helpful discussions. We also thank S. Amselem and R. Gatt for technical assistance and R. Knafo for the drawings.

Supplementary Material Available

Supplementary material including various modifications to Small's model, the conclusion that for long-chain phospholipids the PC–bile salt mixed micelles are probably curved, and calculation of the N radius of curvature as a function of the chain length and R (6 pages). Ordering information is given on any current masthead page.

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Comparison of Fluorescence Energy Transfer and Quenching Methods to Establish the Position and Orientation of Components within the Transverse Plane of the Lipid Bilayer. Application to the Gramicidin A–Bilayer Interaction†

Elizabeth A. Haigh, Keith R. Thulborn, and William H. Sawyer*

ABSTRACT: Fluorescence quenching and resonance energy transfer methods have been used to investigate the position of fluorophores in the lateral and transverse planes of the lipid bilayer. A series of n-(9-anthroyloxy) fatty acids (n = 2, 6, 9, and 12) have been used as energy-transfer acceptors so that apparent transfer distances from a membrane-bound donor (N-stearoyltryptophan) have a transverse as well as a lateral component. Both theory and experiment show that the energy-transfer method is not precise enough to discriminate between the positions of the fluorophores in the transverse plane of the bilayer. The n-(9-anthroyloxy) fatty acids are also susceptible to quenching by the indole moiety of tryptophan. The relative quenching efficiency can provide a semiquantitative measure of the position of quenching molecules in the lipid bilayer. The quenching techniques are applied to the determination of the orientation of gramicidin A in lipid bilayers. The tryptophan residues of gramicidin appear to be located near the membrane surface in agreement with the head-to-head dimeric structure proposed by D. W. Urry et al. [(1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672–676].

The molecular details of structure–function relationships in biomembranes must involve a description of the topographical distribution of membrane components. This is especially true for such complex processes as membrane transport and energy transduction where the disposition of the participating molecules in the membrane can have an important influence on the kinetics of the processes. The position and orientation of a membrane component in the transverse plane of the membrane, together with the proximity of one molecule to another in the lateral plane, are features of recognized importance; yet, relatively few techniques are available for their examination (for reviews, see De Pierre & Ernster, 1977; Peters & Richards, 1977).

An alternative approach, which is particularly suited to the study of natural as well as model membrane systems, employs spectroscopic probes which locate at different depths in the lipid bilayer and whose spectral characteristics change in response to the proximity of a given membrane component. In this paper we explore the use of fluorescence quenching and

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resonance energy transfer to determine the relative positions of fluorophores in the transverse plane of the membrane. Potentially, the resonance transfer of fluorescence, analyzed according to the Förster theory (Förster, 1948) is capable of measuring distances between appropriate donor–acceptor pairs in lipid bilayers. On the other hand, the quenching of a membrane bound fluorophore provides a measure of the accessibility of the fluorophore to the quenching molecule and therefore an indication of how deeply the fluorophore is buried within the bilayer structure. We examine both types of analyses in model systems and, in particular, determine the suitability of the indole moiety of tryptophan to act as a donor for the transfer of energy to a set of *n*-(9-anthroyloxy) fatty acid fluorescent probes (n = 2, 6, 9, and 12)[1] as well as a quencher of their fluorescence. The fluorophores of the fatty acid probes take up a graded series of positions from the surface to the center of the lipid bilayer. Their relative positions have been established previously by fluorescence quenching and NMR methods (Podo & Blasie, 1977; Thulborn & Sawyer, 1978).

The fluorescence quenching and energy transfer methods are also applied to the study of the gramicidin A–lipid bilayer interaction. Gramicidin A is a linear polypeptide antibiotic of 15 amino acid residues which renders lipid bilayers permeable to alkali cations and protons by forming a transbilayer ion channel. The linear gramicidins exist as several species, the most abundant being gramicidin A which has L-tryptophan residues at positions 9, 11, 13, and 15 but no phenylalanine or tyrosine. The N- and C-terminal residues are blocked with a formyl and an ethanolamide group, respectively. Several models have been proposed for the transbilayer structure based on the helical conformations which occur in organic solvents. Urry (1971) and Urry et al. (1971) proposed a head-to-head $\pi_{(L,D)}$ helical dimer (formyl end to formyl end) which places the tryptophan residues closer to the membrane surface than to the center of the bilayer. Veatch et al. (1974) isolated four conformationally distinct species of gramicidin and discussed the probability of these existing as parallel and antiparallel β-double helical dimers which would have the tryptophan residues more or less evenly distributed through one or two bilayer leaflets, respectively, since the pitch of the helix (10–12 Å) is very much greater than in the case of the $\pi_{(L,D)}$ dimer (5 Å). The fluorescence quenching and energy transfer methods are used in an effort to determine the orientation of gramicidin in the bilayer and, in particular, to determine if the tryptophan residues are situated predominantly at the membrane surface or are distributed throughout the transverse plane of the bilayer.

