ENERGY TRANSFER IN LIPID BILAYERS

T. N. ESTEP AND T. E. THOMPSON, *Department of Biochemistry, University of Virginia, Charlottesville, Virginia 22901 U.S.A.*

ABSTRACT The quenching of fluorescence due to energy transfer between a dilute, random array of donor and acceptor chromophores in lipid bilayers was measured and compared to theoretical expressions developed to predict the decrease in emission intensity under these circumstances. The observed intensity was found to be the same function of quencher concentration in both planar, multilamellar dispersions and small, spherical vesicles. The degree of quenching was accurately predicted by a simple relation derived in this paper, as well as a more complex equation previously developed by Tweet, et al. The results suggest that significant quenching may be observed even when the average donor-acceptor separation exceeds the Förster critical distance by severalfold. Application of these results to problems of current interest in membrane research are discussed.

INTRODUCTION

Nonradiative energy transfer between chromophores by the Förster mechanism has long been recognized as a phenomenon of use in elucidating molecular conformation and aggregation (1–3). Recently, several publications have described experiments in which changes in fluorescence intensity due to energy transfer were used as the criterion for detecting the mixing (4) or aggregation (5) of certain membrane constituents or the interaction of proteins with membranes. (6) These efforts were hampered by the lack of a quantitative estimate of the amount of quenching to be expected for a random distribution of donor and acceptor species in two dimensions. A prerequisite for the performance of any such experiment is the demonstration that the degree of quenching for such a random array can be quantitatively predicted. If such prediction is possible, the predictive capability may be used to test for the randomness of chromophore distribution or, alternatively, if it is known that the chromophore distribution is random, for the number of quenchers present.

The purpose of this communication is to demonstrate that the degree of quenching by energy transfer in membranes can in fact be estimated through the use of equations developed on the basis of simple two-dimensional models. These equations are shown to hold for small phospholipid vesicles as well as simple dispersions, despite the differences between the physical properties and geometry of these structures. This work is intended to be applicable to experiments in which intrinsically or extrinsically introduced chromophores comprise only a small fraction of the total membrane mass. Energy transfer in membranes containing high concentrations of chromophores (e.g., chloroplasts) is not considered.

THEORY

The quantity to be derived is the ratio of donor fluorescence intensity in the presence of acceptors to the intensity observed in their absence. It is assumed that the acceptor molecules

affect the donor fluorescence only by the dipole-dipole interaction described by the Förster equation (7):

$$K_T = R_o^6/\tau R^6, \tag{1}$$

where K_T is the rate constant of energy transfer between a donor and acceptor separated by a distance, R. R_o is the distance at which the rate constant for energy transfer equals the rate constant for deactivation of the excited state in the absence of acceptors. The latter rate constant is the inverse of the lifetime of the unquenched donor, τ. R_o may be calculated for a given donor-acceptor pair from the spectral properties of the two chromophores if the relative chromophore orientations are known. Under these circumstances, R_o should be computed on the basis of the quantum yield of donor molecules subject to all deactivation processes that may be operant, except for energy transfer to the added acceptors. In the present discussion, it is assumed that R_o is known and equal for each donor-acceptor pair that is near enough to engage in energy transfer. The assumptions normally made in the derivation of Eq. 1 are discussed in detail by Berlman (8).

The chromophore-containing molecules are idealized as points distributed in a random and mutually independent manner upon a planar surface. It is further assumed that translational motion during the excitation lifetime is negligible and, that there is no donor-donor energy migration. Using these assumptions, Tweet et al. (9) derived an equation describing the quenching of chlorophyll fluorescence in planar monolayers by modifying the Förster (10) treatment for three-dimensional solutions. The resulting expression is:

$$F_A/F_o = \int_0^\infty \exp\,[-x - \Gamma(2/3)\pi c R_o^2 x^{1/3}]\mathrm{d}x, \tag{2}$$

where F_A is the fluorescence intensity in the presence of a random distribution of quenchers, F_o is the intensity in the absence of quenchers, $\Gamma(2/3)$ is the gamma function evaluated for the argument value of 2/3, and c is the average number of quenchers per unit area. This expression was derived with the assumptions that the surface concentration of both chromophores was low, and the mole fraction of acceptor was greater than that of donor. The latter assumption would seem to be somewhat stronger than necessary, because the equation should be valid as long as the acceptor concentration is greater than the concentration of excited donors. As noted by Brand and Witholt (11), in most fluorescence experiments, the concentration of excited species is a minute fraction of the total number of chromophores present, so that the weaker assumption will hold even when the mole fraction of donor exceeds that of acceptor. Under these conditions, quenching will be independent of donor concentration as long as there are no donor-donor interactions. Other equations were derived by Tweet and co-workers (9) for situations in which excitation is not a rare event, and for high concentrations of chromophores. Because in experiments utilizing fluorescent molecules as probes of membrane structure it is desirable to employ a low probe concentration so as to avoid undue perturbation of the system, the latter expressions will not be discussed.

