The Effects of Energy Transfer and Rotational Diffusion upon the Fluorescence Polarization of Macromolecules*

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ABSTRACT: The rotational relaxation time of a macromolecule computed from data of fluorescence polarization is an average of the principal relaxation times of the associated hydrodynamic ellipsoid weighted according to the orientation of the transition moments in absorption and emission with respect to the ellipsoid's axes. Memming's equations, describing the fluorescence polarization of these cases, are transformed so as to express the polarization as an explicit function of the angle θ between the transition moments, and the angle γ between the plane containing them and the equator of the ellipsoid. This transformation is particularly suitable to enumerate the cases arising when an aromatic ligand with $\pi \rightarrow \pi^*$ electronic transitions in absorption and emission is bound to a macromolecule with preferential orientation. The effects of energy transfer among bound ligands may also be easily visualized and calculated. We have made calculations for a typical prolate ellipsoid of axial ratio 4, varying systematically θ, γ, and the fraction transferred when more than one ligand is involved, in the following cases: (a) bound single ligand, (b) two ligands bound in the same plane in fixed relative orientations, and (c) coplanar ligands having no further fixed orientation with respect to each other. These considerations open up the possibility of demonstrating the preferential orientation of multiple bound ligands by the effects of energy transfer upon the apparent rotational relaxation time. A practical case of this type is described in the following paper (Anderson, S. R., and Weber, G. (1969), *Biochemistry 8, 371*).

The polarization of the fluorescence of labeled macromolecules has been extensively used for the determination of rotational relaxation times of proteins in solution (Weber, 1953; Steiner and Edelhoch, 1962). In general each macromolecule may carry one or more fluorescent oscillators but in the development of the theory of rotational depolarization the possibility of energy transfer among the labeling oscillators has been neglected, although the distances among the labels within one macromolecule are often those at which transfer might be expected to occur.

Similar qualitative considerations indicate that energy transfer can modify the character of the rotational depolarization and result in apparent rotational relaxation times very different from those measured in the absence of transfer. To demonstrate this situation in qualitative fashion we shall consider the disorientation by the Brownian motion of a set of molecules with cylindrical symmetry (Figure 1). The rotations of the molecule may be described by specifying the Euler angles and the motion may then be thought as consisting of a rotation about an arbitrary axis through the equatorial plane (axial motion) and a rotation of the equator about the cylinder axis (equatorial motion). It is known from a classical paper of Perrin (1934) that for a prolate ellipsoid of revolution of fixed volume the axial motion becomes increasingly slow as the ratio of the axes increases, while the equatorial motion is not appreciably affected. Let this prolate ellipsoid of revolution carry two oscillators between which energy transfer may take place. Transfer of the excitation from one of the oscillators to the other has exactly the same depolarizing effects as a rotation which would bring the excited donor oscillator into coincidence with the acceptor one. If both oscillators are contained in an axial plane[1] transfer among them is equivalent in its effects to the axial or slow rotational motion. If the two oscillators are contained in equatorial planes, transfer imitates the equatorial or fast rotational motion. Such replacements of the rotational motions by transfer are insensitive to the viscosity and thermal energy of the medium and reveal themselves in the value of the limiting polarization, p_0, observed in the absence of molecular rotations. However, they have a further result: they limit the depolarizing effect of the rotational motion they replace. This is easily visualized by considering a particular case, that in which the transfer effectively leads to a random orientation of the direction of the oscillators either in the axial or the equatorial plane. In the former case the depolarizing effect of the Brownian rotations is limited to the fast equatorial rotations, and the relaxation time of rotation calculated from the dependence of the polarization of fluorescence upon the viscosity will appear to be shorter than in the absence of transfer. On the other hand, in the case in which the oscillators contained in equatorial planes reach random orientation by transfer, only the slow axial rotations can further modify the distribution

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[1] Axial planes are planes containing the axis of revolution. Equatorial planes are at right angles to the axis of revolution.

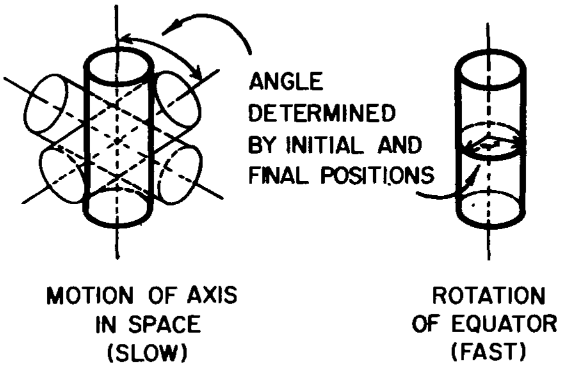

FIGURE 1: Modes of disorientation of the cylinder. (This figure has been reproduced from Weber, 1968.)

of the oscillators in space and the calculated rotational relaxation times will appear longer than in the absence of transfer. In an oblate ellipsoid of revolution, in which axial and equatorial rotations are slowed down to the same extent when the axial ratio increases (Perrin, 1934), energy transfer cannot give rise to the effects described.

In the above discussion we have referred to transfer of the excitation between two oscillators; formally the same considerations apply when these two oscillators are, respectively, the oscillator of absorption and emission of the same molecule, in which case all the excitations of the former are transferred to the latter, and when transfer of a variable fraction of the excitations takes place from the oscillator of absorption of one ligand to the oscillator of emission of another ligand. Throughout the quantitative considerations that follow we will have reason to stress the similarity of both cases. In practice distinction between them will almost always be an easy one since only the transfer among different ligands is dependent upon the number bound.

Depolarization of the Fluorescence of Ellipsoidal Molecules by Brownian Rotations. Perrin (1936) and Memming (1961) have considered the problem of the polarization of the fluorescence of solutions in which the emitting elements are nonspherical. Memming's equations describe explicitly the depolarization of the fluorescence by the Brownian rotations of ellipsoids of revolution having axial relaxation time, ρ_a, and equatorial relaxation time, ρ_e, and transition moments in absorption and emission which lie in fixed directions with respect to the ellipsoid axis. The coordinates of these directions are shown in Figure 2. α_1 and α_2 are, respectively, the angles determined by the absorption and emission oscillators with respect to the axis of revolution, while ϵ is the angle determined by their projections on the equatorial plane. We can define the quantities or partial anisotropies

$$A_{01} = \frac{1}{4}(3\cos^2\alpha_1 - 1)(3\cos^2\alpha_2 - 1)$$

$$A_{02} = \frac{3}{4}\sin^2\alpha_1\sin^2\alpha_2(2\cos^2\epsilon - 1) \qquad (1)$$

$$A_{03} = 3\sin\alpha_1\cos\alpha_1\sin\alpha_2\cos\alpha_2\cos\epsilon$$

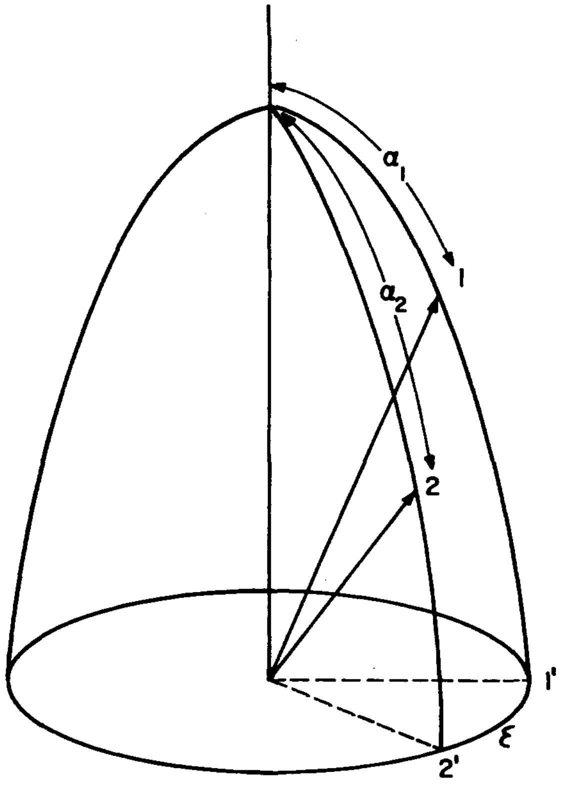

FIGURE 2: The coordinates referred to in eq 1. α_1 and α_2 are the angles formed by the directions of the transition moments with the axes of revolution. ϵ is the angle between their equatorial projections.

