

Effect of the Orientation of Donor and Acceptor on the Probability of Energy Transfer Involving Electronic Transitions of Mixed Polarization[†]

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ABSTRACT: The probability of transfer of electronic excitation energy between chromophores by the Förster mechanism depends, among other factors, on the orientation in space of the donor and acceptor chromophores. The orientational dependence of the transfer efficiency has been previously evaluated for the case in which the donor and acceptor electronic transitions are characterized by a single electric transition dipole moment at the range of spectral overlap between donor fluorescence and acceptor absorption. Polarization data for the chromophores involved show that this is often not the case and that the electronic transitions have mixed polarizations and are characterized by two or three transition dipole moments. In this paper we evaluate the orientational dependence of energy transfer when the chromophores have mixed polarizations. First, we relate the magnitude of the transition dipoles of a chromophore to its fluorescence polarization. Then, we evaluate the dependence of the probability of energy transfer on orientation in terms of the one, two, or three transition dipole moments associated with the electronic transitions of each of the chromophores participating in the energy transfer. Finally,

we calculate the probability for obtaining a given value for the orientational factor in an ensemble of donor-acceptor pairs the chromophores of which have random orientations but are fixed in space. The results obtained are applied to the evaluation of the distribution of end-to-end distances in a series of oligopeptides studied previously in viscous solutions by energy transfer between chromophores attached to the molecular ends. The donor and acceptor used in that study were naphthalene and dansyl, which have effective polarizations of 0.07 and 0.33, respectively, at the spectral range of interest. It is shown that in this case the orientational dependence of the probability of energy transfer affects the calculated end-to-end distribution function of the oligopeptides to a negligible extent. Calculated estimates are presented for the range of error that can occur in the evaluation of distances between chromophores by energy transfer when the orientations of the chromophores are fixed but unknown. The smaller the fluorescence polarization of the donor and acceptor, the smaller the error, which is not likely to exceed $\pm 10\%$ for polarizations smaller than 0.3.

Transfer of electronic excitation energy by the Förster mechanism has been proposed by Stryer & Haugland (1967) to serve as a tool for the measurement of distances between donor and acceptor chromophores in biological systems in the range of 10–50 Å. This approach has found many applications (Stryer, 1968; Steinberg, 1971). A major problem in the interpretation of energy transfer data in terms of the distance between donor and acceptor is that the probability of transfer depends not only on the distance to be evaluated but, among other parameters, also on the orientation of the donor and acceptor chromophores involved relative to the vector \mathbf{r} connecting them. The probability, $n_{A \rightarrow B}$, of energy transfer per unit time is given by (Förster, 1948):

$$n_{A \rightarrow B} = [9000 (\ln 10) \kappa^2 \Phi / 128 \pi^5 n^4 N r^6 \tau] \int_0^\infty \frac{f(\bar{\nu}) \epsilon(\bar{\nu})}{\bar{\nu}^4} d\bar{\nu} \quad (1)$$

where Φ is the quantum yield of the donor in the absence of acceptor, n is the index of refraction of the medium, N is Avogadro's number, r is the distance between donor and acceptor molecules, τ is the lifetime of the donor in the absence of acceptor molecules, $f(\bar{\nu}) d\bar{\nu}$ is the normalized fluorescence intensity of the donor in the wavenumber range $\bar{\nu}$ to $\bar{\nu} + d\bar{\nu}$, and $\epsilon(\bar{\nu})$ is the absorption coefficient of the acceptor at the wave-

number $\bar{\nu}$. κ^2 is the factor that expresses the orientational dependence of the probability of energy transfer and is given by the equation $\kappa = \cos \theta_{AB} - 3 \cos \theta_A \cos \theta_B$, where θ_{AB} is the angle between the donor and the acceptor transition dipole moments, and θ_A and θ_B are the angles between \mathbf{r} and the donor and acceptor dipoles, respectively. It should be emphasized that the above expression for κ is based on the assumption that the emission of the donor and absorption of the acceptor, at the range of their spectral overlap, are characterized each by a single transition dipole moment. Under such conditions κ^2 may assume values between 0 to 4, depending on the orientation of the donor and acceptor. Knowledge of the value of κ^2 is thus necessary in order to evaluate r from $n_{A \rightarrow B}$. Since the value of r , as calculated from energy transfer data, assuming an incorrect value for κ^2 , is inversely proportional to the true value of $(\kappa^2)^{1/6}$, it is obvious that values of κ^2 approaching zero will make the estimation of r most uncertain. Only when the chromophores undergo unrestricted fast Brownian rotation during the donor lifetime, can the orientational factor κ^2 be evaluated by averaging to yield a numerical value of 2/3 (Förster, 1949). It is pertinent to note that, whereas the dependence of $n_{A \rightarrow B}$ on r and on the overlap integral has been verified experimentally (Stryer & Haugland, 1967; Haugland et al., 1969), no such verification for the dependence of $n_{A \rightarrow B}$ on κ^2 has been reported apparently.

Various suggestions have been proposed to deal with the problem of the orientational factor in those cases in which the donor and acceptor do not span all orientations in space during the fluorescence lifetime of the donor. Among these are: the use of different donor-acceptor pairs, which might permit the

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sampling of different orientational combinations for the donor and acceptor (Stryer & Haugland, 1967), or taking advantage of the partial rotational diffusion that the chromophores can undergo (as revealed by the degree of polarization that they exhibit in their fluorescence) in order to set some limits on the range of κ^2 (Dale & Eisinger, 1974, 1975).