Materials and Methods

DL-α-Dimyristoyl- and DL-α-dipalmitoylphosphatidylcholines were purchased from Sigma and migrated as single spots on TLC in several solvent systems. Egg phosphatidylcholine and phosphatidylserine from bovine spinal chord were from Lipid Products. The synthesis and properties of the set of *n*-(9-anthroyloxy) fatty acids (n = 2, 6, 9, and 12) have been described previously together with procedures for preparing vortexed and sonicated dispersions of phospholipids (Thulborn & Sawyer, 1978). 16-(9-Anthroyloxy)palmitic acid was also used in some experiments (Cadenhead et al., 1977). *N*-Stearoyltryptophan and 12-(9-anthroyloxy)-*N*-stearoyltryptophan were prepared by conjugation of the *N*-hydroxysuccinimide ester of the fatty acid with tryptophan (Lipidot et al., 1967; Thulborn, 1979). Gramicidin was a mixture of types A, B, and C and was purchased from ICN Pharmaceuticals. Spin-labeled fatty acids (5-, 12-, and 16-nitroxide stearate) were purchased from Syva Corp. Stock solutions (1 mM) were prepared in methanol and their relative concentrations were checked by EPR and far-UV absorption measurements.

Fluorescence measurements were made with a Hitachi Perkin-Elmer MPF3 spectrofluorometer equipped with a thermostated cell block and polarization accessory. For polarization measurements, the intensities of horizontal and vertical components of the emitted light (I_H and I_V) were corrected for the contribution of scattered light determined independently for an unlabeled reference solution of the same composition (I_V^S and I_H^S). Thus

$$p = \frac{(I_V - I_V^S) - G(I_H - I_H^S)}{(I_V - I_V^S) + G(I_H - I_H^S)} \quad (1)$$

where p is the polarization and G the grating correction factor. Rotational relaxation times (ρ) were calculated according to the Perrin equation for isotropic rotation

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right) \quad (2)$$

where p_0 is the limiting polarization in the absence of rotational motion and τ is the lifetime of the excited state. The p_0 values for the anthroyloxy fatty acids are similar (0.29 ± 0.01) (Thulborn et al., 1978). Equation 2 provides values of apparent rotational relaxation times since the motion of the fluorophore in the lipid bilayer is not isotropic (Chen et al., 1977; Kinosita et al., 1977).

Fluorescence lifetimes were measured by the method of single photon counting by using 363-nm excitation and Schott GG400 and GG420 cut-off filters to observe fluorescence at wavelengths greater than 420 nm. The exciting light was vertically polarized and a polarizer set at an angle of 54.7° (Shinitzky, 1972) to the vertical plane was used in the emission beam to eliminate the effects of polarized emission on the lifetime. The fluorescence decay was deconvoluted from the lamp impulse function as a single exponential by using a nonlinear least-squares fitting program.

Details of the fluorescence quenching experiments are presented in the figure captions. Energy transfer experiments between *N*-stearoyltryptophan and the fluorescent fatty acids were carried out by adding small aliquots (5–10 μL) of the fatty acid (1 mM in methanol) to a liposome suspension containing a fixed concentration of *N*-stearoyltryptophan (5 μM). Additions were made directly to the cuvette and fluorescence intensities were measured 1 h after the addition of each aliquot to allow for complete probe uptake. A similar procedure was followed when gramicidin was used as the donor. A 45° front-surface geometry was essential in these fluorescence experiments to prevent trivial reabsorption of donor fluorescence. Such trivial reabsorption was detected easily because the absorption bands of anthracene (344, 363, 382 nm) overlap the donor emission in such a way that reabsorption of donor fluorescence results in asymmetry and sometimes in multiple peaks in the normally featureless emission band of tryptophan. Values of the spectral overlap integral, J_{da}, were calculated from eq 3 by numerical integration of linear wavelength values of absorption and of fluorescence intensity measured at constant bandwidth (Badley & Teale, 1969)

[1] Abbreviations used: 2-AP, 2-(9-anthroyloxy)palmitic acid; 6-AS, 9-AS, and 12-AS, 6-, 9-, and 12-(9-anthroyloxy)stearic acid; 16-AP, 16-(9-anthroyloxy)palmitic acid; TLC, thin-layer chromatography.

$$J_{da} = \frac{\sum_{\lambda_1}^{\lambda_2} \epsilon_\lambda I_\lambda \lambda^4}{\sum_{\lambda_1}^{\lambda_2} I_\lambda} \quad (3)$$

where I_λ is the tryptophan fluorescence intensity at wavelength λ and ϵ_λ is the molar absorbancy of the fluorescent fatty acid at the same wavelength. Values of J_{da} were between 3.7×10^{-15} and 5.9×10^{-15} mmol^{-1} cm^6 for each donor–acceptor pair and the average value of R_0 (eq 7) was 19.0 ± 0.5 Å.

Theory

Energy Transfer from Multiple Donors to One Acceptor. The rate of energy transfer between a donor–acceptor pair of fixed relative distance and transition dipole orientation is given by

$$k_{da} = 8.71 \times 10^{23} n^{-4} Q_d k_D J_{da} (\kappa^2 / R_{da}^6) \quad (4)$$

where Q_d is the quantum yield of the donor, k_D is the excited state decay constant in the absence of any acceptor, n is the refractive index of the intervening medium, κ^2 is the dipole–dipole orientation factor, and R_{da} is the donor–acceptor distance. By following Sklar et al. (1977), the transfer efficiency from N donors to a single acceptor is given by

$$[(1/T) - 1]^{-1} = J_{da}[8.71 \times 10^{23} n^{-4} Q_d \sum_{i=1}^{N} (\kappa^2 / R_{da}^6)] \quad (5)$$

the assumption being that all donors are spectrally identical. The apparent transfer distance ($R_{da,app}$) for a given geometric distribution of N donors to one acceptor is

$$T = 1/[1 + (R_{da,app}/R_{da}^\circ)^6] \quad (6)$$

R_{da}° being the distance between chromophores at which 50% of the energy is transferred from donor to acceptor.