The anisotropy of emission, A (Jablonski, 1960; Weber, 1952), characteristic of the light emitted at right angles to the exciting linearly polarized beam is (Memming, 1961)

$$A = \frac{1}{\frac{1}{p} - \frac{1}{3}} = \frac{3}{5}\left(\frac{A_{01}}{1 + \frac{3\tau}{\rho_a}} + \frac{A_{02}}{1 + \frac{4\tau}{\rho_e} - \frac{\tau}{\rho_a}} + \frac{A_{03}}{1 + \frac{2\tau}{\rho_a} + \frac{\tau}{\rho_e}}\right) \qquad (2)$$

where p is the polarization of the emission and τ the lifetime of the excited state of the fluorescent molecules, the exponential decay of which is assumed. In the absence of Brownian rotations, that is, when τ/ρ_a and τ/ρ_e are negligible in comparison with 1

$$A = A_0 = \frac{3}{5}(A_{01} + A_{02} + A_{03}) \qquad (3)$$

If θ is the angle determined by the transition moments in absorption and emission

$$\cos\theta = \cos\alpha_1\cos\alpha_2 + \sin\alpha_1\sin\alpha_2\cos\epsilon$$

On introduction of the last equation, eq 3 rearranges to the well-known form

$$A_0 = \left(\frac{1}{p_0} - \frac{1}{3}\right)^{-1} = \frac{3}{5}\left(\frac{3}{2}\cos^2\theta - \frac{1}{2}\right) \qquad (4)$$

which permits the calculation of θ from the experimental value of the limiting polarization, p_0.

It is known that the anisotropies of emission A are additive quantities (Weber, 1952) so that in a system in which the coefficients A_{01}, A_{02}, and A_{03} result from the contribution of ligands differing in orientation with respect to the axes of ellipsoids of identical relaxation times, eq 2 applies with

$$\begin{aligned} A &= \bar{A} \\ A_{01} &= \sum_{i=1}^{i=N} A_{01}(i) f_i = \Sigma_1 \\ A_{02} &= \sum_{i=1}^{N} A_{02}(i) f_i = \Sigma_2 \\ A_{03} &= \sum_{i=1}^{N} A_{03}(i) f_i = \Sigma_3 \end{aligned} \qquad (5)$$

f_i being the fractional contribution of the ith component to the total emitted intensity.

Equation 4 is still valid with

$$\cos^2\theta = \sum_{i=1}^{N} \cos^2\theta_i f_i$$

In analyzing the effects to be expected in the case of prolate ellipsoids carrying preferentially oriented oscillators of absorption and emission, it is convenient to introduce in eq 2 the dimensionless variable $x = \tau/\rho_0$, where ρ_0 is the relaxation time of the rotation of a sphere having the same volume, V, as the ellipsoid. With this variable and the quantities defined by eq 5, eq 2 becomes

$$\bar{A} = \frac{3}{5}\left(\frac{\Sigma_1}{1 + 3x\frac{\rho_0}{\rho_a}} + \frac{\Sigma_2}{1 + x\left(\frac{4\rho_a}{\rho_e} - \frac{\rho_0}{\rho_a}\right)} + \frac{\Sigma_3}{1 + x\left(\frac{2\rho_0}{\rho_a} + \frac{\rho_0}{\rho_e}\right)}\right) \qquad (6)$$

Performing the indicated sum in the last equation and discarding terms in x^2 and higher orders[2]

$$\frac{1}{p} - \frac{1}{3} = \bar{A}^{-1} = \frac{5}{3}\frac{1}{\Sigma}\left[1 + \left(\frac{C_a}{\Sigma}\frac{\rho_0}{\rho_a} + \frac{C_e}{\Sigma}\frac{\rho_0}{\rho_e}\right)x\right] \qquad (7)$$

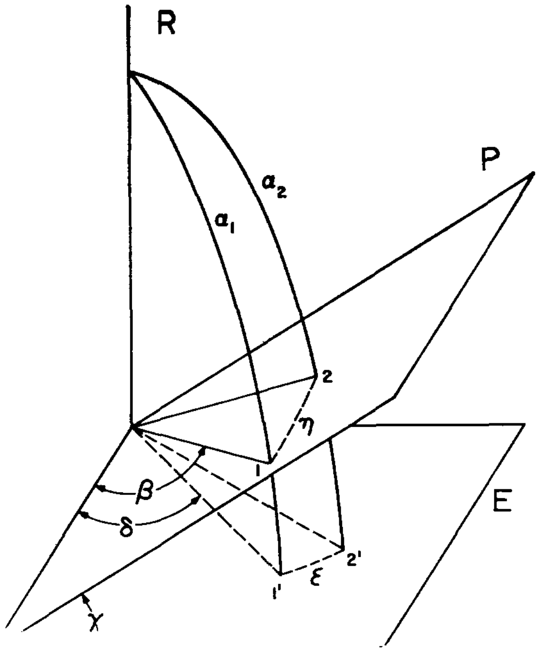

FIGURE 3: The coordinates referred to in eq 12. α_1 and α_2 have the same significance as in Figure 2. η is the angle between the transition moments; γ, the angle between the plane of the transition moments and the equatorial plane; β, the angle between one of the transition moments and the line of nodes; and δ, the projection of β on the equatorial plane.

in which

$$\begin{aligned} C_a &= 3\Sigma_1 - \Sigma_2 + 2\Sigma_3 \\ C_e &= 4\Sigma_2 + \Sigma_3 \\ \Sigma &= \Sigma_1 + \Sigma_2 + \Sigma_3 = \frac{1}{3}(C_a + C_e) \end{aligned} \qquad (8)$$

Experimentally it is customary to determine the apparent relaxation times $\langle\rho\rangle$ of the system from a plot of $1/p$ against T/η where T is the absolute temperature and η the viscosity coefficient of the solvent. If τ is assumed constant, this plot is equivalent to one of $1/p$ against τ/ρ_0, since $\rho_0 = 3\eta V/RT$. Moreover if the plot of the experimental data is linear it may be expressed by the relation

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + 3x\frac{\rho_0}{\langle\rho\rangle}\right) \qquad (9)$$

Comparing eq 7 and 9, we see that the experimental value $\langle\rho\rangle$ is given by

$$\langle\rho\rangle = \frac{3\Sigma}{\frac{C_a}{\rho_a} + \frac{C_e}{\rho_e}} \qquad (10)$$

Thus $\langle\rho\rangle$ is independent of Σ only if the two rotational

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[2] In the study of rotational relaxation times of proteins by means of fluorescence polarization, the value of τ is generally 20 nsec or less while ρ_0 is 50 nsec or larger. Therefore τ/ρ_0 is sufficiently small that neglect of terms in $(\tau/\rho_0)^2$ and higher order is justified as a first approximation.