In the following we propose an alternative approach to the orientational problem, which makes use of the fact that many chromophores show mixed polarizations in their spectral behavior (Albrecht, 1960; Steinberg, 1975); i.e., their absorption or emission across various spectral ranges is characterized not by a single transition dipole moment, but rather by a combination of two or more incoherent dipole moments. The physical basis for the multiplicity of dipole moments may be either the presence of various electronic transitions in the pertinent spectral range of the absorption of the acceptor, or the partial forbidden character (Albrecht, 1960) of the electronic transitions of either donor or acceptor resulting in different polarization properties of the various vibronic transitions in a given electronic band.

As it will be shown below, the consequence of the occurrence of mixed polarizations for the angular dependence of the orientational factor in energy transfer may be far reaching, i.e., in a great number of cases a zero value of κ^2 is excluded, and its range of variation is markedly decreased as compared with corresponding cases in which the donor and acceptor are characterized by a single dipole each (Steinberg, 1975). In the extreme case that both donor and acceptor have three equal perpendicular transition moments, the probability of energy transfer does not depend at all on the orientation of the chromophores. In other cases, the effect is not as dramatic; nevertheless, the problem of the orientational factor in the use of nonradiative energy transfer for the estimation of distances between chromophores is markedly alleviated.

In the following we calculate the dependence of κ^2 on the orientation of the donor and acceptor chromophores when the electronic transitions involved in the energy transfer process are of mixed polarizations. The data thus obtained concerning the angular dependence of κ^2 enabled the evaluation of the probability that a donor-acceptor pair attains a given value of κ^2 when the chromophores are randomly oriented but fixed in space. Finally, we could refine the distribution functions for the distances between donor-acceptor pairs that have been previously derived from energy transfer data (Haas et al., 1975), and found that the effect of the orientational factor on these distribution functions is negligibly small. Included are also results of calculations which permit the estimation of the error in the determination of distances by energy transfer measurements for various pairs of chromophores which have a fixed but an unknown orientation. Throughout it will be assumed that transfer of energy can take place within each donor-acceptor pair belonging to the same molecule, and that the solution is sufficiently dilute to prevent energy transfer from the donor of one molecule to the acceptor of another.

Theory

The fluorescence polarization, p , of a chromophore in a frozen system is expected to be 0.5 if its electronic transitions are characterized by single transition dipole moments, and if the absorption and emission dipoles are parallel. The latter condition is usually fulfilled upon excitation at the long wavelength absorption band of the chromophore. Yet it is not often that a value of 0.5 is obtained for p even upon excitation at the long wavelength edge of the absorption. Values of p that are lower than 0.5 are often obtained even at very low temperatures in glasses of extremely high viscosity; occurrence of

Brownian rotation of the chromophores during the lifetime of the excited state can thus be eliminated. A low value of p thus indicates that the pertinent transitions have mixed polarizations; i.e., they are characterized by more than one electronic transition. This may be due to degeneracy in energy levels, as in spherically symmetric ions, or to vibronic mixing of levels if the transitions under consideration are weak (Albrecht, 1960). The vibronic mixing is particularly pronounced if there is a strong band in the vicinity of the weak band.

The evaluation of the orientational factor κ^2 in cases that the electronic transitions responsible for the energy transfer from donor to acceptor are of mixed polarization will be carried out in three consecutive steps. (a) The estimation of the magnitude of the mutually perpendicular noncoherent transition dipoles of the donor emission and the acceptor absorption in the spectral range of their overlap. The fluorescence polarization of the chromophores is used to derive this information. (b) The formulation of an expression for the magnitude of κ^2 in terms of the transition moments of the donor and acceptor derived according to step a. (c) The scanning, by computer simulation, over all orientations of the donor and acceptor at discrete intervals of rotation, and enumeration of the number of times different values of κ^2 are obtained. The probability distribution for obtaining a certain value for κ^2 is thus evaluated.

(a) *Estimation of the Transition Dipole Moments of a Chromophore from Its Polarization.* Since the long wavelength absorption band and the emission band presumably involve the same electronic transition, we assume that the same transition dipoles are involved in these two transitions. If a change in molecular conformation takes place upon electronic excitation, this assumption may not hold; however, it has been shown that in rigid media such changes tend to be arrested (Gafni et al., 1975). Furthermore, such changes are not expected to occur in many of the chromophores. Let us treat the general case in which three mutually perpendicular, noncoherent dipoles, V_1 , V_2 , and V_3 , characterize the transition under discussion, and let us choose the molecular x , y , and z axes so that they are parallel to V_1 , V_2 , and V_3 , respectively. The polarization, p , is given by:

