Dynamic Behavior of Fluorescent Probes in Lipid Bilayer Model Membranes†

R. A. Badley,‡ W. G. Martin,* and H. Schneider

ABSTRACT: A technique has been developed, based on polarized fluorescence intensity measurements, for studying the orientation and motion of fluorescent probes incorporated into lipid bilayers. The behavior of a series of seven such probes, chosen for their differences in rigidity, shape, and size, has been studied in multibilayers prepared from a selection of phospholipids. It was found that the probes were all oriented to a greater or lesser extent. All showed a distribution of orientations which in many cases was sensitive to structural changes caused by cholesterol addition, lipid type, or temperature. Most of the probes displayed a superimposed rapid, but restricted, oscillatory motion which was also sensitive to structural changes in the bilayers. However, the probe's own structure influenced its behavior in a particular lipid. The use of such molecules for studying membranes is discussed with special reference to the local perturbations thought to be caused by the probes, the application to intrinsic fluorophores and the study of single cell membranes.

Physical techniques have provided much useful information about lipids in natural and model membranes. In particular nuclear magnetic resonance (Lee *et al.*, 1972; Chan *et al.*, 1972; Oldfield and Chapman, 1971a; Darke *et al.*, 1972), electron spin resonance (Butler *et al.*, 1970; Kornberg and McConnell, 1971; Griffith *et al.*, 1971), X-ray diffraction (Luzzatti, 1968), and calorimetry (Chapman *et al.*, 1967) have enabled the motion and geometry of the component lipids to be examined as well as the effects of such biologically significant molecules as cholesterol (Butler *et al.*, 1970), anaesthetics (Patterson *et al.*, 1972) and antibiotics (Hauser *et al.*, 1970). Since it is now certain that phospholipid bilayers are an important component of biological membranes their study can contribute much to our understanding of the whole membrane.

The utility of fluorescent molecules in membrane studies is well established (Chance *et al.*, 1971). A frequent approach has utilized the dependence of fluorescence parameters of molecules such as ANS[1] or TNS on the apparent solvent polarity (Chance *et al.*, 1971) or solvent correlation time (Mazurenko and Bakhshiev, 1970; Anufrieva *et al.*, 1970) as well as their binding dependence on membrane ionic state. These studies have sought to correlate changes in fluorescence intensity with membrance associated events such as electron transport (Chance, 1970), structural transitions (Jones *et al.*, 1969), and photosynthesis (Azzi *et al.*, 1971). In a previous paper (Badley *et al.*, 1971) we presented a new method for examining suitable two-dimensional systems such as lipid multibilayers to determine the orientation and motion of fluorescent molecules included therein. The present work was undertaken to determine the relation between the behavior of fluorescent probes and lipid bilayers containing them. The orientation and motion of several probes in a variety of phospholipid multibilayers were examined. The probes were chosen in an effort to sample different regions of the bilayers and in many cases contained a lipid anchor. A subtle combination of size, rigidity, chemical, and shape properties appeared to determine their behavior, in addition to the properties of the bilayer lipids.

For several aromatic probes a considerable degree of freedom was observed which probably reflects their ability to create or utilize space within bilayers. Nevertheless the motion of most of these probes was still very severely restricted in contrast to that in hydrocarbon solution.

Materials and Methods

Egg lecithin, phosphatidylserine, and sphingomyelin were obtained from Lipid Products Ltd. (Epsom, England). Egg lecithin was also prepared from hen eggs by chromatography on alumina and silicic acid. Dipalmitoyllecithin was a product of Mann Research Laboratories, New York, N. Y. Cholesterol (Steraloids Inc., Pawling, N. Y.) was recrystallized from methanol. All lipid solutions were stored in chloroform at $-30°$ and were pure as judged by thin-layer chromatography.

The compound 2,2′-(vinylenedi-*p*-phenylene)bisbenzoxazole was generous gift from Dr. G. McGraw of Tennessee Eastman Co. *N*-Dansylphosphatidylethanolamine, 12-(9-anthroyl)stearic acid, and *N*-octadecylnaphthyl-2-amino-6-sulfonic acid were prepared according to Waggoner and Stryer (1970). *N,N*′-Di(octadecyl)oxacarbocyanine was prepared according to Czikkely *et al.* (1969). 1-Anilino-8-naphthalenesulfonate (ammonium salt) was a product of Sigma Chemical Co. (St. Louis). *d*-3-Aminodesoxyequilenin was prepared according to Bachmann and Dreiding (1950) from *d*-equilenin (Steraloids Inc.). Structural formulae of the probes are shown in Table I.