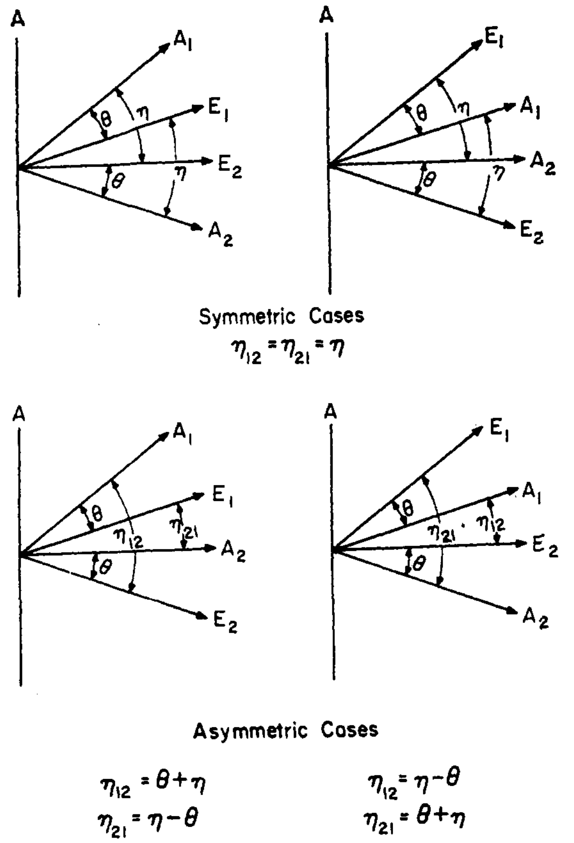

FIGURE 4: The four possible dispositions of two coplanar ligands when the orientation of the four oscillators with respect to the major axis (A) of the elliptical cross section containing them is given and θ is fixed. The reference angle referred to in Figures 5–8 corresponds to the angle AA_1 in the upper left-hand figure or to the angle AE_1 in the upper right-hand figure.

relaxation times are identical or at least experimentally indistinguishable.

Effects of Energy Transfer upon the Polarization of the Fluorescence. In the quantitative discussion of the effects of energy transfer we shall assume that the binding protein is a prolate ellipsoid of revolution carrying aromatic ligand molecules rigidly bound to it. In such aromatic molecules, absorption and emission arise in general from allowed $\pi \rightarrow \pi^*$ transitions, the moments of which are contained in the plane of the aromatic ring system. We shall consider the general case in which aromatic ligands lie in planes at an angle γ to the equator. If this angle is zero (equatorial ligands) or $\pi/2$ (axial ligands), transfer from the oscillator of absorption to that of emission, whether of the same or another ligand, changes only the equatorial or axial component-respectively. From the qualitative remarks of the introduction we expect these to be limiting cases in which the effects of Σ upon $\langle\rho\rangle$ will be most conspicuous. Results qualitatively similar, though quantitatively less striking, may be expected when γ is neither zero nor $\pi/2$ and transfer from the oscillator of absorption to that of emission changes both axial and equatorial components, although one of them to a greater extent than the other.

For the description of the effects to be observed with coplanar ligands, it is convenient to express the quantities A_{01}, A_{02}, and A_{03} in terms of the angle γ of the plane of the oscillators with respect to the equatorial plane and the angle η between the oscillators. The relevant angles are shown in Figure 3. R is the axis of revolution of the ellipsoid, E the equatorial plane, P the plane containing the ligands and therefore the oscillators 1 and 2 responsible for the vectorial character of the absorption and emission. η is the angle between them and β the angle between the first oscillator and the line of nodes where the planes P and E intersect at an angle γ. δ and $\delta + \epsilon$ are the equatorial components of vectors 1 and 2 and their difference is ϵ, the equatorial angle of eq 1. The following relations are easily worked out from the figure

$$\left.\begin{aligned}
\cos \alpha_1 &= \sin \beta \sin \gamma \\
\cos \alpha_2 &= \sin (\beta + \eta) \sin \gamma \\
\sin \alpha_1 &= \cos \beta / \cos \delta \\
\sin \alpha_2 &= \cos (\beta + \eta) / \cos (\delta + \epsilon) \\
\tan \delta &= \tan \beta \cos \gamma \\
\tan (\delta + \epsilon) &= \tan (\beta + \eta) \cos \gamma
\end{aligned}\right\} \quad (11)$$

Utilizing the above relations the coefficients A_{01}, A_{02}, and A_{03} may be written as a function of β, η, and γ instead of α_1, α_2, and ϵ.

$$\left.\begin{aligned}
A_{01} &= \frac{1}{4}(3 \sin^2 \beta \sin^2 \gamma - 1) \times \\
&\qquad (3 \sin^2 (\beta + \eta) \sin^2 \gamma - 1) \\
A_{02} &= \frac{3}{4}[(\cos^2 \beta - \sin^2 \beta \cos^2 \gamma) \times \\
&\quad (\cos^2 (\beta + \eta) - \sin^2 (\beta + \eta) \cos^2 \gamma) + \\
&\quad 4 \sin \beta \cos \beta \sin (\beta + \eta) \cos (\beta + \eta) \cos^2 \gamma] \\
A_{03} &= 3[\sin \beta \cos \beta \sin (\beta + \eta) \cos (\beta + \eta) \\
&\quad \sin^2 \gamma + \sin^2 \beta \sin^2 (\beta + \eta) \sin^2 \gamma \cos^2 \gamma]
\end{aligned}\right\} \quad (12)$$

A_{01}, A_{02}, and A_{03} are symmetric functions of β and $\beta + \eta$ which may be exchanged without changing the resulting equations. Thus a pair of directions in the ellipsoid defines a characteristic anisotropy independent of which of these two directions belongs to the emission or absorption oscillator.

Although eq 1 and 12 as originally derived apply to the oscillators of absorption and emission of a single molecule, the equations are equally applicable to a manifold of oscillators bound to a macromolecule. If the subscripts i and j assign the absorption and emission oscillator, respectively, to given ligands on the same macromolecule, A_{ii} represents the anisotropy expected when ligand i absorbs the exciting radiation and emits without transfer while A_{ij} is the anisotropy when ligand i is excited and ligand j emits after transfer. For a collection of N such ligands having identical probabilities of ab-

