of the two overlapping absorption bands estimated from the polarization spectra of dilute solutions. The identity of the emission spectra found upon excitation in the range 270–440 nm rules out interference from a fluorescent impurity (Table II).

Clearly this represents a second example of energy transfer dependent upon the wavelength of excitation. This phenomenon, which seems to be general, is presently under study. For our present purpose it is sufficient to know that transfer fails to occur upon excitation at 436 nm.

The dependence of transfer upon exciting wavelength was found in 1-anilino-8-naphthalenesulfonate–bovine serum albumin adsorbates just as in concentrated solutions of 1-anilino-8-naphthalenesulfonate in propylene glycol (Figures 5 and 6). The polarization on excitation at 436 nm varies only 3% throughout the range of $\bar{n}$; concurrently $\langle\rho\rangle/\tau$ becomes independent of the number bound (Table I and Figure 2). Therefore, the intrinsic correlation between increase in relaxation time and transfer is confirmed.

The value of $\langle\rho\rangle/\tau$ found upon excitation by the 436-nm where transfer does not take place is virtually independent of $\bar{n}$ and conspicuously higher than the value for $\bar{n} \rightarrow 0$ found on excitation by the 366-nm wavelength of Hg (Figure 2). This suggested that the lifetime of the excited state found upon excitation at 436 nm is appreciably shortened; the directly measured value is in fact 13.3 nsec (Table I). Therefore, the corresponding value of $\langle\rho\rangle$ is 105 nsec, in good agreement with the value at 366 nm when $\bar{n} \rightarrow 0$. The differences in τ may reflect the heterogeneity of the adsorbed population as regards its absorption spectrum and lifetime, or a property of the 1-anilino-8-naphthalenesulfonate molecule. Experiments to determine this point are being presently carried out.

The Decrease in Limiting Polarization with $\bar{n}$. To ensure that migration of the excited state is the only variable source of depolarization, energy-transfer calculations should be based on measurements of the limiting polarization, p_0. In their analysis, Weber and Daniel (1966) assumed that the decrease in polarization with $\bar{n}$ found upon excitation at 366 nm was accounted for entirely by variation in p_0. Since our observations on the variation $\langle\rho\rangle$ with $\bar{n}$ show that the polarization actually involves two parameters, we have repeated the analysis using the extrapolated values of $A_0(\bar{n})$ instead of $A(\bar{n})$ (Figure 7). The reader is referred to the paper of Weber and Daniel (1966) for the details of the calculations.

We shall only restate here that the analysis permits determination of two values: A_T, the anisotropy of the fluorescence emitted after one transfer, and $\epsilon = \upsilon/\lambda$, the average ratio of the rates of transfer, υ, and emission, λ. These values are used to construct an equivalent system in which actual distance, r_e, and orientation, θ_e, are as-

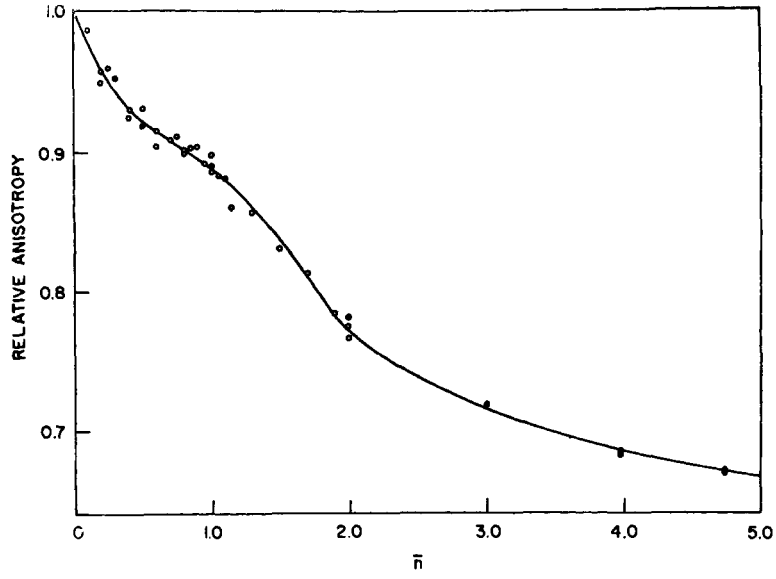

FIGURE 7: Plot of $A_0(\bar{n})/A_0(\bar{n} \rightarrow 0)$, the ratio of the limiting anisotropies, for various values of $\bar{n}$. Wavelength of exciting light: 366 nm. The absolute value of $A_0(\bar{n} \rightarrow 0)$ is 0.478. The other experimental conditions are given under Figure 6. ●, Sucrose was added (25°); ○, the temperature was varied.

signed to an average pair of oscillators occupying two binding sites. In this equivalent system, the depolarizing effects would be the same as those observed in the experiments. While Weber and Daniel (1966) estimated $\epsilon \simeq 0.58$ from the set of values of $A(\bar{n})$, calculations made from the limiting polarizations lead to $\epsilon \simeq 0.75$ (Figure 8) and $A_T = 0.264$. Nevertheless, the new values of θ_e and r_e, 33° and 20 Å, do not differ appreciably from the old ones of 33° and 21 Å. Hence, the conclusions of Weber and Daniel are vindicated.

The average angle, λ, between the oscillators of absorption and emission can be calculated from the limiting polarizations (Jablonski, 1935) since

$$\left(\frac{1}{p_0} - \frac{1}{3}\right) = \frac{5}{3}[2/3(\cos^2 \lambda - 1)]$$

The average angle between oscillators of absorption and emission calculated from the limiting anisotropy observed in the absence of transfer is 21° 30′ for excitation by the 366-nm wavelength of Hg. Similarly, the average angle between donor and acceptor oscillators in transfer estimated from A_T is 37° 40′.