A simpler expression for energy transfer quenching for dilute concentrations of chromophores in planar membranes may be derived from the following considerations: we first note that we need only consider those acceptors that are relatively close to a given donor because the $(R_o/R)^6$ dependence of the energy transfer rate constant insures that quenching will

decrease rapidly for distances beyond R_o. We therefore consider the quenching due to n acceptors arrayed within a distance, R_d, of a donor, where R_d is small but greater than R_o. Ultimately we will choose R_d to be the approximate distance beyond which quenching is insignificant. As has been customary in previous treatments of similar problems, we assume that various excited-state deactivation processes compete by parallel first-order kinetics, so that for a given donor (1, 3, 12):

$$\begin{aligned} F_n/F_o &= (1/\tau)\Big/\Big(1/\tau + 1/\tau \sum_{i=1}^{n} R_o^6/R_i^6\Big) \\ &= 1\Big/\Big(1 + \sum_{i=1}^{n} R_o^6/R_i^6\Big), \end{aligned} \tag{3}$$

where F_n is the fluorescence intensity in the presence of n acceptors, and R_i is the distance from the donor to the i^{th} acceptor. The probability, P, of a specific distribution of n acceptors at distances R_i ($i = 1, \ldots, n$) within a circle of radius R_d centered on the donor under the assumptions mentioned above is:

$$P = (2^n/R_d^{2n})\Big(\prod_{i=1}^{n} R_i dR_i\Big).$$

For a large number of donors we may calculate the average relative fluorescence intensity, $F_A(n)/F_o$, for n acceptors within R_d of a donor by taking the weighted average over all possible distributions, the weighting factor being F_n/F_o. Thus:

$$\begin{aligned} F_A(n)/F_o &= \frac{\int_0^{R_d} \cdots \int_0^{R_d} \Big[2^n\Big/\Big(1 + \sum_{i=1}^{n} R_o^6/R_i^6\Big)R_d^{2n}\Big]\Big[\prod_{i=1}^{n} R_i dR_i\Big]}{\int_0^{R_d} \cdots \int_0^{R_d}\Big[2^n/R_d^{2n}\Big]\Big[\prod_{i=1}^{n} R_i dR_i\Big]} \\ &= [2^n/R_d^{2n}] \int_0^{R_d} \cdots \int_0^{R_d} \Big[1\Big/\Big(1 + \sum_{i=1}^{n} R_o^6/R_i^6\Big)\Big]\Big[\prod_{i=1}^{n} R_i dR_i\Big]. \end{aligned}$$

To convert this expression to one more amenable to analysis, we let $x_i = R_i^2$, giving:

$$F_A(n)/F_o = (1/R_d^{2n}) \int_0^{R_d} \cdots \int_0^{R_d} \Big[1\Big/\Big(1 + R_o^6 \sum_{i=1}^{n} x_i^{-3}\Big)\Big]\Big[\prod_{i=1}^{n} dx_i\Big]. \tag{4}$$

To integrate Eq. 4 for $n > 1$, it is necessary to approximate the integrand and decide upon an appropriate value for R_d. In treating the analogous three-dimensional problem, Jabloński (12) was able to achieve good agreement with experiment by an approximation that is equivalent in the present case to letting $x_i = R_o^2$ for all i. Substituting this approximation into Eq. 4 and integrating yields: $F_A(n)/F_o = 1/(1 + n)$. To compute the fraction of unquenched fluroescence intensity actually observed experimentally, we sum over all products of $F_A(\mathrm{n})/F_o$

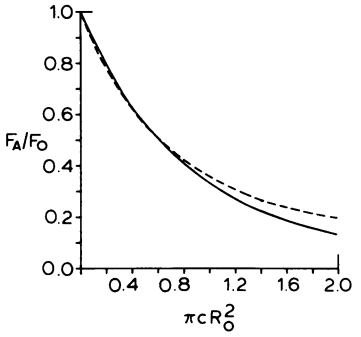

FIGURE 1 Relative fluorescence intensity as a function of acceptor concentration as predicted by theoretical expressions. The solid line was generated from Eq. 2 and the broken line from Eq. 5.

multiplied by the probability that n acceptors are within R_d of a donor. For a random array of acceptors, the probability distribution is Poisson and we have:

$$F_A/F_o = \sum_{n=0}^{\infty} [1/(n+1)][e^{-a_1} a_1^n/n!] = \sum_{n=0}^{\infty} e^{-a_1} a_1^n/(n+1)! = \frac{1 - e^{-a_1}}{a_1}, \qquad (5)$$

where a_1 is the average number of acceptors within R_d of a donor. If the average number of acceptors is c per unit area, $a_1 = \pi c R_d^2$. Jablónski treated R_d as an adjustable parameter that was to be varied to give the best fit to a given set of experimental data. We believe that for low concentrations of quencher there should be a best choice for R_d that will correctly predict the quenching observed experimentally à priori. In this regard we note that Eq. 5 can be made to fit Eq. 2 reasonably well for dilute acceptor arrays if we let $R_d = \sqrt{2.6}\, R_o$. This is illustrated in Fig. 1, in which Eqs. 2 and 5 are plotted as a function of $\pi c R_o^2$, with $a_1 = 2.6\, \pi c R_o^2$ in the latter expression. We therefore select $\sqrt{2.6}\, R_o$ as the most appropriate value for R_d.

It is also necessary to consider the quenching due to a random distribution of donors and acceptors on a spherical surface because small phospholipid vesicles are frequently used in membrane studies and because many natural membranes possess regions having a radius of curvature on the order of 100 Å (13). Without loss of generality we may assume that R_s, the radius of our model sphere, exceeds R_o, because few, if any, chromophore pairs exhibit R_o values 100 Å (8, 14). Quenching for the case in which R_s is less than or equal to R_o has previously been considered by several groups (1, 3, 15).

We again note that for the quenching of fluorescence of a given donor, we need only consider the transfer of energy to those acceptors occupying some part of the spherical surface about the donor, due to the rapid diminution of energy transfer with distance beyond R_o. We see, referring to Fig. 2, that the probability of a particular arrangement of n acceptors upon the curved surface of a spherical segment of height h_d is:

$$P = 1/(R_s h_d)^n \prod_{i=1}^{n} r_i dC_i.$$

The fractional decrease in fluorescence intensity for this arrangement is given by Eq. 3.