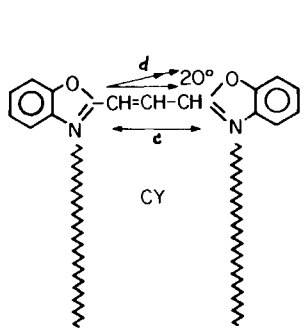
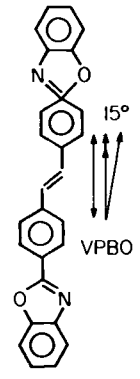
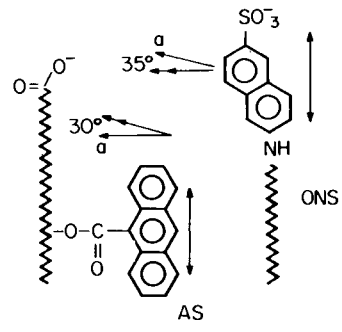
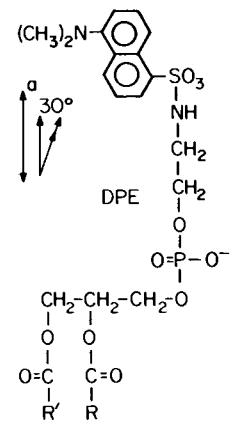
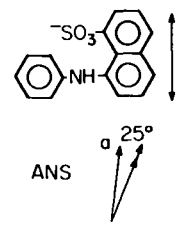
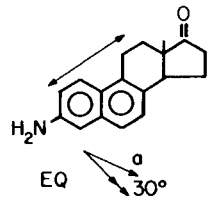
Phospholipid multibilayers were prepared by two methods. In one, a chloroform solution of lipid (1 mg in 0.1 ml) + probe (if any) was slowly dried down onto one long face of a $3 \times 3 \times 1$ cm polished quartz block using a stream of wet nitrogen. The film was then kept in a vacuum for 2 hr to remove residual chloroform. Hydration was carried out either by exposure for 2–3 hr to nitrogen saturated with water vapor

† From the Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada K1A 0R6. *Received August 24, 1972.* Issued as NRCC No. 12987.

‡ Present address: Unilever Research Laboratory, The Frythe, Welwyn, Herts., England.

[1] Abbreviations used are: ANS, 1-anilino-8-naphthalenesulfonate; TNS, 6-toluidino-2-naphthalenesulfonate; CY, N,N′-di(octadecyl)-oxacarbocyanine; VPBO, 2,2′-(vinylenedi-*p*-phenylene)bisbenzoxazole; AB, 12-(9-anthroyl)stearic acid; ONS, *N*-octadecylnaphthyl-2-amino-6-sulfonic acid; DPE, *N*-dansylphosphatidylethanolamine; EQ, *d*-3-aminodesoxyequilenin.

TABLE 1: Collected Data for the Fluorescent Probes in Egg Lecithin and Egg Lecithin + Cholesterol (1:1) Multibilayers.

Probe	CY	VPBO	AS	ONS	DPE	ANS	EQ
							
Fluorescence intensity matrix	$\begin{vmatrix} 1.00 & 0.53 & 0.34 \\ 0.53 & 1.00 & 0.34 \\ 0.34 & 0.34 & 0.26 \end{vmatrix}$	$\begin{vmatrix} 1.00 & 0.50 & 0.55 \\ 0.50 & 1.00 & 0.55 \\ 0.55 & 0.55 & 1.50 \end{vmatrix}$	$\begin{vmatrix} 1.00 & 0.84 & 0.75 \\ 0.84 & 1.00 & 0.75 \\ 0.75 & 0.75 & 0.80 \end{vmatrix}$	$\begin{vmatrix} 1.00 & 0.64 & 0.65 \\ 0.64 & 1.00 & 0.65 \\ 0.65 & 0.65 & 0.60 \end{vmatrix}$	$\begin{vmatrix} 1.00 & 0.78 & 0.80 \\ 0.78 & 1.00 & 0.80 \\ 0.85 & 0.85 & 0.80 \end{vmatrix}$	$\begin{vmatrix} 1.00 & 0.85 & 0.80 \\ 0.85 & 1.00 & 0.80 \\ 0.80 & 0.80 & 0.90 \end{vmatrix}$	$\begin{vmatrix} 1.00 & 0.80 & 0.85 \\ 0.80 & 1.00 & 0.85 \\ 0.85 & 0.85 & 1.00 \end{vmatrix}$
Model	2	1	2	2	4 (and 2)	2	2
Distribution of θ	80-90°	0-80°	30-90°	30-90°	25-90°[b]	30-90°	30-90°
Distribution of long axes	0-10°	0-80°	0-60°	0-60°	25-90°	30-90°	0-60°
Range of γ	0-30°	0-30°	0-60°	0-45°	50°	0-55°	0-60°
Estimated actual motion	0-10°	0-15°	0-30°	0-20°	20°	0-30°	0-30°
Probable location	surface	interior	interior	surface	glycerol region	surface	surface
Fluorescence lifetime	$\leq$ 1 nsec	$\leq$ 1 nsec	12.6 nsec	9.0 nsec	14.0 nsec	7.8 nsec	11.0 nsec
Angle between vectors	20°	15°	30°	35°	30°	25°	30°
Effect of cholesterol (1:1)	stops rapid motion	restricts θ to 0-67° γ unchanged	θ = 33-90° γ = 0-55° τ = 13.0 nsec	θ = 25-90° γ unchanged τ = 9.5 nsec	θ = 15-90° γ unchanged	θ unchanged γ = 0-45° τ = 7.0 nsec	unchanged
Excitation wavelength (nm)	470	380	380	380	380	380	370
Emission filter (F_2)	Kodak 16	Kodak 2C+2E	Kodak 2C+2E	Kodak 2C	Kodak 2C+2E	Kodak 2C+2E	Kodak 2C
Type of film support	slide	both	both	slide	slide	both	both
[Lipid]/[Probe]	5000	5000	1000	300	300	300	1000

