FLUORESCENCE DEPOLARIZATION AND ORIENTATION FACTORS FOR EXCITATION ENERGY TRANSFER BETWEEN ISOLATED DONOR AND ACCEPTOR FLUOROPHORE PAIRS AT FIXED INTERMOLECULAR SEPARATIONS*

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The energy transfer efficiencies and transfer depolarization factors are calculated for both heterogeneous and homogeneous excitation energy transfer in the very weak coupling limit between isolated donor and acceptor fluorophore pairs having static isotropic orientational distributions. Effective average values for the orientation factors are obtained and shown to be closely approximated by linear functions of the transfer efficiencies themselves. The use of these approximations is shown to result in simple quadratics relating the transfer efficiencies to the sixth power of the inter-fluorophore separations, rather than the linear forms found in the equivalent dynamically averaged cases. For homogeneous transfer, similar approximations in terms of the overall observed depolarization factor are demonstrated.

1. Introduction

The phenomenon of quenching and sensitization of fluorescence by energy transfer between unlike fluorescent molecules of suitable spectral properties statistically distributed in isotropic solution has long been rather well described, both on the basis of virtual dipole-dipole interaction in the very weak coupling limit (Förster, 1949, 1965), and phenomenologically by the active sphere model of Jabłoński (1954, 1955a, 1957a). The allied effect of concentration depolarization in (solid) solutions containing only one kind of emitter is also dealt with quite well by the active sphere treatment (Jabłoński, 1955b, 1958; Bojarski, 1958), or again by applying the dipole-dipole interaction mechanism, in two completely different approximations (Ore, 1959, 1971; Hemenger and Pearlstein, 1973).

At the other end of the scale from such statistical donor and acceptor ensembles, energy transfer between isolated donor (D) and acceptor (A) pairs bound specifically to

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a stationary macromolecular substrate at a fixed mutual separation has been of interest to polymer chemists and biochemists as a "spectroscopic ruler" (Stryer and Haugland, 1967) to enable definition of various intramolecular dimensions of interest. The Förster very weak coupling treatment is generally appropriate, whether the transfer be between unlike fluorophores (heterogeneous) or like ones (homogeneous). As in the solution ensemble cases, the former will be identified by quenching of donor and sensitization of acceptor emissions, the latter (if at all) by depolarization of emission from the pair. To date, only the heterogeneous case has been exploited experimentally.

Usually the donor and acceptor may be expected to be bound anisotropically to the substrate, i. e. with some fairly restricted range of mutual dipole orientations upon which, as well as upon their separation, the transfer efficiency depends quite strongly (Förster, 1951). Within the approximation that these relative orientations are sampled rapidly in comparison with the transfer and emission rates (dynamic averaging), it has been demonstrated that, by making use of the "dynamic" depolarization of emission, both sensitized and direct, due to these reorientational processes, realistic (and unfortunately usually quite large) limits may be set on derived intramolecular separations (Eisinger and Dale, 1976; Eisinger, 1976). If, on the other hand, the averaging regime is static, i. e. if instead of an ensemble of identical D, A pairs each exhibiting the same energy transfer efficiency, there exists a continuous stationary statistical orientational distribution of D, A pairs, the problem is more complicated since the effects of mutual orientation and separation on the average transfer efficiency cannot in general be separated (Dale and Eisinger, 1976). In the following, and admittedly more for heuristic than immediately practical purposes, the effect of a strict static averaging regime on the energy transfer properties (including depolarization) of D, A pairs at fixed separations and with isotropically distributed orientations (in the ensemble) is investigated and compared with the simple results obtained under dynamic averaging conditions.

2. General theory

2.1. Excitation energy transfer

In the very weak coupling limit, the rate constant μ of (one-way) energy transfer has been shown (Förster, 1951, 1965) to be given by:

$$\mu = \frac{1}{\tau}\left(\frac{R_0'}{R}\right)^6, \tag{1}$$

where τ is the first order decay time of D emission in the absence of A, R the D, A separation and R_0' a characteristic separation defined by:

$$R_0'^6 = C\kappa^2 n^{-4}\Phi J, \tag{2}$$

where C is made up of numerical and universal constants, n is the refractive index of the intervening "medium", Φ the quantum yield of D in absence of A, and J the normalised

spectral overlap integral of D emission with A absorption.[1] The dipole-dipole orientation factor κ^2 carries the dependence of R'_0 on the mutual orientations of D and A and is usually expressed as:

$$\kappa^2 = (\cos\theta_T - 3\cos\theta_D \cos\theta_A)^2$$
$$= (\sin\theta_D \sin\theta_A \cos\varphi - 2\cos\theta_D \cos\theta_A)^2 \quad (3)$$

where θ_T is the angle between D emission and A absorption dipole moment vectors, θ_D and θ_A the angles between these respectively and the separation vector, and φ is the azimuth of the planes containing $\boldsymbol{D}$ and $\boldsymbol{A}$ about the separation vector $\boldsymbol{R}$ (Figure 1). An alternative formulation, which will prove more useful here, considers the interaction of the donor