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (2)$$

and I_{\parallel} and I_{\perp} are the fluorescence light intensities detected along the y axis and polarized along the z and x axis, respectively. The excitation light is assumed to travel along the x axis and be polarized along the z axis. I_{\parallel} and I_{\perp} are given by the following expressions (Jablonski, 1935):

$$\begin{aligned} I_{\parallel} = & \langle [(a_{31}V_1)^2 + (a_{32}V_2)^2 + (a_{33}V_3)^2][(a_{31}V_1')^2 \\ & + (a_{32}V_2')^2 + (a_{33}V_3')^2] \rangle = \frac{1}{5} (V_1^2V_1'^2 + V_2^2V_2'^2 \\ & + V_3^2V_3'^2) + \frac{1}{15} (V_1^2V_2'^2 + V_1'^2V_2^2 + V_1^2V_3'^2 \\ & + V_1'^2V_3^2 + V_2^2V_3'^2 + V_2'^2V_3^2) \quad (3) \end{aligned}$$

$$\begin{aligned} I_{\perp} = & \langle [(a_{31}V_1)^2 + (a_{32}V_2)^2 + (a_{33}V_3)^2] \\ & \times [(a_{11}V_1')^2 + (a_{12}V_2')^2 + (a_{13}V_3')^2] \rangle \\ = & \frac{1}{15} [V_1^2V_1'^2 + V_2^2V_2'^2 + V_3^2V_3'^2 + V_1^2V_2'^2 + V_1'^2V_2^2 \\ & + V_2^2V_3'^2 + V_2'^2V_3^2 + V_1^2V_3'^2 + V_1'^2V_3^2] \end{aligned}$$

where V_i ($i = 1, 2, 3$) are the dipole moments involved in the absorption; V_i' are the dipole moments involved in the emission; and a_{ij} are the elements of the transformation matrix for

TABLE I: Two Sets of Transition Dipole Moments (V_1 ; V_2 ; V_3) Which Might Be Assigned to an Electronic Transition Showing a Fluorescence Polarization p .^a

p	model A			model B		
	V_1	V_2	V_3	V_1	V_2	V_3
0.50	1.00	0.0	0.0	1.00	0.0	0.0
0.45	0.98	0.20	0.0	0.98	0.14	0.14
0.40	0.96	0.29	0.0	0.96	0.20	0.20
0.35	0.93	0.36	0.0	0.94	0.25	0.25
0.30	0.90	0.42	0.0	0.91	0.29	0.29
0.25	0.87	0.49	0.0	0.89	0.33	0.33
0.20	0.83	0.56	0.0	0.85	0.37	0.37
0.15	0.75	0.66	0.0	0.83	0.39	0.39
0.10	0.69	0.69	0.24	0.78	0.44	0.44
0.05	0.66	0.66	0.23	0.73	0.48	0.48
0.00	0.58	0.58	0.58	0.58	0.58	0.58

^a Table I was compiled by use of eq 3, assuming $V_i = V_i'$; i.e., the results apply to the emission dipoles and the absorption dipoles at the wavelength absorption band. It is obvious that the assignment of the vectors V_1 , V_2 , and V_3 to a given value of p is not unequivocal; however, any combination of dipole moments compatible with a given p value was found to yield very similar probability functions $P(\kappa^2)$, or $Q(r'/r)$ [see below].

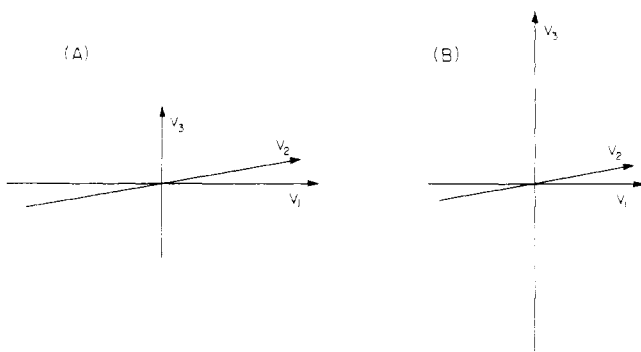


FIGURE 1: Two models describing the transition moments of the naphthalene chromophore which are compatible with the measured polarization of 0.07 of the fluorescence of this chromophore. In model A, $V_1 = V_2 = 0.67$ and $V_3 = 0.32$; in model B, $V_1 = V_2 = 0.46$ and $V_3 = 0.75$. Both models yield similar results for the angular dependence of κ^2 when the dansyl chromophore is used as an acceptor.

rotation and are functions of the Euler angles which describe a specific rotation. The symbol $\langle \rangle$ denotes taking the average of the enclosed expression over all rotation angles of the chromophore in space. Equations 3 follow from the fact that terms of the form $a_{3i}V_i$ express the components of the vectors V_i along the z axis, while terms of the form $a_{1i}V_i$ express the components of the vectors V_i along the x axis. The probability of absorption of light polarized along the z axis is proportional to the sum of the terms of the form $(a_{3i}V_i)^2$, while the probability of emission of light polarized along the x axis is proportional to the sum of the terms of the form $(a_{1i}V_i)^2$. The dipoles V_1 , V_2 , and V_3 should be normalized to yield a total absorption or emission intensity equal to that of a single dipole of unit magnitude, so that

$$V_1^2 + V_2^2 + V_3^2 = 1 \quad (4)$$

and a similar expression holds for V_i' . If the fluorescence excitation is performed at the long wavelength absorption band, which involves the same electronic levels as the fluorescence (S_0 and S_1), we may assume that, at least approximately, $V_i = V_i'$. Equations 2, 3, and 4 are not sufficient to define completely the magnitude of V_1 , V_2 , and V_3 , if all three exist. Under such circumstances various models may be tried which are compatible with these equations. For example, in the case of the naphthalene chromophore both models shown in Figure

1 are compatible with the value of 0.07 obtained experimentally for the polarization of this chromophore. The out of plane dipole might result from vibronic mixing (Albrecht, 1960), from overlap with an electronic transition that has such a transition dipole moment (Valeur & Weber, 1977), or from librations (Zinsli, 1977). This might affect the results to some extent by modifying the relation $V_i = V_i'$; however, as will be illustrated below, the different models for calculating V_i lead to values for the orientational factor that are rather similar. In some cases one can have independent theoretical information about the chromophore and limit the number of dipoles to two if $p > 1/7$. Equations 2–4 then define the magnitude of these dipoles unequivocally. Table I describes various magnitudes that can be assigned to V_1 , V_2 , and V_3 for given values of p .