[a] Absorption vector direction estimated by comparison to related compounds. [b] Calculated from model 2 to give an estimate of the dstribution of θ. [c] Assigned long axis. [d] Absorption and emission vectors are represented by single and double headed arrows.

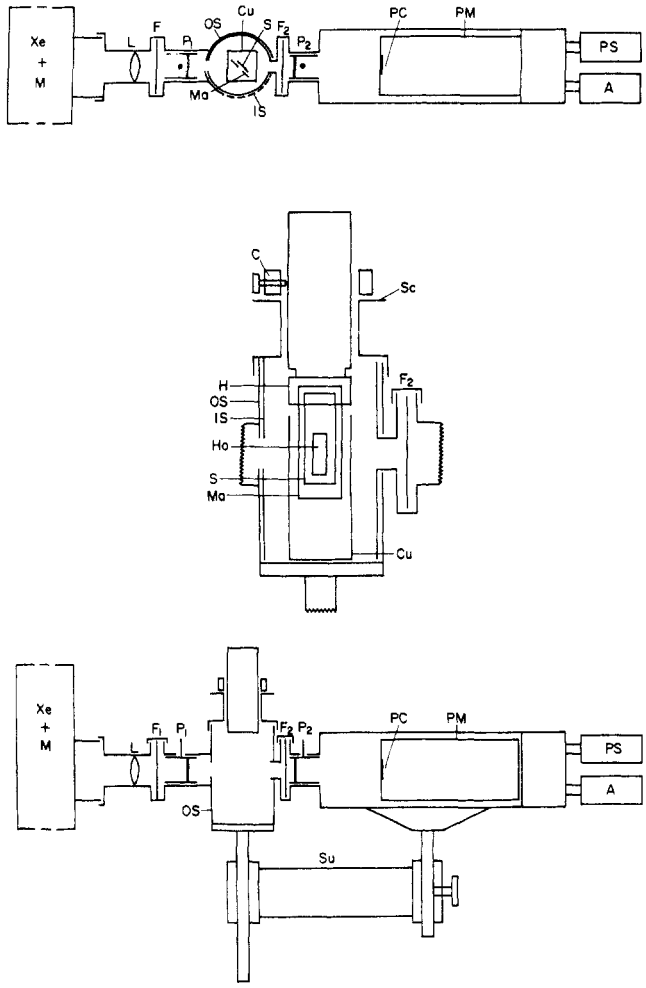


FIGURE 1: Diagram of the apparatus used to make the polarized fluorescence intensity measurements; from the top (a), the cell compartment (b), and from the side (c). Xenon arc (Xe), monochromator (M), lens (L), filter (F_1), polarizer (P_1), outside shell (OS), inside shell (IS), cuvet (Cu), slide (S), mask (M), filter (F_2), polarizer (P_2), photocathode (PC), photomultiplier (PM), power supply (PS), microammeter (A), locking collar (C), scale (Sc), slide holder (H), hole in mask (Ho), movable support (Su).

or by dipping in buffer solutions. The film was then compressed between two quartz blocks, care being taken to ensure optical contact between lipid and quartz and also that no excess water was present. In the other method the lipid solution was similarly dried down, but onto a glass cover slip (40 × 22 × 0.15 mm), dried under vacuum and hydrated in buffer solution. All the probes were present in the bilayers at sufficiently low concentrations that energy transfer did not occur (probe diluted until I_{21}/I_{22} yielded a constant low value; see below).