$$R_{da}^\circ = 9.79 \times 10^3 (J_{da} Q_d n^{-4} \kappa^2)^{1/6} \quad (7)$$

Energy Transfer from One Donor to Multiple Acceptors. The transfer efficiency from one donor to N acceptors is given by the sum of the pairwise rates (Gennis & Cantor, 1972)

$$T/(1 - T) = \sum_{i=1}^{N} (R_{da}^\circ / R_{da})^6 \quad (8)$$

The apparent transfer distance for this system can be calculated as before, by using eq 6.

Equations 4–8 apply to donors and acceptors whose relative positions are fixed within the decay time of the donor. If the donor and acceptor are on different components in a lipid bilayer, it is possible that translational diffusion might bring them to within the transfer distance during the donor lifetime. The effect of such diffusion on energy transfer has been examined by Steinberg & Katchalski (1968), and recently Fung & Stryer (1978) and Thomas et al. (1978) have shown that extremely long donor lifetimes (ms) would be required to make this effect significant in the lipid bilayer. In the present study, the effect is likely to be insignificant due to the short lifetime of tryptophan (1–2 ns).

Distribution of Donor and Acceptors in the Lipid Bilayer. We now examine the dependence of the apparent transfer distance on the surface density and transverse position of donor and acceptor groups in the lipid bilayer. Consider molecules of a spectroscopic membrane probe distributed in a square array on a membrane surface at a surface density of N molecules/1000 Å^2. Each $1000/N$ Å^2 unit contains one molecule of probe and the average minimum distance between them is $(1000/N)^{1/2}$ Å, by assuming that the probe molecule occupies little area itself. As the surface density of probe molecules increases, their intermolecular distance decreases.

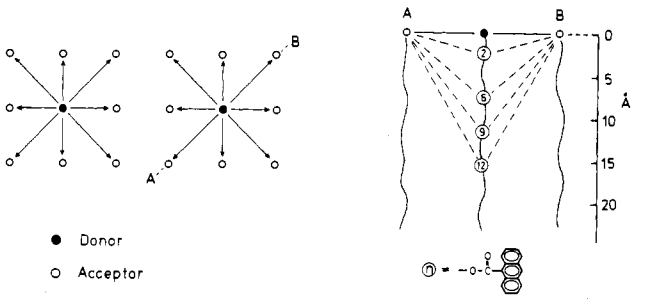


FIGURE 1: A diagram of the lateral (left) and transverse (right) distribution of donor and acceptor chromophores in a lipid bilayer. The donor and acceptor molecules are arranged in a square array at a surface density which precludes any one acceptor from receiving energy from more than one donor. The dotted lines on the right indicate the transfer distances which arise from the placement of the acceptor chromophore at different depths in the lipid bilayer. In the experimental system described in the text, the donor is *N*-stearoyltryptophan and the acceptors are the *n*-(9-anthroyloxy) fatty acids.

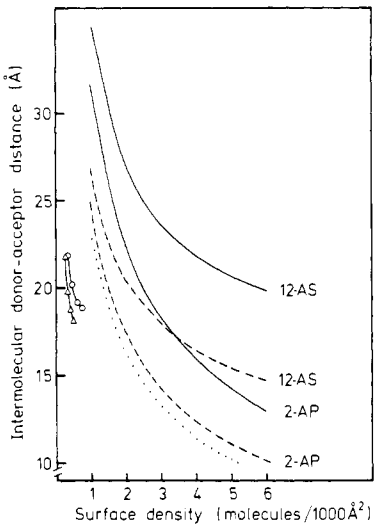


FIGURE 2: Variation of the minimum intermolecular distance between donor and acceptor with surface density of a square array of molecules in a lipid bilayer. The solid lines are the true intermolecular distances for 2-AP and 12-AS. The broken lines are the corresponding apparent transfer distances ($R_{da,app}$) calculated by using eq 6 and 8. The distances for 6-AS and 9-AS fall intermediate between the extreme members of the series. The dotted line is the apparent transfer distances for 2-AP calculated for a hexagonal rather than a square array of probe molecules in the bilayer. Calculations take account of transfer from one donor to the first and second nearest-neighbor shells. The experimental points are those for 12-AS (○) and 2-AP (△); the concentration of donor (*N*-stearoyltryptophan) was 5 μM and the acceptor concentrations were within the range 3–15 μM. The concentration of dimyristoylphosphatidylcholine was 500 μM.

If a proportion of the molecules are donors and the remainder acceptors, the distance between donor and acceptor fluorophores increases as the acceptor chromophore is moved deeper into the bilayer. These relations are depicted in Figures 1 and 2. The dominant feature of Figure 2 (solid lines) is that the differences in donor–acceptor distances originating from the varying transverse positions of the acceptor chromophore are greatest at high surface density of probe. These calculations assume that the distribution of donor and acceptor molecules is mutually independent. The separation calculated is the minimum distance and is therefore independent of the relative proportion of donor and acceptor present. Thus, the abscissa of Figure 2 refers to the total probe concentration (i.e., donor plus acceptor).