The polarization p of either donor or acceptor may in some instances vary across the spectral range at which the donor fluorescence overlaps the acceptor absorption. Under such circumstances one has to evaluate V_1 , V_2 , and V_3 of the corresponding chromophore across the spectrum and average them with proper weighting by the corresponding values of $f(\bar{\nu})\epsilon(\bar{\nu})/\bar{\nu}^4$ (see eq 1). The procedure to be followed may be illustrated for the case of the dansyl chromophore, the polarization data of which are shown in Figure 2. The value of p at the edge of the absorption band is 0.45. By use of eq 3 and using $V_i = V_i'$, one obtains $V_1' = 0.98$ and $V_2' = 0.20$. By use of these values for the emission dipoles, one can then calculate V_1 and V_2 at other wavelengths of the absorption spectrum, using the corresponding values of p . If p does not vary across the overlap region to a great extent, the value of V_1 , V_2 , and V_3 at the wavelength at which $f(\bar{\nu})\epsilon(\bar{\nu})/\bar{\nu}^4$ is maximum may be taken as a good approximation for their average values.

If the donor or acceptor chromophores under given conditions undergo some Brownian rotatory motion during the lifetime of the excited state, the polarization of their fluorescence will of course be lower than their limiting polarization in a fully frozen solution. The effect of such rotatory diffusion on κ^2 has been treated in much detail by Dale & Eisinger (1974, 1975) by averaging procedures over regions of rotational space that are compatible with the fluorescence depolarization data. It should, however, be noted that the fluorescence depolarization phenomenon by rotational diffusion may be viewed in a formal way as due to the occurrence of additional transition dipole moments in the emission process which

are nonparallel to, and noncoherent with, the dipole moments that occur in fully frozen systems. The approach outlined above can thus be extended to systems which are not fully frozen and in which the chromophores exhibit some rotatory Brownian motion.

(b) *Formulation of the Expression for κ^2 .* Each of the oscillating dipoles V_1 , V_2 , and V_3 of the donor chromophore A produces an oscillating electric field at the location of the acceptor B . Without loss of generality we can define the direction of r , the vector connecting A and B , as the z axis of the laboratory coordinate system (see Figure 3); whereas V_1 , V_2 , and V_3 coincide in direction with the x , y , and z molecular axes, respectively, as indicated above. When the donor rotates, the electric field produced by each of the dipoles V_1 , V_2 , and V_3 changes; their value may, however, be readily obtained by using the Euler transformation matrix. For example, the x , y , and z components of V_1 in the laboratory frame of reference are $a_{11}V_1$, $a_{21}V_1$, and $a_{31}V_1$, respectively. The x , y , and z components of the electric field produced by V_1 at the location of the acceptor will thus be proportional to $a_{11}V_1$, $a_{21}V_1$, and $2a_{31}V_1$, respectively. (The proportionality factor may be ignored, since it is not relevant to the evaluation of the dependence of κ^2 on molecular orientation.) If W_1 , W_2 , and W_3 are the three dipoles that characterize the acceptor absorption, the probability of energy transfer from donor to acceptor will be proportional to the square of the scalar product of each of these with the electric field produced by each of V_1 , V_2 , and V_3 . Thus, the contribution to energy transfer of W_1 and V_1 is proportional to $V_1(a_{11} + a_{21} + 2a_{31}) \cdot W_1(A_{11} + A_{21} + A_{31})$, where the A_{ij} 's are the elements of the Euler matrix for the orientation of the acceptor. Generalizing to all three dipoles of both the donor and acceptor, one obtains for κ^2 :

$$\begin{aligned} \kappa^2 = & (V_1W_1)^2(a_{11}A_{11} + a_{21}A_{21} + 2a_{31}A_{31})^2 \\ & + (V_1W_2)^2(a_{11}A_{12} + a_{21}A_{22} + 2a_{31}A_{32})^2 \\ & + (V_1W_3)^2(a_{11}A_{13} + a_{21}A_{23} + 2a_{31}A_{33})^2 \\ & + (V_2W_1)^2(a_{12}A_{11} + a_{22}A_{21} + 2a_{32}A_{31})^2 \\ & + (V_2W_2)^2(a_{12}A_{12} + a_{22}A_{22} + 2a_{32}A_{32})^2 \\ & + (V_2W_3)^2(a_{12}A_{13} + a_{22}A_{23} + 2a_{32}A_{33})^2 \\ & + (V_3W_1)^2(a_{13}A_{11} + a_{23}A_{21} + 2a_{33}A_{31})^2 \\ & + (V_3W_2)^2(a_{13}A_{12} + a_{23}A_{22} + 2a_{33}A_{32})^2 \\ & + (V_3W_3)^2(a_{13}A_{13} + a_{23}A_{23} + 2a_{33}A_{33})^2 \quad (5) \end{aligned}$$