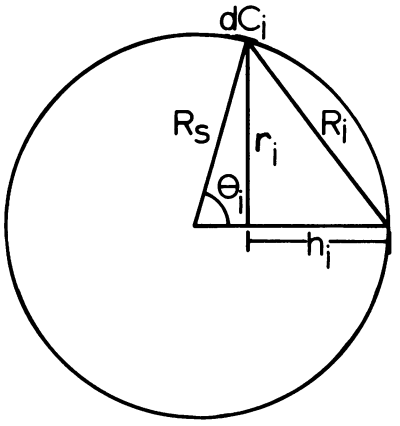

FIGURE 2 Diagram of parameters used in the calculation of quenching on spherical surfaces.

Combining these expressions and converting to polar coordinates gives:

$$\frac{F_A}{F_o} = \frac{2^n}{(1 - \cos\theta_d)^n} \int_0^{\theta_d} \cdots \int_0^{\theta_d} \frac{\prod_{i=1}^{n} \sin(\theta_i/2) \cos(\theta_i/d)\, d\theta_i}{1 + (R_o/2R_s)^6 \sum_{i=1}^{n} \sin^{-6}(\theta_i/2)}, \tag{6}$$

where θ_d is the angle corresponding to h_d. Letting $x_i = 4R_s^2 \sin^2(\theta_i/2)$ and $R_d = 2R_s \sin(\theta_d/2)$, we obtain, upon substitution into Eq. 6, an expression that is identical to Eq. 4. Proceeding in an analogous fashion to the derivation for the planar array, we find that the fractional decrease in fluorescence intensity is given by Eq. 5 with a_1 still corresponding to the average number of acceptors within R_d of a donor. Because the function describing the quenching due to a spherical distribution of acceptors is identical to that derived for a planar array, the optimal choice for R_d should correspond as well. We therefore allow R_d to equal $\sqrt{2.6}\, R_o$ for calculations involving both spherical and planar distributions. This implies that a_1 should also be defined identically for the two cases, because the area of spherical segment within R_d of a point on a sphere is equal to the area of a circle with radius R_d. Thus, Eq. 5 should hold equally well for planar and spherical membranes, with a_1 defined as $2.6\,\pi c R_o^2$, if the minimum radius of curvature exceeds $\sqrt{2.6}\, R_o/2$, and if R_o is not itself a function of this radius.

Recently, Hauser et al. derived a general expression for the time dependence of donor fluorescence after excitation by a short, intense flash in the presence of a random distribution of acceptors (16). This expression may be integrated to yield Eq. 2 for the case of a two-dimensional planar array. These authors also derived an equation describing the time dependence of acceptor fluorescence upon excitation of the donor, although the only two-dimensional arrangement that was specifically discussed was that of a single donor-acceptor pair confined to a small disk. Although the quantitation of energy transfer by its effect on the excited-state lifetime does offer certain advantages over intensity measurements

(16, 17), it also requires a more specialized apparatus. For this reason, we have chosen to focus our attention upon changes in the steady-state fluorescence intensity.

Shaklai et al. have considered the fluorescence quenching due to a random two-dimensional array of acceptors arranged on or above a planer surface containing the donor molecules, but restricted their analysis to the case in which the distance of closest approach of donor and acceptor was 1.68 R_o (18).

EXPERIMENTAL

To test the validity of Eqs. 2 and 5, the quenching of anthracene fluorescence by perylene was measured in both small unilamellar vesicles and large multilamellar liposomes of egg phosphatidylcholone. Lipid was isolated from hen egg yolks by the procedure of Litman (19), as modified by Roseman et al (2). Perylene was Aldrich "gold label" quality (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and was used without further purification. Anthracene was purchased material (Matheson, Coleman & Bell, East Rutherford, N.J.) and was purified by sublimation. Anthracene and perylene were stored in acetone solutions at −20°C. The concentration and integrity of these compounds were assessed by absorption spectra taken with Cary 14, Cary 15, or American Instruments DW-2 spectrophotometers (Cary Instruments, Fairfield, N.J.; American Instrument Co., Inc., Silver Springs, Md.) Organic solvents were either spectral grade or redistilled before use. All other compounds were of the highest available purity. Lipid was stored in chloroform under argon at −20°C. Aqueous solutions were made from deionized doubly distilled water.

Multilamellar liposomes were formed by shaking an aqueous solution of 13% sucrose, 50 mM KCl, 1 mM ethylenediaminetetraacetate, and 1 mM ethyleneglycol-bis-(β-aminoethyl ether)–N,N′–tetra-acetic acid, pH 7, over a film of phospholipid evaporated out of chloroform. The sucrose and chelating agents were used to minimize aggregation and settling of the liposomes. Vesicles were produced by the sonication of dispersions formed as described above in 50 mM KCl, followed by centrifugation at 100,000 g for 3 h to remove titanium particles and any remaining large lipid aggregates. Phosphatidyl-choline concentrations were determined by a modified Bartlett assay (19).