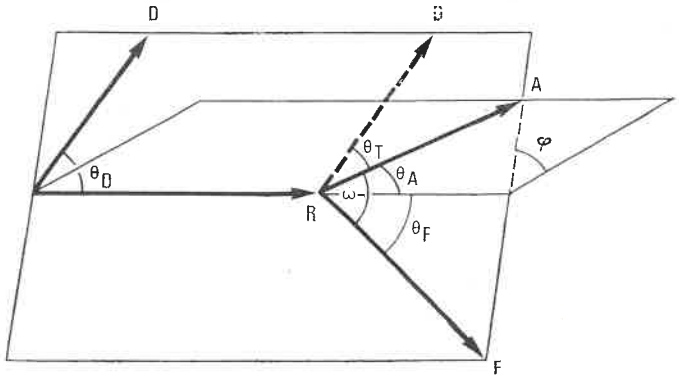

Fig. 1. Donor-acceptor pair geometry depicting the angular relationships of two definitions of the orientation factor

electric field vector with the acceptor (Galanin, 1955; Maksimov and Rozman, 1962; Steinberg, 1968). Resolving the unit vector $\boldsymbol{D}$ into components along and perpendicular to $\boldsymbol{R}$ (amplitudes $\cos\theta_D$ and $\sin\theta_D$ respectively), the field vector $\boldsymbol{F}$ can be considered to arise as the sum of the field vectors due to these components, of amplitudes $2\cos\theta_D$ and $-\sin\theta_D$ respectively, where the negative sign indicates the reversal of field direction in the latter case (see Figure 1). On summing these to obtain $\boldsymbol{F}$ and defining as ω the angle it makes with $\boldsymbol{A}$, the orientation factor is finally given by:

$$\kappa^2 = (1+3\cos^2\theta_D)\cos^2\omega. \quad (4)$$

As is readily seen from both Eqs. (3) and (4), κ^2 may take on values in the range of 4 ($\boldsymbol{D}$ and $\boldsymbol{A}$ parallel-in-line along $\boldsymbol{R}$) to zero ($\omega = \pi/2$ or whenever $\cos\varphi = 2\cot\theta_D \cot\theta_A$).

[1] The effect of "pre-relaxation" transfer (Guéron, Eisinger and Shulman, 1967) which may, for very fast and efficient transfer, enhance (or, for heterogeneous transfer in some cases, even diminish) the transfer rate relative to that predicted above on the assumption that complete thermal equilibration of the excited donor has been attained before appreciable transfer can take place (Förster, 1965), is ignored in the following. In principle, it may be taken into account by adjusting the value of J appropriately, as long as the very weak coupling limit is not violated.

An arbitrarily small actual value of κ^2 will lead to a correspondingly small value of R'_0 and therefore of the apparent intermolecular separation derived for a given transfer rate.

As indicated above, two cases of energy transfer will be considered here, the homogeneous and the heterogeneous. In the former case, the one-way transfer rate constant is the same in both directions, i. e. designating the spectrally identical fluorophores 1 and 2: $\mu_{1\to 2} = \mu_{2\to 1} = \mu$. On considering multiple re-transfers to and from the originally excited fluorophore, the overall energy transfer efficiency to the originally unexcited fluorophore is readily derived (Förster, 1948; Ore, 1959) to be:

$$T_s = \mu\tau/(1+2\mu\tau), \tag{5}$$

where the subscript "s" will be used to designate homogeneous ("self") transfer. In the general case of heterogeneous transfer, allowance will need to be made for a finite "back"--transfer rate due to overlap between the emission spectrum of the initially unexcited acceptor and the absorption spectrum of the donor. Calling the initially excited fluorophore 1, the generalised transfer efficiency T_g is similarly given by:

$$T_g = \mu_{1\to 2}\tau_1/(1+\mu_{1\to 2}\tau_1+\mu_{2\to 1}\tau_2). \tag{6}$$

This, of course, reduces immediately to Eq. (5) when the two rate constants and lifetimes become identical. When the back-transfer rate is vanishingly small it reduces to the well--known expression for one-way energy transfer quenching or sensitization efficiency (e. g. Jabłoński, 1971b) on which its derivation was based:

$$T_q = \mu\tau/(1+\mu\tau). \tag{7}$$

2.2. Fluorescence depolarization

The most convenient description of the polarization of fluorescence of an ensemble of excited fluorophores photoselected (Albrecht, 1961) from an originally isotropic ground state ensemble in solution, is the emission anisotropy (EA) r first defined formally by Jabłoński (1957b, 1960), but also used implicitly much earlier than this (e. g. Jabłoński, 1936; Perrin, 1936), and given in a laboratory coordinate system by:

$$r = (I_V - I_H)/I, \tag{8}$$

where I_V, I_H are the components of emission polarized in the vertical and horizontal plane respectively and observed perpendicularly to the direction of propagation of the exciting light in the horizontal plane, while I represents the sum of any three mutually orthogonally polarized intensities, i. e. is proportional to the total emission intensity. As shown by Jabłoński (1960), I is given by (I_V+2I_H) for linearly V-polarized excitation and by $(2I_V+I_H)$ for e. g. "natural" (unpolarized) or circularly polarized excitation. In the absence of any depolarizing influence, the maximum values of 0.4 and 0.2 will be observed under these two excitation conditions reflecting the angular dependence of the photoselection process and also the fact that, in general, $r_n = (r/2)$ where the subscript denotes "natural" excitation (Jabłoński, 1960).