The plots of $\langle\rho\rangle/\tau$ and of the limiting anisotropy *vs.* $\bar{n}$ reveal a distinct inflection near $\bar{n} = 1$ (Figures 2 and 7). The position of this inflection coincides with that found in the titration curves of bovine serum albumin and 1-anilino-8-naphthalenesulfonate (Daniel and Weber, 1966). However, the changes in p_0 and ρ_0 compensate each other so that the polarization measured at 20–25° in 0.1 M phosphate buffer simply decreases continuously with increasing $\bar{n}$ (Figure 6). Thus

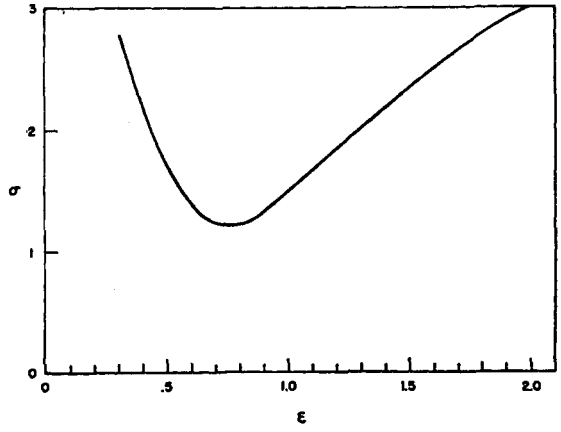

FIGURE 8: Plot of σ, the average standard deviation between the anisotropies due to specified systems of equivalent oscillators and the experimental data given in Figure 7 (see Weber and Daniel, 1966). ϵ is the average ratio of the rates of transfer and emission.

FIGURE 9: The experimental data of Figure 2 replotted to show the dependence of $\langle\rho\rangle/\tau$ upon the average degree of transfer calculated from the experimental values of $\bar{n}$ and a value for ϵ of 0.75. Random distribution of the ligand among binding sites was assumed; 366-nm excitation.

Weber and Daniel (1966) could not observe this detail in their measurements.

Discussion

Measurements of $\langle\rho\rangle$ and τ of 1-anilino-8-naphthalenesulfonate–bovine serum albumin absorbates show that as $\bar{n} \rightarrow 0$, $\langle\rho\rangle$ approaches 105–110 nsec. This value is significantly lower than the value of 130 nsec obtained from measurements of $\langle\rho\rangle$ in 1-dimethylaminonaphthalene-5-sulfonate conjugates, for which the observed relaxation time is close to the harmonic mean of the principal relaxation times of rotation (Weber, 1952; Moser *et al.*, 1966). This value is obtained either by variation of temperature or isothermal increase in viscosity and is therefore free of effects arising from thermally activated ligand rotations (Wahl and Weber, 1967). The simplest explanation of the low relaxation time is found in the preferential equatorial orientation of the bound ligand. In fact, the value of ρ_0 reported by Moser *et al.* (1966) is 110 nsec.

The increase in rotational relaxation time from 103 nsec at $\bar{n} = 1$ to 128 nsec at $\bar{n} = 5$, where 75% of the fluorescence is emitted after transfer, agrees well with the increase expected for ligands equatorially placed on a prolate ellipsoid of axial ratio 4 if the ligands are coplanar but otherwise not preferentially oriented (compare Figure 9 with Figure 10 of Weber and Anderson, 1969). A monotonic increase in $\langle\rho\rangle$ with $\bar{n}$ could also be expected for axial orientation if the oscillators of absorption and emission are astride the axis of revolution (Figure 5b of Weber and Anderson, 1969). From Figure 5b of Weber and Anderson (1969) it is easy to calculate that such a model would give $\langle\rho\rangle = 180$ nsec at $\bar{n} = 0$ and $\langle\rho\rangle = 220$ nsec at $\bar{n} = 5$. Clearly the absolute values of the rotational relaxation times are sufficient to exclude this model.

We are therefore led to the conclusions that: (1) the increase in $\langle\rho\rangle$ with $\bar{n}$ reflects the preferential orientation of the bound ligands with respect to the axis of revolution of the hydrodynamic ellipsoid. (2) Apparently the only model which can account for the increase in $\langle\rho\rangle$ with $\bar{n}$ and for the absolute values observed is one in which the ligands are located in planes closely if not rigorously parallel to the equator (Figure 10). It is not necessary to postulate any further orientation of the ligands with respect to each other to obtain qualitatively and quantitatively the results observed.

Acknowledgment

We thank Mr. Richard D. Spencer, who kindly undertook the measurements of fluorescence lifetimes for us.

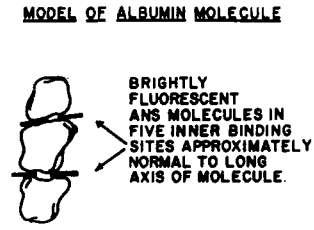

FIGURE 10: Hypothetical model demonstrating the preferential equatorial orientations of the molecules of 1-anilino-8-naphthalenesulfonate bound to bovine serum albumin.

Added in Proof

Recent observations of fluorescence lifetime of the anilinonaphthalenesulfonate–bovine serum albumin complexes (T. Pasby and G. Weber, unpublished data) have shown a small but significant decrease of about 8% in the interval $\bar{n} = 1$ to $\bar{n} = 0.1$. Thus, $\langle\rho\rangle$ is constant at approximately 105 nsec over this interval.

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