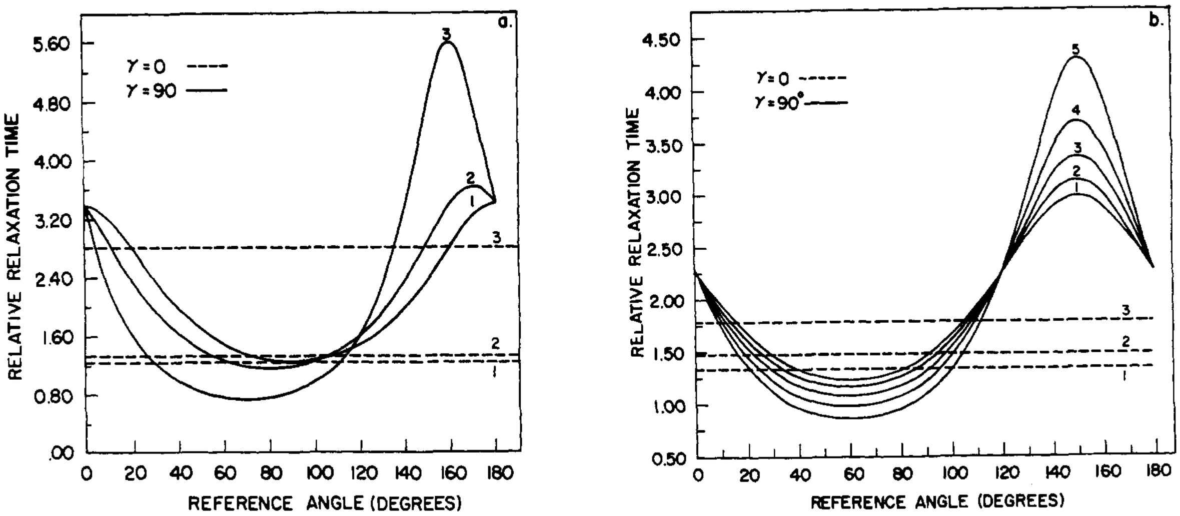

FIGURE 5: Mathematical studies. (a) Relative relaxation time $\langle\rho\rangle/\rho_0$ for single ligand in a plane at an angle γ to the equator of a prolate ellipsoid of axial ratio 4. When $\gamma = 90°$ (—), the major axis of the elliptic cross section is the axis of revolution of the ellipsoid. The ligands are equatorially placed when $\gamma = 0$ (- - - -). In each case, curves 1, 2, and 3 correspond to $\theta = 0$, 20, and 40°, respectively. (The values of $\langle\rho\rangle$ were calculated from the initial slopes of the Perrin plots in the region $0 \leq \tau/\rho_0 \leq 0.25$. Under these conditions, terms in $(\tau/\rho_0)^2$ and higher order may be neglected and deviations from linearity are less than 10%.) (b) $\langle\rho\rangle/\rho_0$ for a pair of symmetrically placed ligands (Figure 4) in a plane at angle γ to the equator when f, the fraction of the excitation transferred, is varied. θ and η are fixed at 21° 40′ and 37° 40′, respectively, since these angles describe the limiting polarizations observed for the complexes of 1-anilino-8-naphthalenesulfonate with bovine serum albumin upon excitation at 366 mm (refer to the following paper). The solid lines represent two axially placed ligands ($\gamma = 90°$). Curves 1–5 correspond to $f = 0$, 0.25, 0.50, 0.75, and 1, respectively. The dotted lines describe two equatorially placed ligands ($\gamma = 0$) with $f = 0$ (1), 0.5 (2), and 1 (3).

sorption the average anisotropy $\bar{A}$ is given by

$$\bar{A} = \frac{1}{N}\left(\sum_{i=1}^{N} f_{ii}A_{ii} + \sum_{i\neq j}^{N} f_{ij}A_{ij}\right) \qquad (13)$$

f_{ij} is the probability that transfer from i to j will follow excitation, so that

$$f_{ii} = 1 - \sum_{j\neq i=1}^{N} f_{ij}$$

The values of A_{ii} or A_{ij} are calculated by eq 2 and 12. The excited absorption oscillator determines β while η is given by the angle between the absorption and emission oscillators of the same ligand for A_{ii}, or the angle between the absorption oscillator of i and the emission oscillator of j for A_{ij}.

The values of f_{ij} form a square matrix, not necessarily symmetric

$$\begin{array}{l} f_{11}\, f_{12} \ldots\ldots f_{1N} \\ f_{21}\, f_{22} \ldots\ldots f_{2N} \\ f_{N1}\, f_{N2} \ldots\ldots f_{NN} \end{array} \qquad (14)$$

The matrix elements are *forward or effective transfer fractions*, quantities calculable in principle from the experimental data. We will not be concerned here with the possibility of back-transfers since these are equivalent to the absence of transfer and introduce therefore no observable effects. Back-transfers have to be considered in attempting a derivation of the f values from a given mechanism for energy transfer that specifies not only the angular and radial dependence of the probability of transfer but, most important and often forgotten, changes in these and in the intrinsic probability of transfer during the lifetime of the excited state.

Specific Models

Our purpose is to examine the effects of energy transfer in cases of ligand distributions which are relatively simple and demonstrate effects likely to be found in practice. We shall consider two circumstances: (1) transfer effects in a system of two ligands occupying fixed positions in the same plane; and (2) transfer effects in a system of ligands occupying the same plane but otherwise randomly oriented.

In our calculations we shall give f, the probability of transfer, all values from 0 to 1, in order to render evident the effects of energy transfer upon the polarization. If n molecules are present and electronic energy exchanges take place rapidly among them the probability of emission after transfer may reach the value $n/(n + 1)$ (Förster, 1947), so that for two molecules values of f greater than $^1/_2$ would not appear realistic. Nevertheless in cases in which more than two ligands are bound, the "second molecule" of the calculations could be considered as representative of the several molecules to which transfer may occur, and in such cases f can conceivably approach 1.

Transfer between Two Coplanar Ligands with Fixed Orientations. Figure 4 shows the four possible arrangements of the oscillators of absorption and emission of identical coplanar ligands when the four directions are

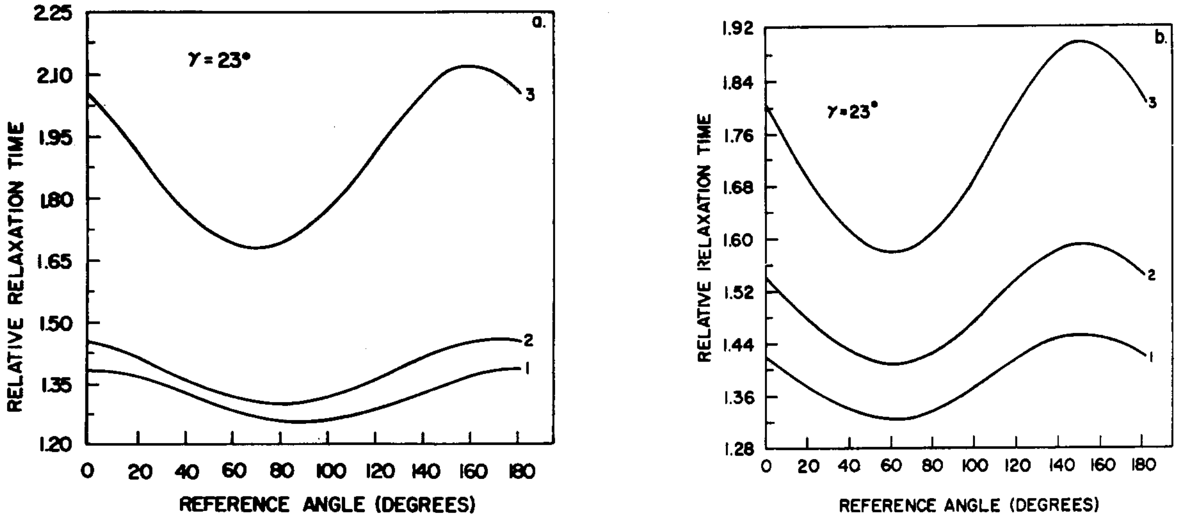

FIGURE 6: Mathematical studies. (a) Same as Figure 5a. $\gamma = 23°$. (b) Same as Figure 5b. $\gamma = 23°$.

fixed and the oscillators of absorption and emission of the same molecule are required to determine a given angle θ. Two of the arrangements are symmetric with respect to a line that bisects the system, while the other two are asymmetric. In the symmetric arrangements $\eta_{12} = \eta_{21} = \eta$, and since the probability of transfer is dependent upon η, $f_{12} = f_{21} = f$. In the asymmetric cases, on the other hand, $\eta_{12} = \eta \pm \theta$ and $\eta_{21} = \eta \mp \theta$. Therefore $f_{12} \neq f_{21}$ and the matrix (eq 14) is asymmetric. In the asymmetric cases, as shown in Figure 4, the observed effects will be the average of those expected from two symmetric cases in which the values of η differ by the amount 2θ. Therefore, the asymmetric cases will not be substantially different from the symmetric cases.