Further depolarization occurs if, for any reason, e. g. through torsional oscillations (Jabłoński, 1950) or due to the existence of an effectively anisotropic three-dimensional transition moment (Jabłoński, 1935) there be a difference in orientation of the dipole moment vector associated with emission compared with that associated with the initial absorption process. Provided that all azimuths are equally probable for this reorientation, the resulting depolarization may be represented by a depolarization factor d multiplying the initial anisotropy (Soleillet, 1929; Perrin, 1936). Confining further discussion to linearly V-polarized excitation for simplicity, the EA after one such process may be written:

$$r = 0.4\, d, \tag{9}$$

where

$$d = (\tfrac{3}{2}\cos^2\theta - \tfrac{1}{2}) \tag{10}$$

in which θ is the reorientational angle. When a number of such processes occur independently their effects are multiplicative, as shown by Soleillet (1929), and the EA becomes:

$$r = 0.4 \prod_i (\tfrac{3}{2}\langle\cos^2\theta_i\rangle - \tfrac{1}{2}) = 0.4 \prod_i \langle d_i\rangle, \tag{11}$$

where the averages are included to express possible variation in the θ_i for the i'th clsas of depolarizing event.

In the following, the mechanism of depolarization by energy transfer will be the only one of concern and further discussion will be limited to transfer depolarization factors:

$$d_T = \tfrac{3}{2}\cos^2\theta_T - \tfrac{1}{2} \tag{12}$$

where θ_T, the transfer angle, has been defined above in connection with the orientation factor given in Eq. (3). Thus, strictly, only the case of identical (or parallel) D, and likewise identical A, absorption and emission transition moment vectors is under discussion. If either or both are not, the two θ_T values of Eqs. (3) and (12) are not identical and they cannot be related, in general, by the Soleillet theorem because the requirement for azimuthal averaging is not fulfilled.

2.3. Relationships between the orientation and depolarization factors

As can be seen from the geometry indicated in Figure 1, and also from Eq. (3), θ_T may be expressed as a function of the three independent angles θ_D, θ_A and φ:

$$\cos\theta_T = \sin\theta_D \sin\theta_A \cos\varphi + \cos\theta_D \cos\theta_A \tag{13}$$

and the relationship between d_T and κ^2 in these terms is clear. However, if all azimuths of A about F are equally likely, then the Soleillet theorem may be applied so that, referring to Figure 1:

$$d_T = [\tfrac{3}{2}\cos^2(\theta_D + \theta_F) - \tfrac{1}{2}]\,[\tfrac{3}{2}\cos^2\omega - \tfrac{1}{2}]. \tag{14}$$

Invoking the geometry of Figure 1 again, it can be seen that:

$$\cos\theta_F = 2\cos\theta_D/\sqrt{1+3\cos^2\theta_D}$$

$$\sin\theta_F = \sin\theta_D/\sqrt{1+3\cos^2\theta_D}$$

$$\cos(\theta_D+\theta_F) = \cos\theta_D\cos\theta_F - \sin\theta_D\sin\theta_F \tag{15}$$

so that, eventually:

$$d_T = \left[\tfrac{3}{2}\left\{\frac{(3\cos^2\theta_D-1)^2}{1+3\cos^2\theta_D}\right\}-\tfrac{1}{2}\right]\left[\tfrac{3}{2}\cos^2\omega-\tfrac{1}{2}\right]. \tag{16}$$

It is noted that, as in the expression for κ^2 given in terms of the same parameters in Eq. (4), the two angular variables defining d_T are completely separable. Furthermore, the product of the orientation and depolarization factors, the significance of which will become evident, reduces to quite a simple expression:

$$d_T\kappa^2 = \tfrac{1}{4}(2-3\cos^2\theta_D)(1-9\cos^2\theta_D)(3\cos^2\omega-1)\cos^2\omega. \tag{17}$$

2.4. Averaging regimes

As has already been indicated, only isotropic angular distributions of the transition moments will be considered here. The same results would obtain for a number of pseudo--isotropic distributions e. g., trivially, random orientations within hemispheres whose axis of symmetry lies along the separation vector. When the reorientation rates for ***D*** and ***A*** within these distributions are fast compared with the emission and transfer rates, dynamic isotropic averaging obtains, all orientations are sampled during the transfer and emission times and unique values of $\langle d_T\rangle$ and $\langle\kappa^2\rangle$, independent of the D, A-separation, obtain. Such a convenient case, in terms of determining intramolecular separations, is also effectively provided by the highly triply-degenerate transitions of some metal ions. The experimental use of such probes in "spectroscopic ruling", now in its infancy (Latt et al. 1970; Birnbaum et al. 1977; Leung and Meares, 1977), holds great promise in that it eliminates most or all of the inherent uncertainty in κ^2 referred to above and associated with the more classical organic fluorophores having essentially linear transition moments.