Consider now a surface density of one donor or acceptor molecule per 1000 Å^2. If the molar ratio of donor to acceptor

Table I: Apparent Intermolecular Distances (d) between Donor and Acceptor Calculated for a Square Array of Donor and Acceptor Molecules

probe	actual d^a (Å)	excess acceptor: theor transfer efficiency^b (%)	excess acceptor: app donor–acceptor d^c (Å)	excess donor: theor transfer efficiency^d (%)	excess donor: app donor–acceptor d^c (Å)
2-AP	31.6, 44.7	22.4	24.6	65.2	18.0
6-AS	32.5, 45.3	19.8	25.2	61.7	18.5
9-AS	33.5, 46.1	17.1	26.0	57.3	19.1
12-AS	35.0, 47.2	14.0	27.1	51.4	19.8

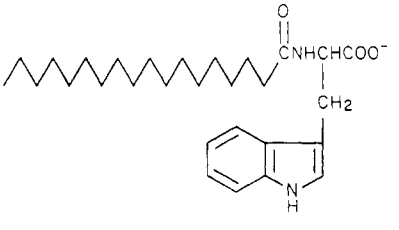
^{a} The two distances (d) result from the square array at a probe (donor + acceptor) density of 1 molecule/1000 Å^2. ^{b} Calculated according to eq 8. ^{c} Calculated according to eq 6. ^{d} Calculated according to eq 5.

is large, transfer will only occur between nearest-neighbor donor–acceptor pairs, all other pairs being well outside the energy transfer distance ($>2R_0$). Such a situation is depicted in Figure 1. One donor transfers energy to several surrounding acceptors but any one acceptor receives energy from only one donor. If the members of each donor and acceptor population are spectrally similar and have similar dipole orientations, eq 8 may be used to calculate the theoretical transfer efficiency and eq 6 used to calculate the apparent intermolecular distance. These values are listed in Table I together with the actual donor–acceptor distances calculated for the model depicted in Figure 1. Clearly, transfer from one donor to several acceptors has the effect of increasing the transfer efficiency and therefore of reducing the apparent intermolecular distance. The difference in $R_{da,app}$ values between 2-AP and 12-AS is about 2.5 Å but may increase to 4.7 Å at high surface densities (6 molecules/1000 Å^2) as depicted by the broken lines in Figure 2. A hexagonal rather than a square array gives slightly closer packing geometry and the values of $R_{da,app}$ are correspondingly lower as shown by the dotted line in Figure 2 which has been calculated for 2-AP. In these calculations, we have assumed that the transition dipole directions of donor and acceptor can take up all orientations within the donor lifetime and the appropriate value of the orientation factor for this dynamic limit is therefore 2/3 (Dale & Eisinger, 1976). A value of 1.5 was taken for the refractive index of the lipid phase (Chong & Colbow, 1976). The model does not take into account the possibility of transfer between donor and acceptor molecules in different leaflets of the bilayer.

Similar calculations were made by using eq 5 and 6 for the case of multiple donors transferring to a single acceptor. The conclusions are similar to those reached for the case of excess acceptor discussed above except that values of $R_{da,app}$ are lower and there is less difference between the 2- and the 12-positioned fluorophores (Table I).

Results and Discussion

Quenching. Preliminary experiments indicated that the indole ring of tryptophan was capable of quenching the fluorescence of molecules containing the anthracene fluorophore. In order to position the indole ring at a fixed location in the lipid bilayer, tryptophan was conjugated to stearic acid to form *N*-stearoyltryptophan (I). We would expect the long



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axis of this molecule to align itself parallel to the phospholipid acyl chains with the carboxyl group directed toward the bilayer surface.

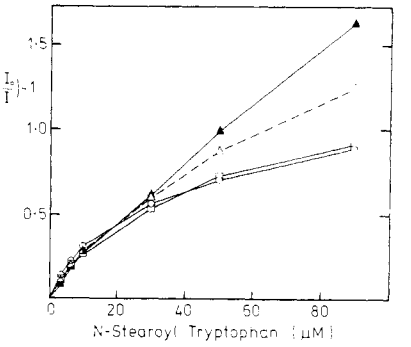

FIGURE 3: Stern–Volmer plot of the quenching of fluorescent fatty acids by *N*-stearoyltryptophan in vortexed dispersions of dimyristoylphosphatidylcholine at 20 °C. Intensities were measured 1 h after the addition of each aliquot of quencher to allow for uptake into the lipid phase. The concentrations of phospholipid and of the fluorescent fatty acids were 500 μM and 3 μM, respectively. (▲) 2-AP; (△) 6-AS; (□) 9-AS; (○) 12-AS. Excitation and emission wavelengths were 366 and 440 nm (band passes 6 nm each).

Certainly, this molecule was readily incorporated into liposomes since measurements of fluorescence polarization (excitation 290 nm; emission 340 nm) were capable of detecting the correct phase transition temperature of dimyristoylphosphatidylcholine dispersions (23 °C), the polarization decreasing from the gel to the fluid side of the transition. Moreover, the position of its emission maximum in these lipsomes (340 nm) indicated that the tryptophan was situated in an environment less polar than water but more polar than *n*-hexane (Cowgill, 1967).