Dyes were added to the lipid by injecting aliquots of their acetone solutions directly into the aqueous phosphatidylcholine dispersions. The dispersions were vigorously agitated immediately after injection. In the case of multilamellar liposomes, chromphore addition was followed by an incubation of at least 1 h in a shaking bath to allow for complete equilibration. This procedure was followed in several of the vesicle experiments as well, although it was subsequently discovered that similar results could be obtained 5 min after the addition of perylene solution directly to cuvettes containing lipid suspensions. For the purpose of calculation, it was assumed that all anthracene molecules partitioned into the lipid bilayers under these conditions. Two experimental results indicate that this assumption is valid. In one instance, dispersions that had been equilibrated with dye were centrifuged for 3 h at 104,000 g and the supernatants examined for fluorescence. The emission intensity from these supernatants was 2% of that of uncentrifuged controls which were otherwise identical. This figure should be considered as an upper limit to the relative contribution of fluorescence from anthracene in the aqueous medium, because most of the intensity in the centrifuged samples may have originated from dye contained in small vesicles that could not be pelleted. In a second experiment, the absorption spectrum of anthracene in vesicle suspensions was compared to spectra of anthracene dispersed in water or dissolved in organic solvents. It was noted that aqueous dispersions of dye exhibit spectra in which the absorption maxima are shifted approximately 15 nm to longer wavelengths, compared to spectra taken in benzene solutions, resulting in a characteristic peak at 392 nm. The absorptivity of the 392-nm maximum is comparable to that of the longest wavelength peak of spectra of anthracene in organic solvents ($\epsilon \sim 7 \times 10^3$ M^{-1} cm^{-1}). No evidence for this 392-nm band was seen in spectra of anthracene in vesicle suspensions, these spectra being virtually idential to those of anthracene dissolved in benzene.

On the other hand, spectral measurements of perylene in the presence of lipid indicate that this dye does not partition completely into bilayers when the ratio of lipid to perylene is $< 500:1$. As was the case

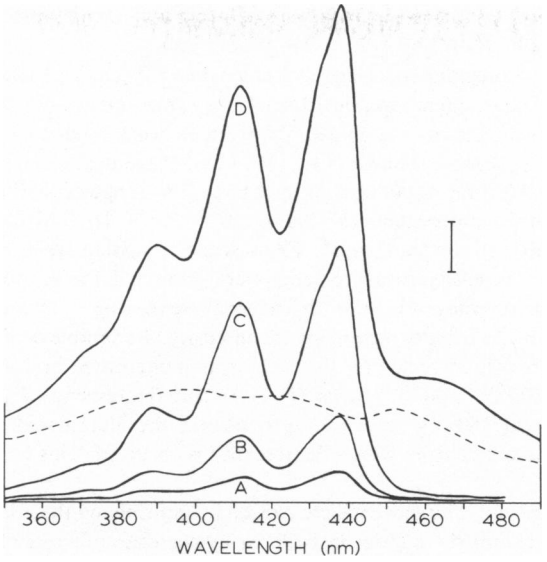

FIGURE 3 Absorption spectra of perylene dissolved in egg phosphatidylcholine vesicles (solid lines) and dispersed in water (broken line). The bandpass was 3.0 nm and the vertical bar represents an optical density of 0.01 for the vesicle samples and 0.05 for the aqueous dispersion of perylene. The lipid concentration was 400 μM in all of the vesicle dispersions. The total perylene concentration was (*A*) 0.17, (*B*) 0.51, (*C*) 1.54, and (*D*) 4.63 μM for the vesicle suspensions, and 10 μM for the aqueous dispersion.

with anthracene, aqueous dispersions of perylene exhibit a red shift of 15 nm in the absorption spectrum when compared to benzene solutions; however, the band structure of the perylene spectrum is broadened much more extensively by dispersion in water than is the band structure of anthracene. In lipid-containing samples in which the ratio of lipid to perylene was $>$ 500:1, the absorption spectra were nearly identical to those obtained in benzene solutions. At higher relative perylene concentrations, a broad absorbance extending to longer wavelengths could be detected underneath the usual band structure and the overall peak spectral intensity was less than expected. For samples having a lipid to total perylene ratio of$<$ 100:1, the observed intensity was only about one-third of that expected on the basis of the amount of dye added to the solution, assuming all of the perylene had partitioned into the bilayer. When such samples were extracted with 2:1 (vol:vol) chloroform-methanol and the extracted solids redissolved in organic solvent, the maximum expected intensity was observed. These effects are illustrated by the absorption spectra of perylene in vesicle suspensions and aqueous solution shown in Fig. 3. Evidently, those perylene molecules that do not rapidly partition into the bilayers remain suspended in the aqueous medium in a form that does not readily exchange into the lipid phase. This effect has been observed in at least one other laboratory.[1] For the purposes of the energy transfer experiments, the amount of perylene actually in the lipid bilayers was determined from the absorption spectra of representative samples. In determining the absorption of lipid-solubilized dye at higher perylene concentrations, measurements were taken from the 439-nm maximum to a base line drawn between the points at 335 and 465 nm on the absorption spectra to subtract that absorbance due to dye in the aqueous solution. Attempts to enhance perylene partitioning into the lipid bilayers by adding dye to the chloroform solutions of lipid before solvent removal were unsuccessful, apparently because the differential solubility of dye and lipid in this solvent precluded their coprecipitation.

Control experiments indicated that the maximum amount of acetone added along with anthracene

[1]Barenholz, Y. Personal communication.

and perylene had no effect on fluorescence intensity. During long incubations the suspensions were kept under an inert atmosphere.