At the other extreme, neither D nor A reorient appreciably and static isotropic averaging obtains. Under these circumstances, neither κ^2 nor d_T are separable from the inter-fluorophore spacing except at very low transfer efficiencies where κ^2 effectively approximates its dynamic value while d_T approaches a limit which, as will be shown below, is not identical with its dynamic isotropic average value. The case in which static isotropic averaging obtains for one fluorophore, dynamic or effectively dynamic for the other is also considered below. If reorientation of ***D*** and/or ***A*** occurs on the same time scale as emission and transfer, the time-dependence of κ^2 in the rate equation, as also that of d_T, would need to be taken into account, a task which will not be attempted here.

3. *Orientation and depolarization factors for transfer between isolated donor and acceptor pairs*

3.1. Some limiting values

From the definitions of Eqs. (3) or (4) it is readily seen that the well-known dynamic isotropic average of the orientation factor, $\langle\kappa^2\rangle$, is 2/3. Inspection of Eqs. (5) to (7) shows that this value will effectively be closely approximated in the static isotropic case when the transfer efficiency is very low so that e. g. $\mu \ll 1$. The dynamic isotropic average value of the transfer depolarization factor, which must of course be zero since under these conditions all memory of the original photo-selection is immediately lost, can be seen directly to be such from Eq. (12), as also from Eq. (16) in which the average of the second factor is similarly identically zero.

The often quoted "static isotropic average" value of 0.476 for the orientation factor (Galanin, 1955; Maksimov and Rozman, 1962; Steinberg, 1968) is actually $(\langle\kappa\rangle)^2 = 0.690^2$, the square of the average of κ, which was derived for heterogeneous transfer to a rigid ensemble of acceptors which are statistically randomly distributed about the donor with respect to both distance and orientation. The transfer depolarization factor for this case has not been calculated directly, although Jabłoński (1971b) has obtained estimates for it within the framework of a combined active-sphere/Förster transfer model.

For the case of homogeneous transfer in a similar ensemble, for whose Förster transfer and depolarization properties no direct closed solution of the differential master equation appears to exist (Knox, 1968), the orientation factor has been replaced by its dynamic isotropic average value of 2/3 in most cases (Förster, 1951; Knox, 1968). Transfer depolarization has usually also been considered complete, i. e. the dynamic isotropic average value $\langle d_T\rangle = 0$ assumed, although a weighting due to the orientation factor has also been introduced (Galanin, 1950; Ketskeméty, 1955; Weber, 1966):

$$\langle d_T\rangle = \langle d_T\kappa^2\rangle/\langle\kappa^2\rangle. \tag{18}$$

This value of 0.04, which is readily obtained from Eqs. (3), (12) and (13) or Eqs. (4) and (17), actually corresponds to the static isotropic average for isolated donor-acceptor pairs (either like or unlike) in the limit of low transfer efficiency and will not strictly apply elsewhere. Jabłoński (1970, 1971a) has also obtained estimates for the value of the depolarization factor for homogeneous transfer in a solution ensemble, again within a combined active-sphere/Förster model. These and his corresponding estimates for the heterogeneous case indicate an upper limit for the average transfer depolarization factor of about 0.025 which, as a function of the model, is independent of the acceptor concentration.

3.2. Full static averaging

3.2.1. Heterogeneous and homogeneous transfer efficiencies

On separating out the orientation factor, the one-way transfer rate constant defined in Eqs. (1) and (2) may be written:

$$\mu = \tfrac{3}{2}\kappa^2\left(\frac{A}{\tau}\right), \tag{19}$$

where $A = (R_0/R)^6$ with R_0^6 defined in the classical way as

$$R_0^6 = \tfrac{2}{3}\, Cn^{-4}\Phi J. \tag{20}$$

Transfer efficiencies averaged over the static ensemble of isotropically distributed orientations are most readily derived by substituting Eqs. (4) and (19) into Eqs. (7) and (5) for the heterogeneous and homogeneous isolated donor-acceptor pair cases respectively:

$$T_q = \left\langle \frac{\frac{3}{2}\kappa^2 A}{1+\frac{3}{2}\kappa^2 A} \right\rangle \tag{21}$$

and

$$T_s = \left\langle \frac{\frac{3}{2}\kappa^2 A}{1+3\kappa^2 A} \right\rangle. \tag{22}$$

It may be noted from these relationships, as also directly from Eqs. (5) and (7), that homogeneous transfer efficiencies are always precisely one half the heterogeneous efficiencies observed for twice the transfer rate (e. g. at $2^{-(1/6)} \simeq 0.89$ of the separation):

$$T_s(\mu) = 0.5\, T_q(2\mu) \tag{23}$$

or, in the orientationally isotropically averaged cases considered here:

$$\langle T_s \rangle (A) = 0.5 \langle T_q \rangle (2A). \tag{24}$$

Substituting $x = \cos\theta_D$, $y = \cos\omega$ and remembering that the angular weighting elements for integration are $\sin\theta_D\, d\theta_D$ and $\sin\omega d\omega$,[2] the average of Eq. (21) becomes:

$$\langle T_q \rangle = \int_0^1 \int_0^1 \left(\frac{\frac{3}{2} A(1+3x^2)y^2}{1+\frac{3}{2} A(1+3x^2)y^2} \right) dx dy = 1 - \int_0^1 \frac{1}{\sqrt{a}} \arctan(\sqrt{a})\, dx, \tag{25}$$

where $a = \frac{3}{2} A(1+3x^2)$. Similarly for the homogeneous case:

$$\langle T_s \rangle = \tfrac{1}{2}\left(1 - \int_0^1 \frac{1}{\sqrt{b}} \arctan(\sqrt{b})\, dx \right), \tag{26}$$

where $b = 3A(1+3x^2) = 2a$. Numerical evaluation of these integrals then yields the desired efficiencies.