Each of the coefficients Σ_1, Σ_2, and Σ_3 of eq 6 is an average resulting from two contributions, one from the directly excited ligands, and therefore a function of θ alone, the other resulting from emission after transfer, a function of η alone

$$\Sigma_1 = (1 - f)\Sigma_1(\theta) + f\Sigma_1(\eta) \qquad (15)$$

and similar equations for Σ_2 and Σ_3.

The effect of the transfer is simply to alter the weight of the two contributions and the over-all effects may be simply visualized by considering the variation of $\langle\rho\rangle$ with θ *for a single isolated ligand* at fixed values of β and γ.

Figures 5a–8a show a series of plots of $\langle\rho\rangle/\rho_0$ against the reference angle (α) described in Figure 4. The general behavior of the curves is apparent from Figure 5a. For $\theta = 0$, that is, when the two oscillators are parallel, the maximum relaxation time is ρ_a when $\alpha = 0$ and the minimum ρ_e when $\alpha = \pi/2$. When the angle θ differs from zero, the maximum value of the relaxation time may be longer than ρ_a and the minimum shorter than ρ_e. The maximum value is reached when the axis bisects the angle between the two oscillators, and the minimum when this angle is bisected by the equator. The maxima occur therefore at $\alpha = \pi - (\theta/2)$ and the minima at $\alpha = (\pi/2) - (\theta/2)$.

In the case of two ligands occupying the axial planes, the results are those qualitatively expected from the behavior of one ligand for various angles of θ. As shown by eq 15, the increase of the fraction of transfers f from 0 to 1 is equivalent to a continuous variation of the angle between the oscillators of absorption and emission from θ to η. In comparing Figures 5–8a and 5–8b, it should be remembered that in the case of two ligands α is the angle made by one of the oscillators of one of the two ligands. In the calculations both ligands are supposed to be excited in equal proportions so that for the purpose of comparison, the angle with the elliptic axis in Figures 5a–8a should be changed from α to $\alpha + [(\theta + \eta)/2]$.

The figures show that transfer between two fixed, axially placed ligands may give rise to decrease or increase in $\langle\rho\rangle$ with increased energy transfer. The increase requires a more strict alignment of the oscillators with respect to the macromolecular geometry, and we shall see that for the case of random axial distribution, transfer always leads to a decrease in $\langle\rho\rangle$ for θ such that $\cos^2 \theta < 1/2$. Transfer among equatorially placed ligands gives rise always to an increase in $\langle\rho\rangle$ with fraction transferred and the question presents itself as to the possibility of discriminating between this case and the first of the two axial cases discussed. This may be done by the value of the absolute relaxation time recorded in the absence of transfer, which will be different in the two cases.

Random Coplanar Distributions. In cases of multiple binding sites there is clearly the possibility of the ligands lying in the same plane but without further orientation with respect to each other. In calculating such models of random coplanar orientation it is necessary to take into account the dependence of the probability of transfer upon the angle determined by the oscillators involved in the transfer. If two such oscillators determine an angle η in the plane thef raction of the excitations transferred

$$f(r,\eta) = \frac{\nu(r,\eta)}{\lambda + \nu(r,\eta)} \qquad (16)$$

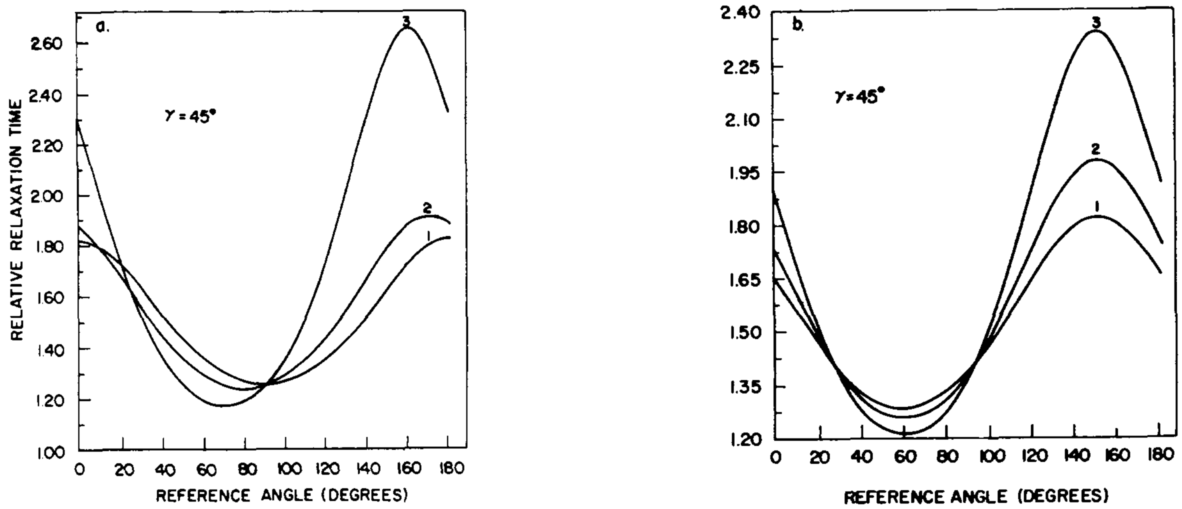

FIGURE 7: Mathematical studies. (a) Same as Figure 5a. $\gamma = 45°$. (b) Same as Figure 5b. $\gamma = 45°$.

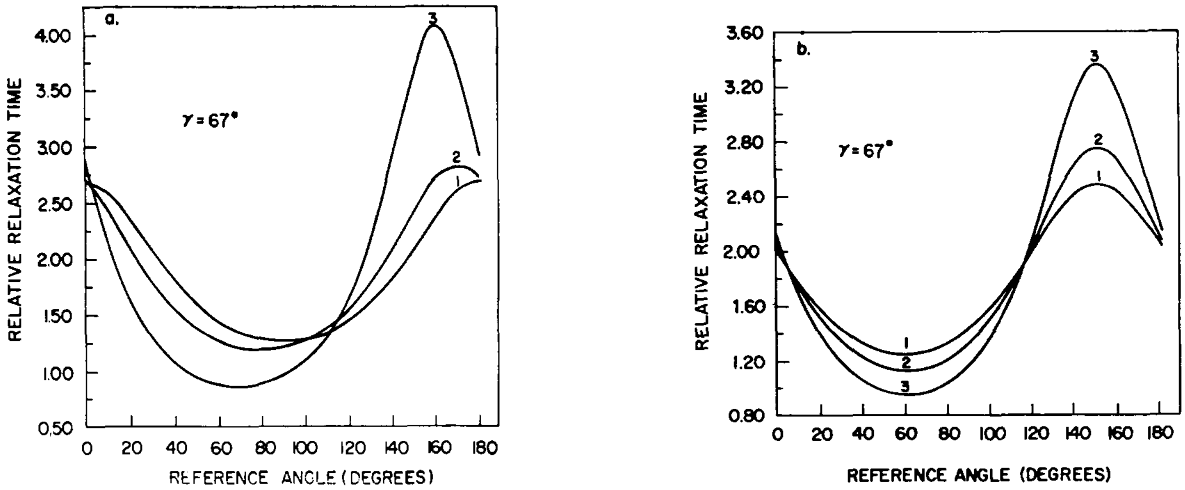

FIGURE 8: Mathematical studies. (a) Same as Figure 5a. $\gamma = 67°$. (b) Same as Figure 5b. $\gamma = 67°$.