Fluorescence intensity measurements were performed on a Hitachi-Perkin-Elmer MPF-3 spectrofluorimeter (Hitachi Ltd., Tokyo, Japan; Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) equipped with a temperature-controllable cuvette holder. Experiments were conducted at ~ 25°C with temperature variations during a typical experiment of < 1°C. Lipid concentrations were 0.4 μmol/ml for vesicle suspensions and 0.2 μmol/ml for dispersions of liposomes. The range of total dye concentration was 8.6 × 10^{-8}–1.7 × 10^{-7} M for anthracene and 8.6 × 10^{-8}–2.3 × 10^{-6} M for perylene. Samples were excited at 330 nm with a 10-nm bandwidth. Fluorescence emission was detected at 380 nm with a bandwidth of 4–10 nm. Intensity measurements were corrected for scattering, base-line drift, and changes in instrumental response with time by utilizing scattering blanks containing all compounds except anthracene, and by repeatedly measuring the intensity of a sample containing anthracene but not perylene. Intensities were also corrected for the reabsorption of emitted radiation by the equation: $F_T = 2.303\, F_m D(x_2 - x_1)/(10^{-Dx_1} - 10^{-Dx_2})$, where F_T is the true fluorescence, F_m is the measured intensity, D is the optical density at 380 nm, and x_2 and x_1 delimit the illuminated volume of the cuvette, as measured from the cuvette face from which fluorescence is observed. This correction was <10% for all samples examined.

Corrected fluorescence emission spectra were plotted by multiplying the relative spectral intensities at various wavelengths as obtained from our instrument by correction factors obtained by comparing the spectrum of anthracene in benzene to that published by Melhuish (21), and the spectra of diphenylhexatriene in cyclohexane, 1,4-*bis*-2(5-phenyloxazolyl)-benzene in methanol, quinine sulfate in ethanol, and indole in ethanol, to spectra published in Berlman (22). To avoid distortion artifacts caused by inner filter effects, only dilute (<10^{-5} M) solutions were utilized, and comparisons made only between those parts of the fluorescence spectra that were well removed from regions of absorption.

The quantum yield, Q_A, of anthracene in lipid bilayers was determined by employing the relationship (23): $Q_A = [I_A Q_S n_A^2(1-10^{-OD_S})]/[I_S n_S^2(1-10^{-OD_A})]$, where Q_S is the known quantum yield of a standard compound, I is the integrated emission spectral intensity, OD refers to the optical density at the wavelength of excitation, and n is the refractive index of the solution. The standard utilized in the present study was anthracene in benzene. The quantum yield for this compound in a benzene solution in equilibrium with atmospheric oxygen was calculated as 0.216 from the data of Melhuish (24). Integrated spectral intensity was calculated as the area under the corrected emission spectrum. Refractive indices were measured with a Bausch & Lomb ABBE-3L refractometer (Bausch & Lomb Inc., Scientific Optical Products Div., Rochester, N.Y.) at 589 nm and corrected to 400 nm to give values of 1.34 for lipid suspensions and 1.51 for anthracene in benzene. Optical densities were determined from absorption spectra. The quantum yield calculated with these values was 0.23.

R_o was calculated from (25): $R_o = (8.785 \times 10^{-25} K^2 Q n^{-4} J)^{1/6}$, where Q is the donor quantum yield, n is the refractive index of the medium between donor and acceptor, K^2 is an orientation factor, and J is a measure of the overlap between donor emission and acceptor absorption defined by $J = [\int_0^\infty F(\lambda)\epsilon(\lambda)\lambda^4 d\lambda]/[\int_0^\infty F(\lambda)d\lambda]$, with $\epsilon(\lambda)$ the molar absorbance of acceptor at wavelength λ and $F(\lambda)$ the fluorescence intensity at the same point. J was evaluated graphically from the corrected emission spectrum of anthracene and the absorption spectrum of perylene in vesicles and was found to equal 4.50 × 10^{-14} cm^6 mol^{-1}. Q was taken to be 0.23 and n was assumed equal to 1.44. K^2, a function of the relative orientation of donor emission and acceptor absorption moments, was assumed to be 2/3, the value resulting when there is rapid, isotropic reorientation of these moments during the donor emission lifetime. This assumption may be rationalized on the basis of the work of Cogan et al (26). These authors studied the fluorescence depolarization of perylene in egg phosphatidylcholine dispersions and found that the motion of this dye in these systems was similar to that observed in isotropic liquids. Thus, there is no preferred orientation of the perylene molecules relative to the bilayer. It was further discovered that the rate of perylene rotation about an axis perpendicular to the plane formed by the aromatic rings was considerably faster than the rotation rate about any axis contained within this plane. No specific rotational rates were determined at 25°C in this study, although estimates were made at −10°C. If one extrapolates the −10°C values to 25°C, assuming the rotational rates vary in proportion to the

reciprocal of the microviscosity reported by perylene for these bilayers at these two temperatures, one finds that perylene undergoes many in-plane rotations and approximately one out-of-plane rotation during the lifetime of anthracene in the absence of acceptors (~ 4 ns) (27). Because anthracene is similar to perylene in size and shape, it would be expected to have the same motional characteristics. These estimates imply that most anthracene and perylene molecules will undergo at least 90° of isotropic rotation during the lifetime of the anthracene excited state, even when this lifetime is reduced due to the presence of acceptors, so that the dynamic averaging approximation will be approached for many donor-acceptor pairs. This supposition is supported by the experimental observation that the fluorescence anisotropy is less than 0.1 for both anthracene and perylene in the lipid bilayers used in our experiments. Using the various parameters quoted above, we calculate an R_o of 33.5 Å for anthracene to perylene transfer.

The average number of acceptors per Å^2 was calculated by taking the inverse of one half of the ratio of lipid molecules to acceptor molecules multiplied by the area occupied by one lipid molecule. An area of 71.7 Å^2 for each lipid molecule was taken from the work of Small (28). For the vesicles used in the present study, this is equivalent to modeling the lipid aggregate as a sphere whose radius is 81.6 Å. This is approximately the distance from the vesicle center to the boundary between the inner and outer lipid monolayers comprising the vesicle surface (29).