3.2.2. Effective average values of the orientation factor

Although in general an average value of the orientation factor cannot be defined independently of the transfer rate, it is instructive to consider effective average values (Jones, 1970) defined for the heterogeneous case by:

$$\langle T_q \rangle = \frac{\frac{3}{2} \langle \kappa^2 \rangle_{eff} A}{1+\frac{3}{2} \langle \kappa^2 \rangle_{eff} A}, \tag{27}$$

[2] It should be noted that, although θ_D and ω are separable in this formulation, ω corresponding to a given A is not independent of θ_D. The order of integration is therefore not generally arbitrary: the average over ω-space for a constant θ_D must usually be performed first.

so that:

$$\langle \kappa^2 \rangle_{\text{eff}} = \frac{\langle T_q \rangle}{\frac{3}{2} A(1-\langle T_q \rangle)} \tag{28}$$

and similarly for the homogeneous case:

$$\langle \kappa^2 \rangle_{\text{eff}} = \frac{\langle T_s \rangle}{\frac{3}{2} A(1-2\langle T_s \rangle)} \tag{29}$$

The effective average values of the orientation factor so defined may then be obtained by substituting into these equations the numerical values of the average transfer efficiencies obtained above. They are plotted as a function of the transfer efficiencies in Figure 2. The

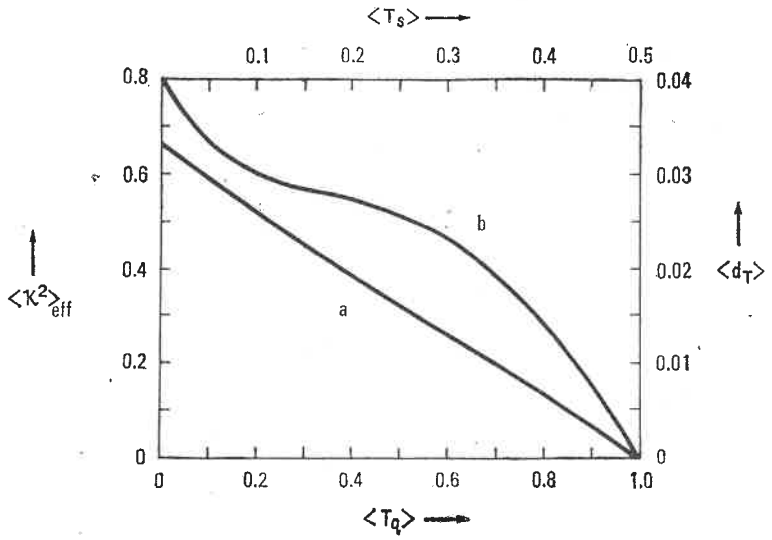

Fig. 2. *a* — Effective average value of the orientation factor $\langle \kappa^2 \rangle_{\text{eff}}$, and *b* — transfer depolarization factor $\langle d_T \rangle$ for static isotropic distributions of donor and acceptor orientations as a function of the heterogeneous or homogeneous transfer efficiencies, $\langle T_q \rangle$ and $\langle T_s \rangle$ respectively

correspondence between the values for heterogeneous and homogeneous transfer as functions of $\langle T_q \rangle$ and $2\langle T_s \rangle$ is a consequence of the relationship between the efficiencies expressed in Eq. (24). It is evident from Figure 2 that, to an equivalent and quite close approximation, the effective average values of the orientation factor are almost linear functions of the transfer efficiencies and may be written:

$$\langle \kappa^2 \rangle_{\text{eff}} \simeq \tfrac{2}{3}(1-\langle T_q \rangle) = \tfrac{2}{3}(1-2\langle T_s \rangle). \tag{30}$$

Thus, as the efficiencies become low, the effective average value of the orientation factor approaches the dynamic isotropic value of 2/3, while as they tend to their maxima, it

approaches zero, both values being confirmed by substituting the appropriate limits ($A \to 0$ and $A \to \infty$ respectively) of the integrals in Eqs. (25) and (26) into Eqs. (28) and (29).