is where $\nu(r,\eta)$ is the rate of transfer determined by their distance, r, and mutual orientation, and λ is the rate of emission. Expressing $\nu(r,\eta)$ as the product of two probabilities, one $\nu(r)$ depending upon r, the other $\nu(\eta)$ upon η, and assuming that distances and orientations are independent

$$f(\eta) \simeq \frac{\epsilon_r \nu(\eta)}{1 + \epsilon_r \nu(\eta)} \tag{17}$$

where $\epsilon_r = \nu(r)/\lambda$ is an average value that would apply if the oscillators were parallel oriented. For coplanar oscillators

$$\nu(\eta) = \cos^2 \eta \tag{18}$$

and

$$f(\eta) = \frac{\epsilon_r \cos^2 \eta}{1 + \epsilon_r \cos^2 \eta} \tag{19}$$

To perform the calculations we assume first that the oscillator of absorption of the excited molecule determines an angle β with the line of nodes. The anisotropy resulting from transfer of a fraction of the excitations $f(\eta)$ to a second ligand the oscillator of emission of which makes an angle η with the absorption oscillator of the first is a function of θ, β, and η, given by

$$A(\theta,\eta,\beta) = A(\beta,\beta \pm \theta)[1 - f(\eta)] + A(\beta,\eta)f(\eta) \tag{20}$$

$A(\beta,\beta \pm \theta)$ is the anisotropy of emission from the ligand directly excited and $A(\beta,\eta)$ is the anisotropy of emission of the ligand emitting after transfer. In random coplanar transfer θ is a fixed parameter while β and η assume random values in the interval $+\pi/2$ to $-\pi/2$. Since β and η are independent variables calculation of the first term in eq 20 involves averaging with respect to β while the second term involves a double average over β and η. The average anisotropy $\overline{\overline{A(\theta)}}$ is a function of θ and f_1 the average fraction transferred alone.

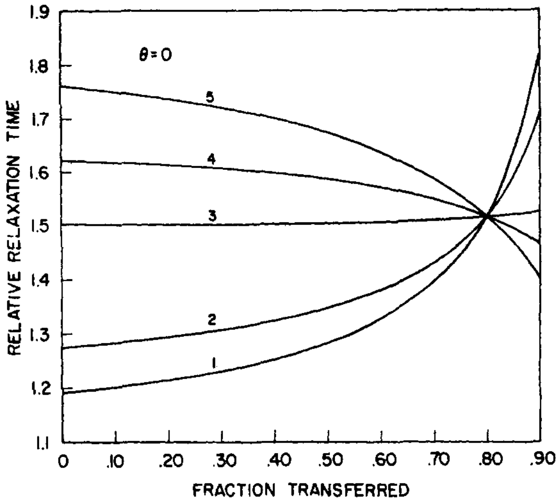

FIGURE 9: Random coplanar oscillators. Variation of $\langle\rho\rangle/\rho_0$ with the average fraction transferred. $\theta = 0°$. Axial ratio of prolate ellipsoid = 4. The curves correspond to different values of γ: 0° (1), 27° (2), 54° (3), 65° (4), and 90° (5).

$$\overline{\overline{A(\theta)}} = \frac{1}{\pi}\left(\int_{-\pi/2}^{+\pi/2} A(\beta,\beta \pm \theta)\mathrm{d}\beta\right)\left(1-\frac{1}{\pi}\int_{-\pi/2}^{+\pi/2} f(\eta)\mathrm{d}\eta\right)+ \frac{1}{\pi^2}\int_{-\pi/2}^{+\pi/2}\int_{-\pi/2}^{+\pi/2} A(\eta,\beta)f(\eta)\mathrm{d}\eta\mathrm{d}\beta = \overline{A(\theta)}\,(1-\bar{\bar{f}}_1) + \bar{A} \quad (21)$$

For any given angle β, $A(\beta,\beta \pm \theta)$ is made up of two contributions, $A(\beta,\beta + \theta)$ and $A(\beta,\beta - \theta)$, in which the emission oscillator has two different directions. Therefore

$$A(\beta,\beta \pm \theta) = \frac{1}{2}[A(\beta,\beta + \theta) + A(\beta,\beta - \theta)] \quad (22)$$

Similarly each of the anisotropies $A_{01}(\beta,\beta \pm \theta)$, $A_{02}(\beta,\beta \pm \theta)$, and $A_{03}(\beta,\beta \pm \theta)$ is made up of two terms which may be calculated by successively replacing η by $+\theta$ and by $-\theta$ in eq 12. Adding the two contributions, after developing the trigonometric functions of $(\beta + \theta)$ and $(\beta - \theta)$ into the products of functions of β and θ, and replacing $\cos^2\beta$ and $\cos^4\beta$ by their respective averages $^1/_2$ and $^3/_8$

$$\overline{A_{01}(\theta)} = \frac{1}{32}[8 - 24z + 9z^2 + 18z^2\cos^2\theta]$$

$$\overline{A_{02}(\theta)} = \frac{3}{32}[-8 + 8z + z^2 + (16 - 16z + 2z^2)\cos^2\theta] \quad (23)$$

$$\overline{A_{03}(\theta)} = \frac{12}{32}[-z^2 + (4z - 2z^2)\cos^2\theta]$$

$$\overline{A_0(\theta)} = \overline{A_{01}(\theta)} + \overline{A_{02}(\theta)} + \overline{A_{03}(\theta)}$$

$$z = \sin^2\gamma$$

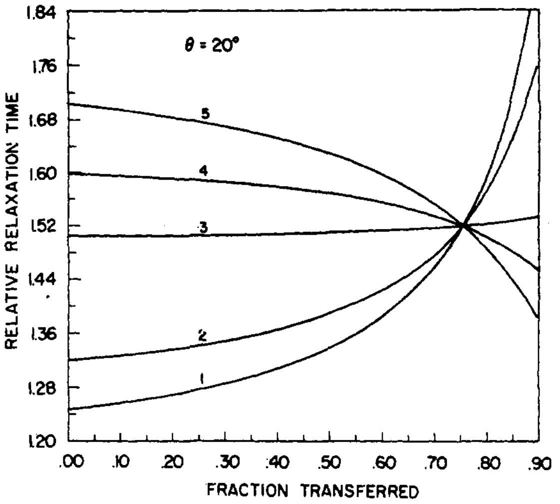

FIGURE 10: Same as Figure 9. $\theta = 20°$.