A few special cases of eq 5 are of interest. If $V_1 = V_2 = V_3$ and $W_1 = W_2 = W_3$, $\kappa^2 = 2/3$ regardless of the orientation of the donor or acceptor. If only one dipole exists for each of the donors and acceptors, κ^2 assumes the angular dependence already known for such a special case. For any orientations of donor and acceptor, rotation of both by a given angle around the vector r (i.e., changing the Euler angles ϕ of both donor and acceptor to the same extent) yields the same value for κ^2 . This result is, of course, intuitively expected. Finally, if both A and B undergo fast rotation during the lifetime of the excited state, one should average the products of the Euler elements over all space. The result yields a value of $2/3$ for κ^2 regardless of the values of V_1 , V_2 , V_3 , W_1 , W_2 , W_3 , provided V_i and W_i are normalized, as required above.

(c) *The Probability Distribution for Obtaining Various Values of κ^2 when the Orientations of A and B Are Isotropically Distributed.* If the system under investigation contains a collection of donor-acceptor pairs each of which is randomly oriented in space, one can evaluate by use of eq 5 the fraction

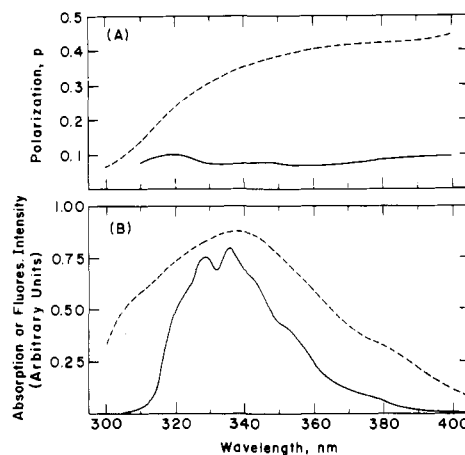


FIGURE 2: Spectroscopic properties of the naphthalene and dansyl chromophores in dansyl-[N^5 -(2-hydroxyethyl)-L-glutamine] $_n$ - N -hydroxyethyl-L- β -naphthylalanine. (A) Polarization spectra in anhydrous glycerol: (—) naphthalene ($\lambda_{ex} = 290$ nm; the low values for p were also obtained upon excitation at 320 nm); (---) dansyl ($\lambda_{em} = 500$ nm). (B) (---) Absorption spectrum of dansyl; (—) emission spectrum of naphthalene. Room temperature ($\sim 23^\circ\text{C}$).

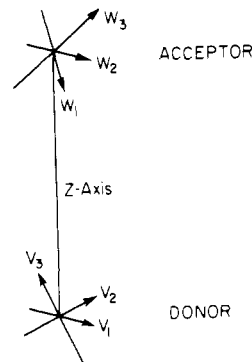


FIGURE 3: Illustration of an arrangement in space of the transition dipoles associated with the donor and acceptor chromophores.

of the number of donor-acceptor pairs, $P(\kappa^2)d(\kappa^2)$, that have an orientational factor whose magnitude falls in the range of κ^2 to $\kappa^2 + d(\kappa^2)$. Obviously $P(\kappa^2)$ gives also the probability density of κ^2 for a frozen system of randomly oriented donor-acceptor pairs. The function $P(\kappa^2)$ was obtained by Jones (1970) and Hillel & Wu (1976) for donors and acceptors each characterized by a single transition dipole moment; in addition Jones (1970) discussed a case corresponding to a planar oscillator. To obtain $P(\kappa^2)$ for the general case we scanned systematically by computer over all orientations of the donor and the acceptor, and calculated the values of the Euler matrix elements a_{ij} and A_{ij} which correspond to the Euler angles which define the orientations of the donor and the acceptor. This permitted the calculation of κ^2 for each orientation by use of eq 5. $P(\kappa^2)$ was derived by enumerating the number of times that the orientational factor attains a value between κ^2 and $\kappa^2 + d(\kappa^2)$. Some details concerning the computational procedure employed are given in the Appendix.

Figure 4 illustrates the results obtained for $P(\kappa^2)$ for some donor-acceptor pairs which possess each, one, two, or three orthogonal transition moments corresponding to various values of p for the participating chromophores. As expected, the lower the values of p , the narrower the distribution of $P(\kappa^2)$. The maximal value of $P(\kappa^2)$ occurs at a value of κ^2 close to $2/3$ when the values of p of the donor and acceptor are relatively low; the maximum occurs at lower values of κ^2 when p of the chromo-