3.2.3. Transfer depolarization factors

Of themselves, of course, the above results for the homogeneous case are of little use, since the emissions of donor and acceptor cannot be distinguished except by their polarizations. In general, the average transfer depolarization factor may be written:

$$\langle d_T \rangle = \langle d_T T \rangle / \langle T \rangle \tag{31}$$

and, again because of the relationships between homogeneous and heterogeneous transfer expressed in Eqs. (23) and (24), the transfer depolarization factors for the two cases are simply related:

$$\langle d_{Ts} \rangle (A) = \langle d_{Tq} \rangle (2A). \tag{32}$$

On combining Eqs. (4), (16) and (21), making the previously used angular substitutions and performing the appropriate analytical integration over ω, the numerator of Eq. (31) becomes, for heterogeneous transfer:

$$\langle d_{Tq} T_q \rangle = \tfrac{1}{4} \int_0^1 \frac{(2-3x^2)(1-9x^2)}{(1+3x^2)} \frac{1}{\sqrt{a}} \arctan(\sqrt{a}) dx$$

$$+ \frac{1}{2A} \int_0^1 \frac{(2-3x^2)(1-9x^2)}{(1+3x^2)^2} \frac{1}{\sqrt{a}} \arctan(\sqrt{a}) dx - \frac{1}{2A} \left(\tfrac{9}{2} - \frac{7\pi}{3\sqrt{3}} \right), \tag{33}$$

with a defined previously. The equivalent expression for $\langle d_{Ts} T_s \rangle$ contains b for a, and divisors of 8 and $8A$ replacing 4 and $2A$ in the above. The average transfer depolarizations, given by numerical evaluation of these integrals along with the results for the transfer efficiencies already obtained, are presented in Figure 2 where it is seen that, like $\langle \kappa^2 \rangle_{eff}$ also displayed there, they correspond as functions of $\langle T_q \rangle$ and $2\langle T_s \rangle$. The limits of $\langle d_T \rangle$ at zero and maximal transfer efficiencies are 0.04 corresponding to the value given by Eq. (18) and zero, as again may be confirmed by taking the appropriate limits of the integrals in Eq. (33). That the value of $\langle d_T \rangle$ for maximal transfer efficiency is the same as the dynamic isotropic average value reflects the fact that all orientations are equally sampled under these two conditions.

Of interest in the homogeneous system is $\langle d_s \rangle$, the average depolarization observed for emission from both members of the pair — that from the originally excited fluorophore retaining its full polarization, that from the partner being depolarized as described above:

$$\begin{aligned} \langle d_s \rangle &= 1 \times (1 - \langle T_s \rangle) + \langle d_{Ts} \rangle \langle T_s \rangle \\ &= 1 - \langle T_s \rangle + \langle d_{Ts} T_s \rangle. \end{aligned} \tag{34}$$

Since the second average factor is very small (always < 0.007), there should be a close to linear relationship between $\langle d_s \rangle$ and $\langle T_s \rangle$ and therefore, according to Eq. (30) above, between $\langle \kappa^2 \rangle_{\text{eff}}$ and $\langle d_s \rangle$. This is confirmed by the appropriate plot (Figure 3) in which the approximate relationship:

$$\langle \kappa^2 \rangle_{\text{eff}} \simeq \tfrac{2}{3}(2\langle d_s \rangle - 1) \tag{35}$$

is visualized.

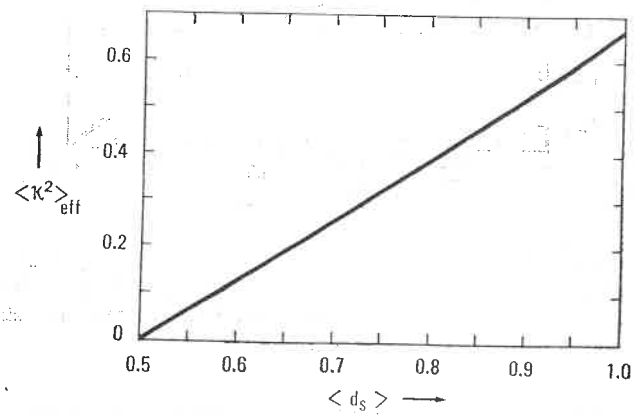

Fig. 3. Effective average value of the orientation factor $\langle \kappa^2 \rangle_{\text{eff}}$ as a function of the overall depolarization factor $\langle d_s \rangle$ for static isotropic distributions of like donor and acceptor orientations over the complete range of homogeneous transfer efficiency

3.2.4. Effect of approximations on derived separations

The extent of the errors in using the approximate relationships of Eqs. (35) and (28), or equivalently (29), are depicted in Figure 4. The limits at maximal transfer efficiency may be obtained by appropriate substitution of the integrals, expansion of the arctangents and neglect of all but the dominant terms:

$$\lim_{A \to \infty} \left[\frac{\frac{2}{3}(1 - \langle T_q \rangle)}{\langle \kappa^2 \rangle_{\text{eff}}} \right] = \tfrac{1}{6} \left[\frac{\pi \log (2 + \sqrt{3})}{\sqrt{3}} \right]^2 \simeq 0.951, \tag{36}$$

and

$$\lim_{A \to \infty} \left[\frac{\frac{2}{3}(2\langle d_s \rangle - 1)}{\langle \kappa^2 \rangle_{\text{eff}}} \right] = \left(\frac{\pi}{4} \right)^2 \left[\frac{\log (2 + \sqrt{3})}{\sqrt{3}} \right] \left\{ 6 - 5 \left[\frac{\log (2 - \sqrt{3})}{\sqrt{3}} \right] \right\} \simeq 1.031. \tag{37}$$

By inspection of Figure 4 it is seen that in neither approximation of $\langle \kappa^2 \rangle_{\text{eff}}$ is the error ever as high as 5%. In terms of derived inter-fluorophore separations, the error in R under these conditions will always be less than 1%, and over the majority of the range of heterogeneous transfer efficiency and the whole of the range of homogeneous transfer efficiency,

it will be less than 0.7%. In practical terms these errors are entirely negligible in comparison with the uncertainties of the validity of the isotropic (or pseudo-isotropic) model investigated here.