Analysis of phosphatidylcholine extracted from the sample solutions after a typical experiment by thin-layer chromatography revealed that no lipid breakdown had occurred. The developing solvent was 65:35:4 (vol:vol:vol) chloroform:methanol:water, and visualization was accomplished by iodine staining.

RESULTS

The experimentally determined fluorescence quenching in multilamellar liposomes and unilamellar vesicles is compared to that calculated on the basis of Eqs. 2 and 5 in Table I. The quenching predicted by Eq. 2 was calculated by numerical integration by using Simpson's

TABLE I
COMPARISON OF THEORETICAL AND EXPERIMENTAL RESULTS

Acceptor concentration			Fractional fluorescence intensity*					
			Vesicles‡‡		Liposomes‡‡		Predicted	
Average number per vesicle or vesicle equivalent‡	Average number within $\sqrt{2.6}\,R_o$ of donor§	Separation**	1 Donor per vesicle‡ (289 Å separation**)	10 Donors per vesicle‡ (91 Å separation**)	3 Donors per vesicle equivalent‡ (167 Å separation**)	10 Donors per vesicle equivalent‡ (91 Å separation**)	Eq. 5	Eq. 2
		Å						
1	0.1094	289	0.95 ± 0.01	0.96 ± 0.01	0.96 ± 0.01	0.96 ± 0.01	0.95	0.95
3	0.3283	167	0.86 ± 0.01	0.87 ± 0.01	0.85 ± 0.03	0.86 ± 0.01	0.85	0.86
7	0.7661	109			0.69 ± 0.02	0.69 ± 0.02	0.70	0.71
8	0.8755	102	0.68 ± 0.03	0.70 ± 0.03			0.67	0.67
9	0.9849	96			0.65 ± 0.04	0.64 ± 0.04	0.64	0.64
13	1.4227	80	0.51 ± 0.06	0.57 ± 0.10			0.53	0.53

*Error limits are estimates of experimental uncertainty or the standard deviation of result averages, whichever is largest. Each data point is calculated from the results of 2–4 experiments.
‡Calculated as the average number of acceptors per 2335 lipids (37).
§Equal to parameter a_1 of Eq. 5.
**Calculated as the square root of average area occupied per chromophore.
‡‡Donor concentrations are averages calculated as for acceptors.

approximation. In both liposomes and vesicles, the relative fluorescence intensity was accurately predicted by Eqs. 2 and 5 over the concentration range of donors and acceptors used in this study. Furthermore, the quenching was equivalent for a given concentration of acceptor in both systems and independent of the donor concentration to within experimental error.

To estimate the possible contributions of collisional quenching and molecular translational motion to the fluorescence intensity diminution, we note that Birks and Georghiou (30) have found these mechanisms to be negligible in solutions where the mean diffusion path length of excited chromophores is less than $R_o/3$. This path length, denoted as r, is given by:

$$r = (2D_T\tau)^{1/2}, \tag{7}$$

where D_T is the sum of the diffusion coefficients of the donor and acceptor molecules and τ is the lifetime of the donor in the absence of quenching. The lateral diffusion coefficient of pyrene has been measured as 1.4×10^{-7} cm^2/s in phospholipid bilayers very similar to those used in the present study (31). We will take this value as typical because pyrene resembles both anthracene and perylene in size and shape. Ware has reported the anthracene lifetime to be 4.1 ns (27). Using these results in Eq. 7 gives a mean diffusion length of 3.4 Å, an order of magnitude less than the critical distance of 34 Å. It therefore appears that neither collisional quenching mechanisms nor translational motion during excitation are contributing to the observed fluorescence intensity decrease.

DISCUSSION

Comparison of the experimental results with those calculated from Eqs. 2 and 5 indicates that one can predict the quenching due to a dilute, random distribution of acceptors in lipid bilayers having various radii of curvature from spectral data, if a reasonable estimate can be made of chromophore motion. Both the Tweet et al. expression given in Eq. 2 and Eq. 5 correctly predict the observed quenching of anthracene fluorescence by perylene in egg phosphatidylcholine bilayers over the concentration range of donors and acceptors used in this study.

Eq. 2 was previously shown to predict the energy transfer quenching of dilute, random distributions of donors and acceptors in planar monolayers (9). Because multilamellar liposomes of egg phosphatidylcholine consist of thin bilayers whose radii of curvature are quite large compared to typical R_o values (32), it was anticipated that Eq. 2 would correctly predict the quenching in these structures; however, the ability of Eq. 2 to predict the quenching in small vesicles is somewhat unexpected. This ability results at least in part from the mathematical similarity in the functions describing quenching in both large and small radius of curvature membranes, a similarity shown more specifically in the derivation of Eq. 5. This hypothesis is supported by the experimental data that show that the quenching for a given concentration of acceptors is the same in vesicles and liposomes. It should be noted that equal quenching in vesicles and liposomes depends on K^2 being independent of the radius of curvature. If K^2 is a strong function of the membrane radius, then neither equation derived above will hold in general. On the other hand, small curvature effects may not be detectable experimentally, because the radius of curvature of even the smallest vesicles exceeds R_o by severalfold.