The quenching data obtained with dimyristoylphosphatidylcholine liposomes at 20 °C are presented in Figure 3 in the form of Stern–Volmer plots and indicate that the quenching efficiency is in the order 2-AP > 6-AS > 9-AS ~ 12-AS. In several experiments some deviation from this order was observed at low quencher concentrations (<15 μM) and may indicate that more than one type of quenching process operates or that the quencher can occupy more than one position in the bilayer. The curvature observed in Figure 3 is of the type expected for a quencher which binds to preexisting sites in the lipid bilayer rather than partitions into the lipid phase (Thulborn & Sawyer, 1978; Haigh et al., 1978). The order of quenching efficiency is consistent with the tryptophan chromophore of structure I being located at the surface of the lipid bilayer. A number of other indole derivatives failed to quench probe fluorescence in dipalmitoylphosphatidylcholine bilayers at 25 °C, suggesting that these molecules could not be taken up into the lipid phase (e.g., tryptophan, *N*-acetyltryptophanamide, *tert*-butyloxycarbonyltryptophan).

The quenching of the fatty acid probes by indole in bilayers of dimyristoylphosphatidylcholine (20 °C) is shown in Figure

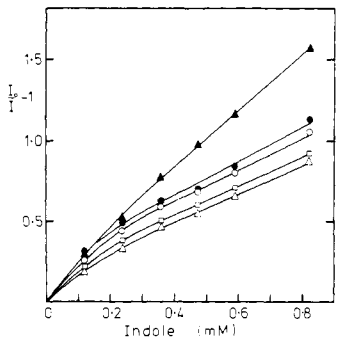

FIGURE 4: Stern–Volmer plot of the quenching of fluorescent fatty acids by indole in vortexed dispersions of dimyristoylphosphatidylcholine at 20 °C. Intensities were measured 1 h after the addition of each aliquot of quencher to allow for uptake into the lipid phase. The concentrations of phospholipid and of the fluorescent fatty acids were 500 μM and 3–4 μM, respectively. (▲) 2-AP; (△) 6-AS; (□) 9-AS; (○) 12-AS; (●) 16-AP. Excitation and emission wavelengths were 366 and 440 nm (band passes 6 and 10 nm, respectively).

4. The order of quenching efficiency is 16-AP > 12-AS > 9-AS > 6-AS. 2-AP appears to be out of sequence having a quenching efficiency greater than that of 16-AP (see below). We have not attempted to determine the mechanism of quenching by indole. Regardless of the mechanism (static or dynamic), the differences in efficiency may result from a high local concentration of indole at the center of the bilayer. However, if the quenching is dynamic, then the bimolecular rate constant k_q is determined by the Einstein diffusion relationship ($k_q = 8RT/3000\eta$ where η is the viscosity and R and T have their usual meanings), and the relevant form of the Stern–Volmer equation is $(I_0/I) - 1 = k_q\tau[Q]$ where [Q] is the concentration of quencher and I and I_0 are the intensities in the presence and absence of quencher, respectively. These relationships apply to collisional quenching in free solution and their application to quenching within a highly anisotropic structure must be viewed with caution. For example, if the quencher seeks a restricted level within the bilayer structure, then quenching takes place in two rather than three dimensions and k_q is determined by diffusion of components within the lateral plane. It also remains possible that indole distributes evenly across the transverse plane of the bilayer and that the relative quenching efficiencies result from the influence of the "microviscosity" gradient which exists from the surface to the center of the lipid bilayer.

The anomalous behavior of 2-AP has been observed previously with respect to quenching by *N*,*N*'-dimethylaniline and may result from the presence of quencher in the aqueous phase or at the bilayer surface (Thulborn & Sawyer, 1978). Use of the quenching efficiency of the fatty acid probes to indicate the relative position of the quencher in the bilayer presumes that all probes have the same *intrinsic* quenching efficiency. To examine this question, the quenching of the probes by indole was studied in free solution in methanol. The results in Figure 5 show that, whereas the quenching efficiencies of 6-, 9-, and 12-AS are the same to within experimental error, those for 16-AP and 2-AP are higher by factors 1.2 and 2.1, respectively. Such behavior may be due to the proximity of the carboxyl group to the anthracene ring in the case of 2-AP and to the greater accessibility of the anthracene ring when it is in the terminal position as in 16-AP. The anomalous behavior of 2-AP discussed above may be explained at least in part by this phenomenon. We have not attempted to correct the quenching data in Figure 4 according to the intrinsic factors discussed above since there is no certainty that these factors determined in methanol would apply within the lipid bilayer. We also note

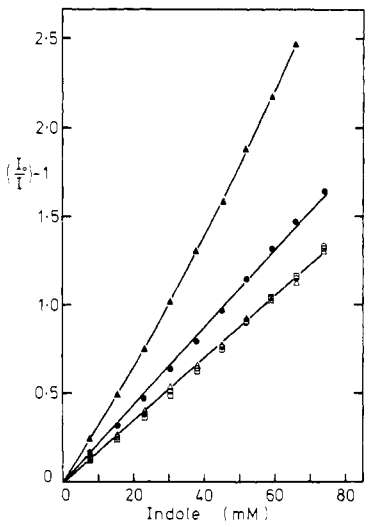

FIGURE 5: Stern–Volmer plot of the quenching of the fluorescent fatty acids (4–10 μM) by indole in methanol at 20 °C. Symbols are as for Figure 4. Excitation and emission wavelengths were 366 and 460 nm (band passes 8 and 10 nm, respectively).

that the concentration range over which significant quenching occurs is approximately ten times greater in free solution than in liposomes, an effect which reflects the partitioning and/or binding of indole to the bilayer. When *N*-steroyltryptophan was used as the quencher (Figure 3), the anomalous behavior of 2-AP was not evident, its effects being to enhance the apparent quenching efficiency without changing the relative order of quenching with respect to the fatty acid probes.