The coefficients $\overline{A_{01}(\beta,\eta)}$, $\overline{A_{02}(\beta,\eta)}$, and $\overline{A_{03}(\beta,\eta)}$ necessary to calculate the second term in the right-hand side of eq 21 involve the following integrals

$$\bar{f}_1 = \frac{\epsilon_r}{\pi}\int_{-\pi/2}^{+\pi/2}\frac{\cos^2\eta}{1 + \epsilon_r\cos^2\eta}\mathrm{d}\eta = 1 - \frac{1}{\sqrt{1 + \epsilon_r}}$$

$$\bar{f}_2 = \frac{\epsilon_r}{\pi}\int_{-\pi/2}^{+\pi/2}\frac{\cos^4\eta}{1 + \epsilon_r\cos^2\eta}\mathrm{d}\eta = \frac{1}{2} - \frac{1}{\epsilon_r}\left(1 - \frac{1}{\sqrt{1 + \epsilon_r}}\right) \quad (24)$$

$$\bar{f}_3 = \frac{\epsilon_r}{\pi}\int_{-\pi/2}^{+\pi/2}\frac{\cos^3\eta\sin\eta}{1 + \epsilon_r\cos^2\eta}\mathrm{d}\eta = 0$$

Introducing these, and the averages $\overline{\cos^2\beta} = {}^1/_2$, and $\overline{\cos^4\beta} = {}^3/_8$

$$A_{01} = \frac{1}{32}[(8 - 24z + 9z^2)\bar{f}_1 + 18z^2\bar{f}_2]$$

$$\overline{A_{02}} = \frac{3}{32}[(-8 + 8z + z^2)\bar{f}_1 + (16 - 16z + 2z^2)\bar{f}_2] \quad (25)$$

$$\overline{A_{03}} = \frac{12}{32}[-z^2\bar{f}_1 + (4z - 2z^2)\bar{f}_2]$$

It will be noticed that

$$\overline{A_0} = \overline{A_{01}} + \overline{A_{02}} + \overline{A_{03}} = \frac{3}{2}\bar{f}_2 - \frac{1}{2}\bar{f}_1$$

for any arbitrary value of z.

This result is of course to be expected since the limiting anisotropy A_0 must be dependent upon the angle between the absorption and emission oscillators, but not on the geometrical relations of the oscillators with the ellipsoid. The physical meaning of $\bar{f}_1$ is that of the

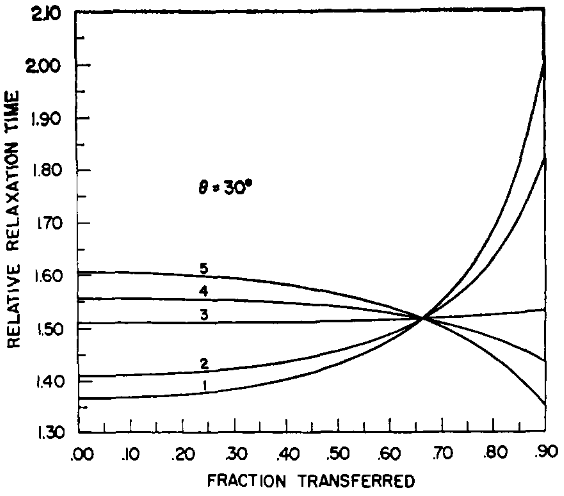

FIGURE 11: Same as Figure 9. $\theta = 30°$.

fraction of excitations transferred over an angle whose average cosine square is f_2. If there is no transfer to another ligand molecule all excitations may be considered as transferred to the oscillator of emission of the same molecule so that $f_1 = 1, f_2 = \cos^2\theta$. This substitution converts eq 25 into 23, and renders clear the reason for the similarity of the two sets of equations.

f_1 and f_2 in the case of random transfer are not independent, since according to eq 24

$$\bar{f}_2 = \frac{1}{2} - \frac{(1-\bar{f}_1)^2}{2-\bar{f}_1} \qquad (26)$$

By means of this relation f_2 may be eliminated from eq 25 giving

$$\overline{A_{01}} = \frac{1}{32}\frac{\bar{f}_1}{(2-\bar{f}_1)}[16 - 48z + 45z^2 - (8 - 24z + 27z^2)\bar{f}_1]$$

$$\overline{A_{02}} = \frac{3}{32}\frac{\bar{f}_1}{(2-\bar{f}_1)}[8 - 8z + 5z^2 - (8 - 8z + 3z^2)\bar{f}_1] \qquad (27)$$

$$\overline{A_{03}} = \frac{12}{32}\frac{\bar{f}_1}{(2-\bar{f}_1)}[6z - 5z^2 - (4z - 3z^2)\bar{f}_1]$$

Equations 27 and 24 permit the calculation of the coefficients Σ_1, Σ_2, Σ_3, and, together with eq 6, the average anisotropy to be expected in the case of random distribution of the coplanar oscillators for fixed values of θ, γ, and f_1. Moreover eq 10 permits the calculation of the relaxation time $\langle\rho\rangle$ that would be measured from the slope and intercept of the plot of $1/\rho$ against T/η

$$\langle\rho\rangle = \frac{3[(1-\bar{f}_1)\Sigma(\theta) + \bar{f}_1\Sigma(\eta)]}{\dfrac{C_a(\theta)(1-\bar{f}_1) + C_a(\eta)\bar{f}_1}{\rho_a} + \dfrac{C_e(\theta)(1-\bar{f}_1) + C_e(\eta)\bar{f}_1}{\rho_e}} \qquad (28)$$

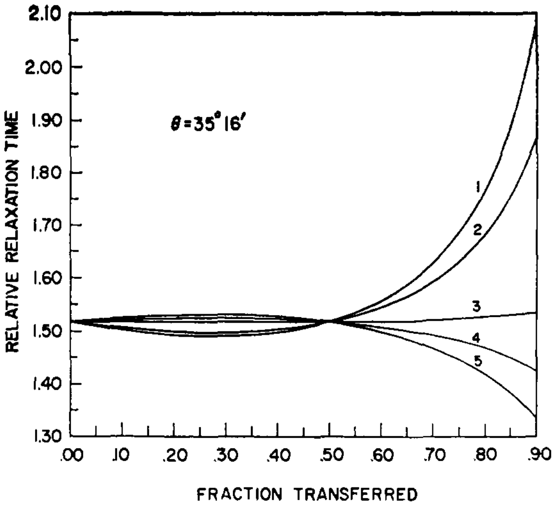

FIGURE 12: Same as Figure 9; $\theta = 35°\ 16'$. Note that for this value of θ, $\langle\rho\rangle$ is relatively constant when $f < 0.5$.

with

$$\left.\begin{aligned}
\Sigma(\theta) &= \frac{3}{2}\cos^2\theta - \frac{1}{2}; \quad \Sigma(\eta) = \frac{1}{4}\frac{(5-4\bar{f}_1)}{(2-\bar{f}_1)}\\
C_a(\theta) &= \frac{3}{2}[1 - \cos^2\theta + z(3\cos^2\theta - 2)]\\
C_e(\theta) &= \frac{3}{2}[4\cos^2\theta - 2 - z(3\cos^2\theta - 2)]\\
C_a(\eta) &= \frac{3}{4}[1 - z(2\bar{f}_1 - 1)]\frac{1}{(2-\bar{f}_1)}\\
C_e(\eta) &= \frac{3}{4}[4(1-\bar{f}_1) + z(2\bar{f}_1 - 1)]\frac{1}{(2-\bar{f}_1)}
\end{aligned}\right\} \qquad (29)$$

The six coefficients (eq 29), together with eq 28, permit the calculations of the limiting polarization and apparent rotational relaxation time to be observed when transfer takes place among ligands randomly distributed over a plane, at an angle γ to the equator, for arbitrary probabilities of transfer. It will be observed that as $f_1 \rightarrow 1$ the limiting polarization p_0 tends to $^1/_7$ independently of z as expected. On the other hand, the apparent rotational relaxation time undergoes large variations with z.