Because a_1 was chosen so that Eq. 5 would approximate Eq. 2 for moderate concentrations of acceptors, it is not surprising that the former expression accurately predicts the observed relative fluorescence intensity as well as the latter. Fig. 1 indicates that the correspondence between the two equations holds for distributions in which $\pi c R_o^2$ is <0.8. This limit is ≈ 1 quencher/100 lipid molecules for an R_o of 30 Å. This does not pose a severe experimental constraint on the use of Eq. 5 in fluorescent probe studies because one can often quantitate the fluorescence intensity originating from a much lower concentration of fluorophores. It is also desirable in many cases to use dye-to-lipid ratios lower than 1:100 so as to minimize perturbation of membrane structure. One advantage in the use of Eq. 5 as opposed to Eq. 2 lies in the ease of computation of expected quenching with the former expression, as the use of Eq. 2 requires that one perform a numerical integration.

The assumptions used in deriving Eqs. 2 and 5 should be valid in many model and natural membrane systems. Even in relatively fluid membranes, such as the egg phosphatidylcholine bilayers employed in this study, the viscosity is high enough to severely reduce collisional quenching between moderate concentrations of fluorescent probes (33). It is therefore relatively easy to create situations in which energy transfer is the dominant quenching mechanism. The equivalence of results obtained with small (vesicle) and large (multilamellar liposome) radius of curvature bilayers implies that Eqs. 2 and 5 should be applicable to highly convoluted natural membrane structures containing dilute concentrations of donors and acceptors. Nonetheless, it must be reiterated that both equations are based on assumptions that may not be met in some membrane systems. In particular, the assumption that R_o is equal for each donor-acceptor pair that is near enough to engage in energy transfer must be carefully evaluated. As is the case with most energy transfer calculations, the most difficult factor to assess is the chromophore motion determining K^2. In some cases, steady-state polarization measurements can define limits as to the range of K^2 values (25). Better still is the use of time-dependent anisotropy measurements to detect and evaluate restricted modes of rotation (34–36). In this regard, the calculations of Dale and Eisinger (25) of K^2 resulting from various types of restricted motion may prove of use for a number of situations likely to be encountered in natural membranes. If it can be shown that K^2 among neighboring donor-acceptor pairs and the chromophore spectral properties are homogeneous throughout the membrane, one may then calculate an R_o that can be used in Eqs. 2 or 5.

To illustrate one potential application of the present work, we apply Eq. 5 to an interesting set of data that has appeared in the literature. Vanderkooi et al. have used fluorescence quenching due to energy transfer between fluorescent labels attached to sarcoplasmic ATPase molecules to determine the state of aggregation of this protein in reconstituted vesicles (5). These authors state that the quenching observed when proteins containing donor labels were mixed with those containing acceptors was probably the result of protein aggregation. The average separation of like chromophores was on the order of 150 Å, for a random distribution, and the critical distance was calculated as 48 Å. Using this information and Eq. 5, we calculate an 18% reduction of donor fluorescence intensity for a randomly distributed nonassociated system. This is similar to that actually observed (15–20%). Although the decrease in intensity predicted by Eq. 5 is possibly an overestimate due to the relatively large excluded volumes of the proteins containing the labels, it is clear that even when the average chromophore separation is several times as large as R_o, significant energy transfer may occur without aggregation.

Besides serving as a criterion for the aggregation of membrane constituents, energy transfer should also prove useful in quantifying binding and exchange phenomena. In addition, by incorporating nonexchangeable donor and acceptor probes into different vesicular or cellular membranes, it should be possible to distinguish between fusion and aggregation, as the former will result in the quenching predicted by Eq. 5, while the latter will decrease donor fluorescence to a lesser extent. Some experiments in this vein have already appeared in the literature and it is hoped that others may be facilitated by the work presented in this communication (4).

The authors would like to thank Doctors R. L. Biltonen, B. J. Litman, and P. W. Holloway for the use of their spectrophotometers, Dr. G. Weber for critical evaluation of the manuscript, and Dr. M. A. Roseman for stimulating interest in the problem of energy transfer within membranes. We also wish to thank the reviewers of this manuscript for a number of helpful criticisms.

This work was supported in part by United States Public Health Service grants GM-14628 and CA-18710.

Received for publication 28 February 1978 and in revised form 20 December 1978.