To summarize thus far, the position of a quenching molecule in the transverse plane of the lipid bilayer may be determined by noting the relative efficiencies with which it quenches the fluorescence of the set of *n*-(9-anthroyloxy) fatty acids.

Energy Transfer. We now examine the possibility that *N*-stearoyltryptophan might act as a suitable donor for the resonance transfer of energy to the fluorescent fatty acids. Although the indole ring quenches probe fluorescence, the quenching of the donor may still be used to determine transfer efficiencies. Indeed, in the systems to be described only weak sensitized fluorescence of the acceptor was observed. The availability of the set of *n*-(9-anthroyloxy) fatty acid probes provides us with a test system to examine the application of energy transfer to the determination of intermolecular distances in lipid bilayers as depicted in Figure 1.

The case of one donor–multiple acceptors is open to experimental test. Aliquots of acceptor may be titrated into a bilayer suspension containing a small amount of donor. However, the amount of acceptor added and, therefore, the surface density of acceptor is limited by two factors. The uptake of acceptor into the bilayer occurs via a binding mechanism such that the amount of free acceptor in the aqueous phase increases as acceptor is added and the amount bound to the bilayer approaches a saturating level (Haigh et al., 1978). Secondly a high surface density of acceptor perturbs the lipid environment and may eventually lead to disruption of the bilayer structure (Cadenhead et al., 1977). We have therefore restricted the amount of acceptor added to a maximum of 15 μM which together with a donor concentration of 5 μM and a phospholipid concentration of 500 μM corresponds to a molar ratio (probe:phospholipid) of 1:25. Assuming a surface area per phospholipid molecule of 60 Å^2, this gives a surface density of one molecule per 1500 Å^2.

Such an experiment was carried out in dimyristoylphosphatidylcholine liposomes at 20 °C, and the results are

included in Figure 2 for comparison with theory. It will be noted that the experimentally accessible region is at low surface densities where differences in the transfer for various donor–acceptor pairs are minimal. Indeed the differences observed experimentally between the 2-, 6-, 9-, and 12-positioned acceptors were within experimental error, although 2-AP gave consistently lower transfer distances as expected. We also note that the experimentally determined transfer distances fall much lower than those predicted on the basis of theory (broken lines, Figure 2). Several factors contribute to this effect. Firstly, the theory assumes a donor:acceptor ratio of not greater than 1:8, whereas in the experiment the ratio was as high as 1:3. Thus, some acceptors will be receiving energy from more than one donor. Secondly, it is possible that, within the time scale of the experiment (see Materials and Methods), the acceptors do not distribute evenly throughout the liposomes but are confined to the outer bilayers of the multilamellar structure. The possibility of transfer between donors and acceptors in opposite bilayer leaflets will depend on the "flip-flop" rate across the bilayer structure and this is an additional factor which must be considered. Thus, although the energy transfer method gives some indication of the separation of donor and acceptor in the lateral plane of the membrane, the differences arising from the location of acceptor chromophores in the transverse plane are too small to be detected at low surface densities of probe. It may prove possible to improve the resolution in the transverse plane by using fluorescent lecithins rather than fluorescent fatty acids, thus avoiding the detergent action of the fatty acids and permitting higher surface densities to be achieved. However, it is likely that the perturbing effect of the bulky anthroyloxy group would remain a problem.

Comparisons in the transverse plane are also made difficult by the bulk of the fluorophore and by the uncertainty of the orientation factor. The dynamic limit of this factor ($\kappa^2 = 2/3$) has been assumed in our calculations. However, it is possible that the magnitude of the orientation factor is different for each donor–acceptor pair since the "microviscosity" of the environment surrounding the acceptor chromophore decreases as the chormophore is moved deeper into the center of the bilayer.

At this point, it is appropriate to emphasize certain precautions which must be taken when using the anthroyloxy fatty acids in energy transfer experiments. If the probes are to be used as acceptors of tryptophan fluorescence (Lenard et al., 1974), only weak sensitized fluorescence may be observed, although the donor quenching may still be used to measure transfer efficiency. Indeed, we have found that, when the donor and acceptor are on the same molecule as in 12-(9-anthroyloxy)-*N*-stearoyltryptophan, only weak donor fluorescence is observed due to efficient resonance energy transfer, and very weak acceptor fluorescence is observed due to donor quenching of the acceptor fluorescence. If the probes are to be used as donors, the acceptor molecule must not contain indole or tryptophan structures as well as the acceptor chromophore. For example, Shaklai et al. (1977) have used energy transfer between 12-AS and heme to measure the binding of hemoglobin to erythrocyte membranes. However, the donor may be quenched not only by energy transfer to the heme but also by the proximity of tryptophan residues in the globin.