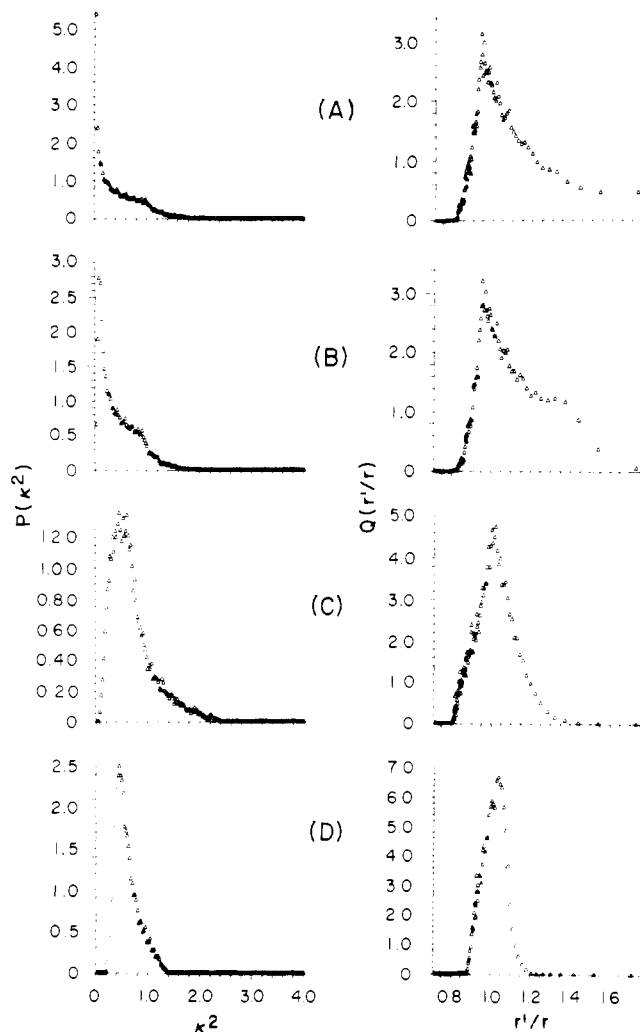


FIGURE 4: The probability functions $P(\kappa^2)$ (left) and $Q(r'/r)$ (right) for donor-acceptor pairs exhibiting different polarizations, p , in their fluorescence. The polarizations of the donor and acceptor are: (A) 0.5 and 0.5; (B) 0.5 and 0.45; (C) 0.3 and 0.3; (D) polarizations corresponding to the naphthalene donor ($p = 0.07$) and the dansyl acceptor (see Figure 2 and text for details).

phores is high.

The information available for $P(\kappa^2)$ [see, for example, Figure 4, left] might be used as follows. Suppose one has a collection of the same donor-acceptor pairs all of which are separated from each other by a distance r , but the donor and acceptor are randomly oriented. Owing to the variation in the value of κ^2 in this collection of donor-acceptor pairs, the efficiency of energy transfer will not be the same for all the chromophore pairs, but will follow the distribution described by $P(\kappa^2)$ in Figure 4. If one interprets the energy transfer data in terms of distances between donors and acceptors, using erroneously a fixed value for κ^2 (say $2/3$), one will obtain an apparent distribution of distances between donors and acceptors. For donor-acceptor pairs for which a certain value κ^2 applies, one will obtain an apparent distance $r' = (2/3/\kappa^2)^{1/6}r$. Using this relation and the data presented in Figure 4, left, one can obtain the apparent distribution of distances between donors and acceptors $Q(r'/r)$ [Figure 4, right]. Do note that the following relation holds: $P(\kappa^2)d(\kappa^2) = Q(r'/r)d(r'/r)$ since $P(\kappa^2)d(\kappa^2)$ and $Q(r'/r)d(r'/r)$ both denote the fraction of the population of donor-acceptor pairs whose orientational factor falls in the range of κ^2 to $\kappa^2 + d(\kappa^2)$. $Q(r'/r)$ is deduced from the above relation and the relation $d(r'/r) = -(2/3)^{1/6}(\kappa^2)^{-$

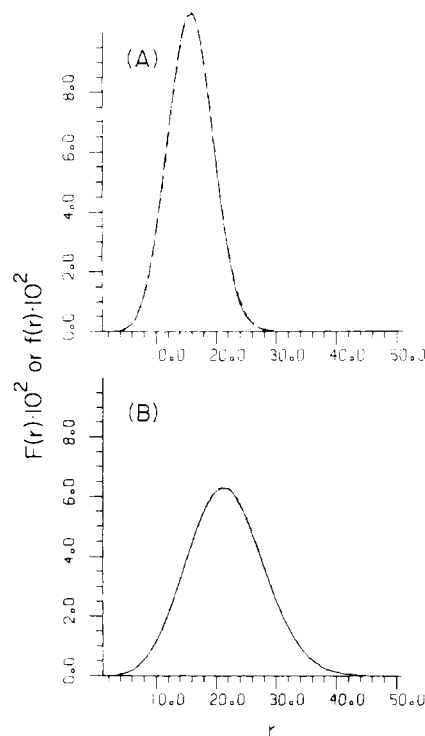


FIGURE 5: The distribution of end-to-end distances of two oligopeptides of the type dansyl- $[N^5$ -(2-hydroxyethyl)-L-glutamine] $_n$ - N -hydroxyethyl-L- β -naphthylalanine. (A) $n = 5$; (B) $n = 9$. Broken lines: the distribution function, $f(r)$, assuming a fixed value of $\kappa^2 = 2/3$. Full lines: refined distribution function, $F(r)$, which takes into account the variation of κ^2 resulting from the random orientation of the donor and acceptor chromophores in the population of oligopeptide molecules (see Figure 4D).

$-7/6d(\kappa^2)$ (the minus sign is of no consequence here; it denotes that increasing κ^2 decreases r'/r).