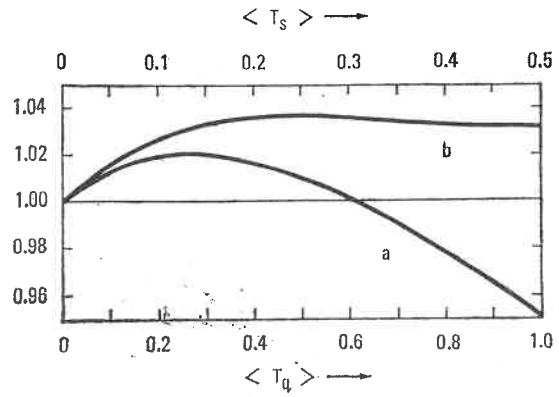

Fig. 4. Ratio of linear approximations a: $\frac{2}{3}(1-\langle T_q\rangle)$ and $\frac{2}{3}(1-2\langle T_s\rangle)$, and b: $\frac{2}{3}(2\langle d_s\rangle-1)$ for the effective average value of the orientation factor to its true value $\langle\kappa^2\rangle_{\text{eff}}$ as functions of the heterogeneous or homogeneous transfer efficiencies, $\langle T_q\rangle$ and $\langle T_s\rangle$ respectively, for static isotropic distributions of donor and acceptor orientations

It is interesting to note that, by substituting the approximate formulas of Eq. (30) for $\langle\kappa^2\rangle_{\text{eff}}$ back into the exact ones of Eqs. (28) and (29), and inverting for A:

$$A \simeq \frac{\langle T_q\rangle}{(1-\langle T_q\rangle)^2} = \frac{\langle T_s\rangle}{(1-2\langle T_s\rangle)^2} \tag{38}$$

compared with the values obtained for A in the dynamic averaging limit of:

$$A = \frac{T_q}{1-T_q} = \frac{T_s}{1-2T_s}, \tag{39}$$

where A in both cases contains the dynamic isotropic average value of 2/3 for κ^2. In terms of the observable depolarization parameter $\langle d_s\rangle \simeq 1-\langle T_s\rangle$, Eq. (38) for homogeneous transfer becomes:

$$A \simeq \frac{1-\langle d_s\rangle}{(2\langle d_s\rangle-1)^2}, \tag{40}$$

but, of course, there is no equivalent expression for the dynamically averaged case in which there is no way that energy transfer between isolated pairs can be detected.

3.2.5. Mixed static and dynamic averaging

For completeness, the case in which one of the fluorophores reorients dynamically, or equivalently is completely three-dimensionally degenerate, the other being static but also with its orientation isotropically distributed in the ensemble, is considered here. Since

either fluorophore may be initially excited with equal probability in the homogeneous case, the overall depolarization factor will always be 0.5, independent of the transfer efficiency, so only the heterogeneous case need be considered. Similarly there, the transfer depolarization factor will be independent of the transfer efficiency, and identically zero since all memory of initial orientation will be lost either immediately after absorption before transfer or immediately after transfer before emission. Taking the case where the acceptor is dynamically averaged and following the methods used previously, but substituting the average value of 1/3 for $\cos^2 \omega$ directly:

$$\langle T_q \rangle = \int_0^1 \frac{\frac{1}{2} A(1+3x^2)}{1+\frac{1}{2} A(1+3x^2)}\, dx = 1 - \frac{2}{3A}\sqrt{B} \arctan(\sqrt{B}), \tag{41}$$

where $B = 3A/(2+A)$. The identical result is obtained using the definition of κ^2 given in Eq. (3) which is completely symmetrical in D and A angular parameters which are independent so that the order of integration is unimportant. An effective average value

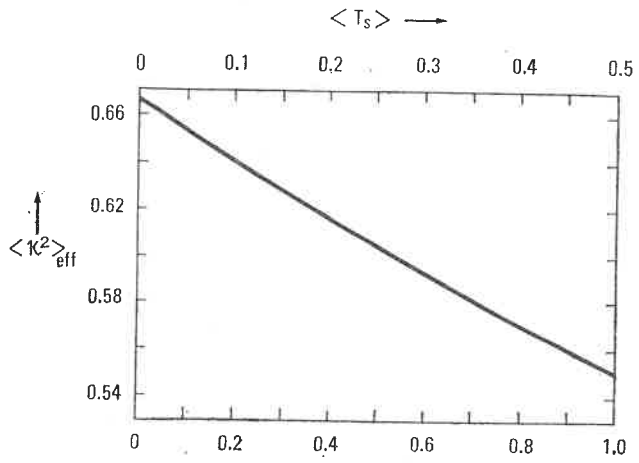

Fig. 5. Effective average value of the orientation factor $\langle \kappa^2 \rangle_{eff}$ for mixed statically and dynamically averaged isotropic distributions of donor and acceptor orientations as a function of heterogeneous or homogeneous transfer efficiencies, $\langle T_q \rangle$ and $\langle T_s \rangle$ respectively