Gramicidin–Bilayer Interaction. Energy transfer distances were determined between the tryptophan residues of gramicidin and the *n*-(9-anthroyloxy) fatty acid probes in bilayers of egg phosphatidylcholine and bovine phosphatidylserine (mole ratio 1:1). Although the transfer distances decreased

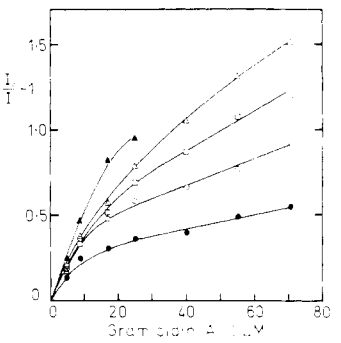

FIGURE 6: Stern–Volmer plot of the quenching of the fluorescent fatty acids (3 μM) by gramicidin in vortexed dispersions of dimyristoylphosphatidylcholine (500 μM) at 20 °C. The excitation and emission wavelengths were 366 and 440 nm (band passes 6 and 10 nm, respectively). Symbols are as for Figure 4.

with increasing surface density of probes, no significant differences were found between the 2-, 6-, and 12-positioned fluorophores. Indeed, based on the results for the model system discussed above, we would not expect the energy transfer method to resolve the small differences in transfer distances involved, particularly when the transfer may take place from several spatially separate donors.

The quenching of the fatty acid probes by gramicidin in dimyristoylphosphatidylcholine liposomes at 20 °C is shown in Figure 6. The order of quenching efficiency is 2-AP > 6-AS > 9-AS > 12-AS > 16-AP. The intrinsic differences in the quenching efficiency of the probes by indole as demonstrated in Figure 5 must be kept in mind when interpreting these results. The differences between 6-, 9-, and 12-AS (Figure 6) whose efficiencies of quenching by indole are the same in methanol solution suggest that the tryptophan residues of gramicidin are located near the membrane surface.

Similar quenching experiments have been carried out on both vortexed and sonicated dispersions of dipalmitoylphosphatidylcholine above and below the transition temperature of 41 °C. In these experiments, the effect of temperature on the conformation of gramicidin in the bilayer may be important. However, in all cases, 2-AP was quenched more efficiently than the other members of the fluorescent fatty acid series, behavior which probably reflects the greater intrinsic quenching efficiency of the probe. However, in most cases there was little difference in the quenching efficiencies of 6-, 9-, and 12-AS. It therefore remains possible that the type of dimer formed is dependent on the physical state of the membrane lipid.

The problem of locating the tryptophan residues of gramicidin in the transverse plane of the membrane can also be studied by using spin-labeled fatty acids as paramagnetic quenchers of the tryptophan fluorescence (Bieri & Wallach, 1975). As with the anthroyloxy fatty acids, a family of spin-labeled analogues is available in which the nitroxide radical is at different positions along the acyl chain. Thus, when incorporated into lipid bilayers, the nitroxide groups locate at different depths within the membrane (Schreier-Muccillo et al., 1976). The Stern–Volmer plots for such an experiment for vortexed dispersions of dimyristoylphosphatidylcholine at 20 °C are shown in Figure 7. The quenching efficiency is in the order 5-nitroxide stearate > 12-nitroxide stearate > 16-nitroxide stearate.

These data support the view that the tryptophan residues of gramicidin are located near the membrane surface and that the dominant structure is the "head-to-head" dimer. Veatch et al. (1974) concluded that three out of four of the conformationally distinct forms of gramicidin are capable of

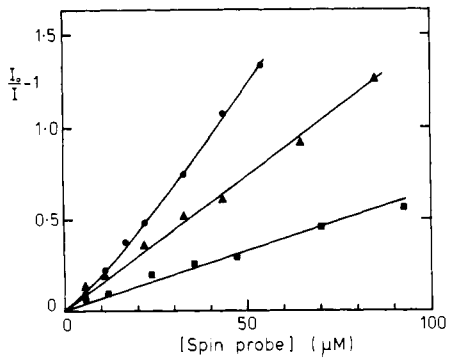

FIGURE 7: The quenching of the tryptophan fluorescence of gramicidin (3.3 μM) by spin-labeled fatty acids in dimyristoylphosphatidylcholine multilayers (500 μM) at 20 °C. (●) 5-Nitroxide stearate; (▲) 12-nitroxide stearate; (■) 16-nitroxide stearate. Excitation and emission wavelengths were 290 and 334 nm, respectively (band passes 6 and 10 nm, respectively).

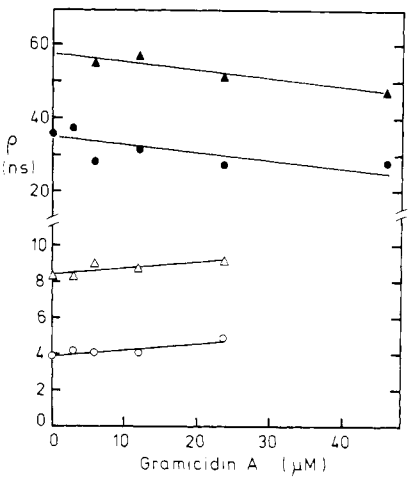

FIGURE 8: The effect of gramicidin on the rotational relaxation time (ρ) of 2-AP (▲, △) and 12-AS (●, ○) in vortexed dispersions of dipalmitoylphosphatidylcholine (500 μM). The filled symbols refer to measurements at 25 °C and the hollow symbols to measurements at 50 °C. The concentration of fluorescent fatty acid was 3 μM. Excitation wavelength was 366 nm. Emission wavelengths were 440 and 448 nm at 25 °C and 50 °C, respectively (band passes were 6 and 12 nm).