As may be noted from Figure 4, $Q(r'/r)$ does not reach a zero value for large values of r'/r if the polarizations of the donor and acceptor are of the theoretical maximal value 0.5. However, reducing the polarization of one of the chromophores by a relatively small amount causes $Q(r'/r)$ to drop to a low value even for r'/r of magnitude of 1.7 (see Figure 4B). Further reduction in p of either donor or acceptor (or both) causes marked narrowing of the bell-shaped curve of $Q(r'/r)$, thus reducing the uncertainty in the value of r measured by energy transfer between such chromophores.

Application of the Above Theoretical Considerations to the Estimation of Distances by Energy Transfer Measurements

(a) *End-to-End Distance Distribution in Oligopeptides.* In a previous study (Haas et al., 1975) we have evaluated the distribution of end-to-end distances of a series of oligopeptides to the ends of which a naphthalene and a dansyl chromophore were attached. The distribution of distances was evaluated from the decay kinetics of the donor fluorescence in a frozen medium assuming a fixed value for κ^2 . In a recent study, Englert & Leclerc (1978) have shown that vectors attached to the ends of even short oligopeptides are very nearly randomly oriented in spaces relative to the vector connecting the ends. From the above theoretical considerations it is evident that the end-to-end distribution thus evaluated is in principle broader than the true distribution, since each subpopulation of the molecules of a defined end-to-end distance appears as a population of molecules with a spread of end-to-end distances as described in Figure 4 (right). The overall apparent distribution of distances, $f(r')$ is given by

TABLE II: The Extreme Limits That r'/r May Attain for Donor-Acceptor Pairs of Fixed Orientation Possessing Various Fluorescence Polarizations.^{a,b}

p of 2nd chromophore	p of 1st chromophore					
	0.5	0.4	0.3	0.2	0.1	0.0
0.5	0.75-∞					
0.4	0.76-∞	0.75-1.75				
0.3	0.78-∞	0.79-1.6	0.8-1.5			
0.2	0.83-∞ ^c	0.81-1.55	0.82-1.44	0.84-1.33		
0.1	0.86-1.5	0.86-1.35	0.87-1.24	0.88-1.21	0.92-1.12	
0.0	0.91-1.12	0.9-1.12	0.93-1.12	0.95-1.1	0.98-1.08	1.0-1.0

^a r is the distance between the donor and acceptor, whereas r' is the distance erroneously obtained from energy transfer measurements by assuming $\kappa^2 = 2/3$. ^b The values of V_i and W_i used to compile this table were taken from Table I, model A. Model B yielded somewhat narrower limits for r'/r . ^c If one of the chromophores has a fluorescence polarization of 0.5 and the other of 0.2, r'/r goes in principle to infinity. However, $Q(r'/r)$ attains already at $r'/r = 1.7$ a very low value of 1.5% of its maximal value.

TABLE III: Values of r'/r at Half-Height of $Q(r'/r)$ for Donor-Acceptor Pairs of Various Fluorescence Polarizations.^a

p of 2nd chromophore	p of 1st chromophore					
	0.5	0.4	0.3	0.2	0.1	0.0
0.5	(0.83-1.12) ^b					
0.4	0.83-1.19	0.83-1.2				
0.3	0.87-1.17	0.88-1.13	0.91-1.12			
0.2	0.88-1.14	0.88-1.14	0.91-1.11	0.91-1.11		
0.1	0.88-1.14	0.87-1.11	0.91-1.08	0.93-1.08	0.93-1.05	
0.0	0.92-1.02	0.9-1.03	0.93-1.09	0.95-1.04	0.98-1.02	1.0-1.0

^a For further details, see footnotes to Table II and text. ^b $Q(r'/r)$ is highly skewed for the case that both donor and acceptor exhibit a fluorescence polarization $p = 0.5$ (see Figure 4A), and has an appreciable magnitude even at $r'/r = 2$ or more. The value of width at half-height of $Q(r'/r)$ cannot be taken in this case as an estimate for the probable error in the measured distance.

$$f(r') = \int_0^\infty F(r) Q\left(\frac{r'}{r}\right) dr \quad (6)$$

where $F(r)$ is the true end-to-end distribution function, and $Q(r'/r)$ is the function described in Figure 4D. The dependence of Q on r'/r shown in Figure 4D relates to the naphthalene and dansyl chromophores as derived from their polarization data (see Figure 2). Assuming for $F(r)$ a functional form of the type $F(r) = 4\pi r^2 e^{-a'(r-b')^2}$ (Edwards, 1965), we have searched by computer the values of a' and b' that fit best to eq 6, using the experimentally obtained functions $f(r')$ as described before (Haas et al., 1975) and $Q(r'/r)$ obtained in this study. The results thus obtained for $F(r)$ for two of the peptide studies before are presented in Figure 5. In this figure, $f(r')$ is also given for comparison. The differences between $f(r)$ and $f(r')$ are strikingly small; i.e., the corrections necessary to introduce in the end-to-end distribution function due to the orientational factor are negligible. This is due to the mixed polarizations in the donor and acceptor used, which result in a relatively weak dependence of κ^2 on the various orientations of donor and acceptor. The criticism (Dale & Eisinger, 1976) of the use of energy transfer measurements to the evaluation of distance distribution functions between the pairs of donor-acceptor chromophores attached to the oligopeptides discussed above is thus shown not to be valid. This theoretical result is in accord with the experimental results reported recently which indicate that the end-to-end distribution function evaluated from experiments conducted in solvents of varying viscosity, in which the chromophores show different degrees of rotational mobility, is practically independent of solvent viscosity (Haas et al., 1978).