of the orientation factor may be calculated as before and is displayed in Figure 5 as a function of $\langle T_q \rangle$ upon which it shows no obvious simple dependence. The limiting value at maximum transfer efficiency is $(\sqrt{3}/\pi) \simeq 0.551$.

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REFERENCES

Albrecht, A C., *J. Mol. Spectrosc.* **6**, 84 (1961).

Birnbaum, E. R., Abbott, F., Gomez, J. E., Darnall, D. W., *Arch. Biochem. Biophys.* **179**, 469 (1977).

Bojarski, C., *Bull. Acad. Pol. Sci. Sér. Sci. Math. Astron. Phys.* **6**, 719 (1958).

Dale, R. E., Eisinger, J., *Proc. Natl. Acad. Sci. USA* **73**, 271 (1976).

Eisinger, J., *Quart. Rev. Biophys.* **9**, 21 (1976).

Eisinger, J., Dale, R. E., in *Excited States of Biological Molecules*, ed. J. B. Birks, J. Wiley and Sons, New York 1976, p. 579.

Förster, Th., *Ann. Phys.* (Germany) **2**, 55 (1948).

Förster, Th., *Z. Naturforsch.* **4a**, 321 (1949).

Förster, Th., *Fluoreszenz Organischer Verbindungen*, Vandenhoek and Ruprecht, Göttingen 1951.

Förster, Th., in *Modern Quantum Chemistry, Pt. III*, ed. O. Sinanoglu, Academic Press, New York 1965, p. 93.

Galanin, M. D., *Trudy Fiz. Inst. Lebedev, Akad. Nauk SSSR* **5**, 339 (1950).

Galanin, M. D., *Soviet Phys.-JETP* **1**, 55 (1955) (*Zh. Eksp. Teor. Fiz.* **28**, 485 (1955)).

Guéron, M., Eisinger, J., Shulman, R. G., *J. Chem. Phys.* **47**, 4077 (1967).

Hemenger, R. P., Pearlstein, R. M., *J. Chem. Phys.* **59**, 4064 (1973).

Jabłoński, A., *Z. Phys.* **96**, 236 (1935).

Jabłoński, A., *Z. Phys.* **103**, 526 (1936).

Jabłoński, A., *Acta Phys. Pol.* **10**, 193 (1950).

Jabłoński, A., *Acta Phys. Pol.* **13**, 175 (1954); **14**, 504 (1955a).

Jabłoński, A., *Acta Phys. Pol.* **14**, 295 (1955b); **17**, 481 (1958).

Jabłoński, A., *Bull. Acad. Pol. Sci. Sér. Sci. Math. Astron. Phys.* **5**, 513 (1957a).

Jabłoński, A., *Acta Phys. Pol.* **16**, 471 (1957b).

Jabłoński, A., *Bull. Acad. Pol. Sci., Sér. Sci. Math. Astron. Phys.* **8**, 259 (1960).

Jabłoński, A., *Acta Phys. Pol.* **A38**, 453 (1970); **A39**, 87 (1971a).

Jabłoński, A., *Bull. Acad. Pol. Sci. Sér. Sci. Math. Astron. Phys.* **19**, 171 (1971b).

Jones, R. E., *Nanosecond Fluorimetry*, Ph. D. Thesis, Stanford University, University Microfilms Ltd., Ann Arbor 1970.

Ketskeméty, I., *Acta Phys. Chem. Szeged* **1**, 29 (1955).

Knox, R. S., *Physica* **39**, 361 (1968).

Latt, S. A., Auld, D. S., Vallee, B. L., *Proc. Natl. Acad. Sci USA* **67**, 1383 (1970).

Leung, C. S.-H., Meares, C. F., *Biochem. Biophys. Res. Commun.* **75**, 149 (1977).

Maksimov, M. Z., Rozman, I. Z., *Opt. Spectrosc.* **12**, 237 (1962) (*Opt. Spektrosk.* **12**, 506 (1962)).

Ore, A., *J. Chem. Phys.* **31**, 442 (1959).

Ore, A., *Physica* **54**, 237 (1971).

Perrin, F., *Acta Phys. Pol.* **5**, 335 (1936).

Soleillet, P., *Ann. Phys.* (Paris) **12**, 23 (1929).

Steinberg, I. Z., *J. Chem. Phys.* **48**, 2411 (1968).

Stryer, L., Haugland, R. P., *Proc. Natl. Acad. Sci. USA* **58**, 719 (1967).

Weber, G., in *Fluorescence and Phosphorescence Analysis*, ed. D. M. Hercules, J. Wiley and Sons, New York 1966, p. 217.