Figures 9–13 show the values of $\langle\rho\rangle$ to be expected for a typical prolate ellipsoid of axial ratio 4, when θ, γ, and f_1 are systematically varied. For values of θ such that $\cos^2\theta > {}^2/_3$, which are those obtaining when the wavelength of excitation falls within the absorption band of least frequency, transfer among axial ligands will decrease the rotational relaxation time while transfer among equatorial ligands will increase it. The plane with $z = {}^2/_3$ separates the "axial" from the '"equatorial" regions.

In conclusion we wish to stress that while there are various possibilities of choice to interpret an increase or decrease in $\langle\rho\rangle$ with transfer we have at our disposal two experimental variables, θ and f, to help in the determination of γ so that in many cases it will be possible to fix this value unequivocally.

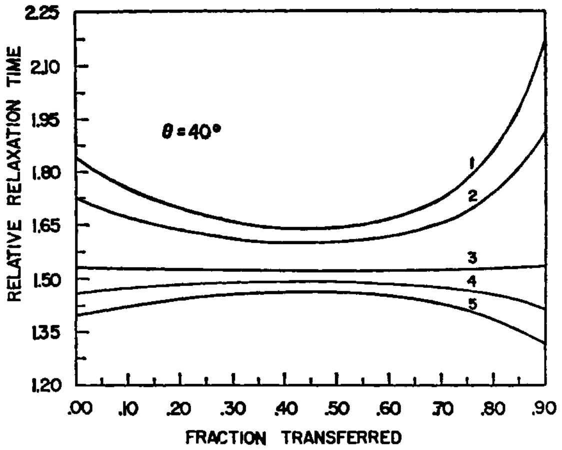

FIGURE 13: Same as Figure 9. $\theta = 40°$. This plot demonstrates inversion of the effects of f on $\langle\rho\rangle$ in the interval $f < 0.5$ for a large value of θ.

Notes on the Derivation of Equations. Three noncoplanar directions 0A, 0B, and 0C with common origin 0 determine the angles θ_{AB}, θ_{BC}, and θ_{CA}, related by the equation

$$\cos\theta_{CA} = \cos\theta_{AB}\cos\theta_{BC} + \sin\theta_{AB}\sin\theta_{BC}\cos\psi \quad \text{(I)}$$

where ψ is the angle between the planes that contain A and B and B and C, respectively.

If A is the axis of revolution, B is the line of nodes, and C the oscillator 1 (Figure 2) in eq I

$$\cos\alpha_1 = \cos\frac{\pi}{2}\cos\beta + \sin\frac{\pi}{2}\sin\beta\cos\left(\frac{\pi}{2} - \gamma\right) = \sin\beta\sin\gamma \quad \text{(II)}$$

If C is the oscillator at 2

$$\cos\alpha_2 = \sin(\beta + \eta)\sin\gamma \quad \text{(III)}$$

The relations

$$\cos\beta = \sin\alpha_1\cos\delta$$

$$\cos(\beta + \eta) = \sin\alpha_2\cos(\delta + \epsilon)$$

are immediate.

From the relations above we have squaring and adding

$$\cos^2\alpha_1 + \sin^2\alpha_1 = 1 = \sin^2\beta\sin^2\gamma + \frac{\cos^2\beta}{\cos^2\delta}$$

Dividing throughout by $\cos^2\beta$

$$1 + \tan^2\beta = \tan^2\beta\sin^2\gamma + 1 + \tan^2\delta$$

$$\tan^2\beta\cos^2\gamma = \tan^2\delta \quad \text{(IV)}$$

$$\tan\delta = \tan\beta\cos\gamma$$

Similarly

$$\tan(\delta + \epsilon) = \tan(\beta + \eta)\cos\gamma$$

To derive the coefficients A_{01}, A_{02}, and A_{03}, we require also some further relations containing $\tan\epsilon$. From eq IV

$$\tan\epsilon = \tan(\delta + \epsilon - \delta) = \frac{\tan(\delta + \epsilon) - \tan\delta}{1 + \tan(\delta + \epsilon)\tan\delta} = \frac{(\tan(\beta + \eta) - \tan\beta)\cos\gamma}{1 + \tan\beta\tan(\beta + \eta)\cos^2\gamma} \quad \text{(V)}$$

$$1 - \tan\delta\tan\epsilon = \frac{1}{\cos^2\delta[1 + \tan\beta\tan(\beta + \eta)\cos^2\gamma]} \quad \text{(VI)}$$

$$1 - \tan^2\epsilon = \frac{(1 - \tan^2\beta\cos^2\gamma)[1 - \tan^2(\beta + \eta)\cos^2\gamma] + 4\tan\beta\tan(\beta + \eta)\cos^2\gamma}{[1 + \tan\beta\tan(\beta + \eta)\cos^2\gamma]^2} \quad \text{(VII)}$$

A_{01} in eq 12 is obtained by replacing $\cos\alpha_1$ and $\cos\alpha_2$ in eq 2 by their values in II and III.

If $\sin\alpha_1$ and $\sin\alpha_2$ are replaced in eq 2 by their values in eq 11, we have

$$A_{02} = \frac{3\cos^2\beta\cos^2(\beta + \eta)}{4\cos^2\delta\cos^2(\delta + \epsilon)}(2\cos^2\epsilon - 1)$$

$$\frac{2\cos^2\epsilon - 1}{\cos^2(\delta + \epsilon)} = \frac{(2\cos^2\epsilon - 1)/\cos^2\epsilon}{\cos^2\delta(1 - \tan\delta\tan\epsilon)^2} = \frac{1 - \tan^2\epsilon}{\cos^2\delta(1 - \tan\delta\tan\epsilon)^2}$$

Replacing $1 - \tan\delta\tan\epsilon$ by its value in VI and $1 - \tan^2\epsilon$ by its value in VII

$$A_{02} = \frac{3}{4}(\cos^2\beta - \sin^2\beta\cos^2\gamma)(\cos^2(\beta + \eta) - \sin^2(\beta + \eta)\cos^2\gamma) + 4\sin\beta\cos\beta\sin(\beta + \eta)\cos(\beta + \eta)\cos^2\gamma$$

Introducing relations 11 into the third equation in eq 2

$$A_{03} = 3\sin^2\gamma\sin\beta\sin(\beta + \eta)\frac{\cos\beta\cos(\beta + \eta)}{\cos\delta\cos(\delta + \epsilon)}\cos\epsilon$$

$$\frac{\cos\epsilon}{\cos(\delta + \epsilon)} = \frac{1}{\cos\delta(1 - \tan\delta\tan\epsilon)}$$

Replacing $1 - \tan\delta\tan\epsilon$ by its value in VII

$$A_{03} = 3\sin^2\gamma\sin\beta\sin(\beta + \eta)\cos\beta\cos(\beta + \eta) + 3\sin^2\gamma\cos^2\gamma\sin^2\beta\sin^2(\beta + \eta)$$

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

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

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

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

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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.