We have also investigated the problem of how the magnitude of the limiting polarizations of donor and acceptor chromophores will affect the evaluation of distribution functions of

distances between the donor and acceptor pairs. The results showed that, even for limiting polarizations that are as high as 0.4, the differences between $f(r')$ and $F(r)$ are rather small, permitting the use of $\kappa^2 = 2/3$ as a good approximation in the evaluation of distance distribution functions by energy transfer experiments. For values of p higher than 0.4 this approximation is less satisfactory.

(b) *Distance between Donor-Acceptor Chromophores of Unknown Orientation.* In many applications of energy transfer to the estimation of distances between chromophores which are assumed to stay fixed within the lifetime of the excited state of the donor, their absolute orientations are not known. Under such circumstances, eq 5 can be used to give the upper and lower limits of κ^2 compatible with the polarization data, and hence the upper and lower limits of the distances between the chromophores. Inspection of Figure 4 clearly shows that, within these upper and lower limits of uncertainty in the distance between the chromophores, the probability of obtaining a given value for r'/r is far from being evenly distributed. In analyzing data one often finds it useful to point out results that, although they cannot be eliminated, are unlikely. In this respect plots of $Q(r'/r)$ vs. r'/r may be of use, since they present the probability of obtaining an erroneous value for r in the analysis of energy transfer data. One may thus take the width of $Q(r'/r)$ at half-height as the likely limits of error in r . Confidence in these limits of error might be considerably enhanced if one uses two different donor-acceptor pairs for the measurement of the same distance chosen in such a way that their relevant transition moments align differently in the binding sites (Stryer & Haugland, 1967). For easy reference we list in Table II the maximum error in the estimation of distances from energy transfer data for donors and acceptors which show different polarizations in the range of overlap of donor fluorescence and acceptor absorption. In Table III we list the values of the width

at half-height of the plots of Q vs. (r'/r) (similar to those shown in Figure 4). These values present the probable ranges of uncertainty in the distances estimated from energy transfer for chromophores of different polarizations. It is thus seen that in case the acceptor is isotropic, then even if the donor has the maximum value of $p = 1/2$, the maximum limit of error in the evaluated distance between the chromophores is about $\pm 12\%$. Even lower values are obtained for the probable ranges of uncertainty if p of the donor and acceptor are less than 0.3.

The above results show clearly that the presence of the orientational factor, κ^2 , in eq 1 is not necessarily an obstacle in determining the distance between donor and acceptor, even in the absence of rotatory Brownian motion, when the spectroscopic transitions involved have sufficiently low polarizations. The choice of chromophores with such spectroscopic properties may thus be recommended for the evaluation of distances by energy transfer measurements for the general cases considered.

Acknowledgment

We are grateful to L. Stryer for important discussions on the topic of the paper.

Appendix: Some Remarks Regarding the Evaluation of κ^2 and $P(\kappa^2)$

Equation 5 can be rewritten in the form:

$$\begin{aligned} \kappa^2 = & (V_1^2 - V_3^2)(W_1^2 - W_3^2) \\ & \times (a_{11}A_{11} + a_{21}A_{21} + 2a_{31}A_{31})^2 \\ & + (V_1^2 - V_3^2)(W_2^2 - W_3^2)(a_{11}A_{12} + a_{21}A_{22} + 2a_{31}A_{32})^2 \\ & + (V_2^2 - V_3^2)(W_1^2 - W_3^2)(a_{12}A_{11} + a_{22}A_{21} + 2a_{32}A_{31})^2 \\ & + (V_2^2 - V_3^2)(W_2^2 - W_3^2)(a_{12}A_{12} + a_{22}A_{22} + 2a_{32}A_{32})^2 \\ & + W_3^2[(V_1^2 - V_3^2)(1 + 3a_{31}^2) + (V_2^2 - V_3^2)(1 + 3a_{32}^2)] \\ & + V_3^2[(W_1^2 - W_3^2)(1 + 3A_{31}^2) \\ & + (W_2^2 - W_3^2)(1 + 3A_{32}^2)] + 12V_3^2W_3^2 \quad (\text{A-1}) \end{aligned}$$

It may be noted that this expression contains an isotropic term, $V_3^2W_3^2$, which is independent of orientation. It may also be noted that, upon scanning over all orientations of the donor and the acceptor, it is by far less time consuming to scan over the terms of the type a_{ij} or A_{ij} which occur in eq A-1 than of the type $a_{ij}A_{kl}$.

As was pointed out in the text, rotation of both donor and acceptor by the same angle around the z axis, other variables remaining the same, should produce no change in κ^2 . This is

indeed verified by changing the Euler angle ϕ of the donor and acceptor to the same extent. Therefore, upon the evaluation of $P(\kappa^2)$, we did not vary the angle ϕ of the donor and let it to be zero. The angle ϕ of the acceptor was varied, as well as ψ and θ of both donor and acceptor.

The intervals used in the variation of the various Euler angles for the evaluation of $P(\kappa^2)$ were in the range of 16.36° to 10° . The smaller intervals obviously yielded a smoother curve for $P(\kappa^2)$, but required longer computation times. For most purposes intervals of the order of 15° yielded acceptable curves for $P(\kappa^2)$.

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