forming "head-to-head" dimeric species. Both single channel conductance measurements and fluorescence energy transfer studies have shown that two molecules of gramicidin are required to form a single ion conducting channel (Bamberg & Läuger, 1973; Veatch & Stryer, 1977). Recently, Bamberg et al. (1977) reported electrical experiments which showed that ion channels failed to form when gramicidin was blocked at its formyl end as in the negatively charged analogue, *N*-pyromellityldeformylgramicidin. However, when the modification was at the ethanolamide end of the molecule as in *O*-pyromellitylgramicidin, ion channels still formed. These results are in agreement with our experiments which have been carried out with unmodified gramicidin and support the view that the conducting channel is made up of two $\pi_{(L,D)}$ monomer units rather than intertwined β helices. Feigenson et al. (1977) have also reported NMR experiments which show that tryptophan resonances in the gramicidin–phosphatidylcholine complex undergo a chemical shift in response to the binding of lanthanide ion. By assuming that the lanthanide binding site is in the interfacial region of the bilayer, their work implies that the tryptophan residues are located near the membrane surface.

Additional information about the gramicidin–bilayer interaction is available from fluorescence polarization measurements. In particular, it should be possible to determine if gramicidin restricts or improves the rotational motion of the fatty acid fluorophores. Such analysis must take into account changes in the lifetime of the fluorophores due to quenching by the gramicidin and for this reason it is more accurate to consider rotational relaxation times rather than polarization values even though the conversion from one to the other requires the use of the Perrin equation and hence the assumption of isotropic rotation. Figure 8 shows the effect of gramicidin on the rotational relaxation times of 2-AP and 12-AS in dipalmitoylphosphatidylcholine bilayers at 25 and 50 °C. Gramicidin decreases the relaxation time above the transition temperature (42 °C) but increases the relaxation time below the transition temperature. The question now arises as to what extent does the change in the motion of the fluorophore reflect the motion of the phospholipid acyl chains in the bilayer. The degree of fluorescence depolarization depends on the rotation of the fluorophore relative to the direction of its emission transition moment. 12-AS is aligned in the lipid bilayer with both the acyl chain and the long axis of the anthracene ring normal to the bilayer surface; the emission moment is 30° to the absorption moment which is parallel to the short axis of the anthracene ring (Badley et al., 1973). Thus, the depolarizing motion sensed by the *n*-(9-anthroyloxy) fatty acids is probably the swinging or reorientation motion of the acyl chain relative to the bilayer normal. This motion may occur within the lifetime of the excited state (10^{-8} s) and is much slower than the rate of trans–gauche isomerizations about C–C bonds (10^{-11} s) (Petersen & Chan, 1977). The simplest interpretation of the data in Figure 8 is that gramicidin decreases this motion in the liquid-crystalline phase but increases the motion in the crystalline phase. Chapman et al. (1977) have examined the gramicidin–lipid interaction using several physical techniques including Raman spectroscopy and calorimetry. They conclude that the action of gramicidin is similar to that of cholesterol in that there is a decrease in the number of gauche conformers above the phase transition but decreased order below the transition. Similar conclusions have been reported by Weidekamm et al. (1977). The fluorescence data discussed above suggest that a change in the rate of reorientation of the acyl chains relative to the bilayer normal is an additional consequence of the gramicidin interaction.

The values of the fluorescence lifetimes which are necessary for the calculation of rotational relaxation time also provide information about the nature of the gramicidin quenching. Below the phase transition, the lifetime was unaffected by gramicidin up to a concentration of 24 μM (gramicidin:lipid ratio, 1:2), suggesting that quenching takes place by a static mechanism involving complex formation between gramicidin and the ground state of the anthroyloxy fluorophore. Above the phase transition, a decrease in lifetime is observed. However, the ratio of the lifetime in the absence and presence of quencher (τ_0/τ) is less than the corresponding ratio of fluorescence intensities (I_0/I), indicating that quenching takes place by both static and dynamic processes. The dynamic process would involve contact between quencher and fluorophore during their laterial diffusion in the bilayer. Such lateral diffusion is markedly inhibited in the crystalline phase and may allow the static mechanism to dominate.

Concluding Remarks

Resonance energy transfer of the Förster type appears to be a useful technique for determining the proximity of donor and acceptor chromophores in the lateral plane of the lipid

bilayer providing account is taken of the possibility of multiple transfers between donors and acceptors. It is less effective in detecting the relatively small differences in donor–acceptor distances which arise due to the placement of the acceptor in the transverse plane of the bilayer. As reviewed recently by Stryer (1978), the spatial resolution of the energy transfer technique is limited by the uncertainty in the orientation factor and by the appreciable size of donor and acceptor groups. On the other hand, measurement of the relative quenching efficiencies of the fluorescent fatty acids is a sensitive method of determining the relative position of quenching molecules in the transverse plane of the bilayer. The method requires that intrinsic differences in the quenching efficiencies of the fluorescent fatty acids be determined in free solution for any given quenching molecule before the interpretation of data for complex membrane systems is attempted. A combination of both quenching and energy transfer methods should aid the study of proximity relationships in membranes, particularly in those cases where selectively labeled components can be reconstituted into the bilayer structure.

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