REFERENCES

1. WEBER G., and F. J. W. TEALE. 1959. Electronic energy transfer in haem proteins. *Discuss. Faraday Soc.* **27**:134.
2. STRYER, L. 1968. Fluorescence spectroscopy of proteins. *Science (Wash. D.C.).* **162**:526.
3. BADLEY, R. A. and F. W. J. TEALE. 1971. Geometry and fluorescence of pepsin-substrate complexes. *J. Mol. Biol.* **58**:567.
4. KELLER, P. M., S. PERSON, and W. SNIPES. 1977. Fluorescence enhancement assay of cell fusion. *Biophys. J.* **17**:30*a* (abstr.).
5. VANDERKOOI, J. M., A. IEROKOMAS, H. NAKAMURA, and A. MARTONOSI. 1977. Fluorescence energy transfer between Ca^{2+} transport ATPase molecules in artificial membranes. *Biochemistry.* **16**:1262.
6. VAZ, W. L. C., K. KAUFMANN, and A. NICKSCH. 1977. Use of energy transfer to assay the association of proteins with lipid membranes. *Anal. Biochem.* **83**:385.
7. FÖRSTER, T. 1948. Zwischenmolekulare energiewanderung und fluoreszenze. *Ann. Physik.* **2**:55.
8. BERLMAN, I. B. 1973. Energy Transfer Parameters of Aromatic Compounds. Academic Press, Inc., New York. 30.
9. TWEET, A. G., W. D. BELLAMY, and G. L. GAINES. 1964. Fluorescence quenching and energy transfer in monomolecular films containing chlorophyll. *J. Chem. Phys.* **41**:2068.
10. FÖRSTER, T. 1949. Experimentelle and theoretische untersuchung des zwischenmolekularen übergangs von elektronenanregungs energie. *Z. Naturforsch. A. Astrophysik, Physik und Physikalische Chemie.* **4**:321.
11. BRAND, L., and B. WITHOLT. 1967. Fluorescence measurements. *Methods Enzymol.* **11**:776.
12. JABLOŃSKI, A. 1957. Yield of photoluminescence of solutions. *Bull. Acad. Pol. Sci. Ser. Sci. Math. Astron. Phys.* **5**:513.
13. THOMPSON, T. E., C. HUANG, and B. J. LITMAN. 1974. Bilayers and biomembranes: compositional asymmetries induced by surface curvature. *In* The Cell Surface in Development. A. A. Moscona, editor. John Wiley & Sons, Inc., New York. 1.
14. FORSTER, T. 1959. Transfer mechanisms of electronic excitation. *Discuss. Faraday Soc.* **27**:7.
15. GENNIS, R. B. and C. R. CANTOR. 1972. Use of nonspecific dye labeling for singlet energy-transfer measurements in complex systems. A simple model. *Biochemistry.* **12**:2509.
16. HAUSER, M., U. K. A. KLEIN, and U. GÖSELE. 1976. Extension of Förster's theory of long-range energy transfer to donor-acceptor pairs in systems of molecular dimensions. *Z. Phys. Chem. N.F.* **101**:255.
17. SCHILLER, P. W. 1975. The measurement of intramolecular distances by energy transfer. *In* Biochemical Fluorescence. F. R. Chen and H. Edelhoch, editors. Marcel Dekker, Inc., New York. Vol. 1. 285.
18. SHAKLAI, N., J. YGUERABIDE, and H. M. RANNEY. 1977. Interaction of hemoglobin with red blood cell membranes as shown by a fluorescent chromophore. *Biochemistry* **16**:5585.
19. LITMAN, B. J. 1973. Lipid model membranes. Characterization of mixed phospholipid vesicles. *Biochemistry.* **12**:2545.

20. ROSEMAN, M. A., P. W. HOLLOWAY, M. A. CALABRO, and T. E. THOMPSON. 1977. Exchange of cytochrome b_5 between phospholipid vesicles. *J. Biol. Chem.* **252**:4842.
21. MELHUISH, W. H. 1960. A standard fluorescence spectrum for calibrating spectro-fluorophotometers. *J. Phys. Chem.* **64**:762.
22. BERLMAN, J. B. 1971. Handbook of Fluorescence Spectra of Aromatic Molecules. Academic Press Inc., New York. 473.
23. PARKER, C. A., and W. T. REES. 1960. Correction of fluorescence spectra and measurement of fluorescence quantum efficiency. *Analyst* **85**:587.
24. MELHUISH, W. H. 1961. Quantum efficiencies of fluorescence of organic substances: effect of solvent and concentration of the fluorescent solute. *J. Phys. Chem.* **65**:229.
25. DALE, R. E., and J. EISINGER. 1975. Polarized excitation energy transfer. *In* Biochemical Fluorescence. R. F. Chen and H. Edelboch, editors. Marcel Dekker, Inc., New York. Vol. 1. 115.
26. COGAN, U., M. SHINITZKY, G. WEBER, and F. NISHIDA. 1973. Microviscosity and order in the hydrocarbon region of phospholipid and phospholipid-cholesterol dispersions determined with fluorescent probes. *Biochemistry*. **12**:521.
27. WARE, W. R., 1961. An experimental study of energy transfer between unlike molecules in solution. *J. Phys. Chem.* **83**:4374.
28. SMALL, D. M. 1967. Phase equilibria and structure of dry and hydrated egg lecithin. *J. Lipid Res.* **8**:551.
29. HUANG, C., and J. T. MASON. 1978. Geometric packing constraints in egg phosphatidylcholine vesicles. *Proc. Natl. Acad. Sci. U.S.A.* **75**:308.
30. BIRKS, J. B., and S. GEORGHIOU. 1968. Energy transfer in organic systems VII. Effect of diffusion on fluorescence decay. *J. Phys. B: At. Mol. Phys.* **1**:958.
31. GALLA, H., and E. SACKMANN. 1974. Lateral diffusion in the hydrophobic region of membranes: use of pyrene excimers as optical probes. *Biochim. Biophys. Acta.* **339**:103.
32. BANGHAM, A. D., and R. W. HORNE. 1964. Negative staining of phospholipids and their structural modification by surface-active agents as observed in the electron microscope. *J. Mol. Biol.* **8**:660.
33. SHINITZKY, M., and Y. BARENHOLZ. 1974. Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing dicetylphosphate. *J. Biol. Chem. 249:2652.*
34. KINOSITA, K., K. SUGURU, and A. IKEGAMI. 1977. A theory of fluorescence polarization decay in membranes. *Biophys. J.* **20**:289.
35. KAWATO, S., K. KINOSITA, and A. IKEGAMI. 1977. Dynamic structure of lipid bilayers studied by nanosecond fluorescence techniques. *Biochemistry* **16**:2319.
36. CHEN, L. A., R. E. DALE, G. ROTH, and L. BRAND. 1977. Nanosecond time-dependent fluorescence depolarization of diphenylhexatriene in dimyristoyllecithin vesicles and the determination of "microviscosity". *J. Biol. Chem.* **252**:2163.
37. NEWMAN, G. C., and C. HUANG. 1975. Structural studies on phosphatidylcholine-cholesterol mixed vesicles. *Biochemistry*. **14**:3363.