Several films containing the fluorescent probes were examined on the cover slips by X-ray diffraction by Dr. A. W. Hanson, whom we thank. They were maintained at high relative humidity in an air-tight cell with X-ray transparent windows. A narrow beam of Cu Kα radiation was allowed to impinge on the specimen at a variable, small angle to the surface and the detector (a scintillation counter protected by a 0.8-mm slit) was placed to measure the Bragg reflections from planes parallel to the surface.

Uncorrected excitation and fluorescence spectra were obtained with a double monochromator instrument of conventional design using a stabilized dc xenon arc (150-W Bausch and Lomb), EMI 6256A photomultiplier, and Keithly 610A electrometer. Spectra were corrected according to Weber and Teale (1957). Fluorescence lifetimes were measured either with a TRW decay time fluorometer (El Segundo, Calif.) or with a single photon counting instrument, for which thanks are due to Dr. F. R. Lipsett. Fluorescence polarizations were measured in the variable geometry instrument (see below) using a conventional right-angle arrangement together with a 4.5 × 1 × 1 cm cuvet.

Polarized fluorescence intensities were measured in an apparatus in which the configuration of detector, light source, and sample are easily varied. A diagram of the apparatus is shown in Figure 1. The lamp (Xe), monochromator (M), filter (F_1), lens (L), and polarizer (P_1) are used to produce a clean high-intensity, narrow, parallel beam of polarized light. P_1 and P_2 can be set in both horizontal and vertical positions. The sample compartment was machined from brass and allows both the quartz sample blocks (*via* a magnetic holder) and cover slips (inserted into a spring clip and incorporating a partial mask to cut out scattered light from the edges of glass and film) to be mounted, vertically, from above. Their direction can be set from outside the compartment to any desired angle (±2°). Normal 4.5 × 1 × 1 cm quartz cuvets can be used in a small adapter when necessary. The cover slips were immersed in a buffer solution contained in a 3 × 3 × 6 cm glass cuvet. This reduces the refractive index difference between medium and lipid (or glass) and hence the angle for grazing incidence of the light beams. The photomultiplier was an EMI 6256A which has a high sensitivity and very low dark current, as well as a small (1-cm) photocathode to reduce the cone of light collection. Provision was made in its housing for a filter (F_2) and polarizer (P_2). The whole unit was mounted on a swinging arm that could be turned through 90°.

Corrections were applied to the measured intensities to allow for the variation in monochromator transmission of horizontally and vertically polarized light. The transmission of polarized light beams through plane surfaces not at a right angle to the light beam depends on the direction of polarization, the angle, and the refractive index difference between the two sides of the surface. The necessary corrections (less than 20%) were calculated from the equations found in Jenkins and White (1957) and tested by using a random rigid array of probe in a solid, amorphous film (a viscous, cold (0°) solution of VPBO in glycerol). Measurements were always taken by comparing the intensity of interest to that with both polarizers vertical (I_{vv}). Since I_{vv} can be measured for any combination of geometries, the changes due to sampling angle, reflections, and trivial absorption of exciting and emitted light are cancelled out.

Polarized Fluorescence Intensity Experiment

The fourth moments of the orientation distribution function of a fluorescent molecule incorporated into an ordered structure can be determined from the polarized fluorescence intensities. As shown by Desper and Kimura (1967) all the intensity information is contained in a matrix or grid of nine intensity values, I_{ij} (i, j = 1–3), obtained by taking the different combinations of excitation (i) and emission (j) polarization directions parallel to the i and j axes of a Cartesian coordinate system, $X_1X_2X_3$ (Figure 2), set in the sample.

$$I = \begin{bmatrix} I_{11} & I_{12} & I_{13} \\ I_{21} & I_{22} & I_{23} \\ I_{31} & I_{32} & I_{33} \end{bmatrix}$$

TABLE II: Definition of the Calculated Models.

Model	δ^a	ϕ^a	γ^a	θ^a	Deviation Type[b]
1	0–2 π	0–2 π	0–γ_{max}	0–θ_{max}	iv
2	0–2 π	0–2 π	0–γ_{max}	θ_{min}–90°	iv
3	0–2 π	0–2 π	0–γ_{max}	Fixed θ	iv
4	0–2 π	0–2 π	Fixed γ	0–θ_{max}	i–iii
5	0–2 π	0–2 π	Fixed γ	Fixed θ	i–iii

[a] Angles defined in the text. [b] See text.

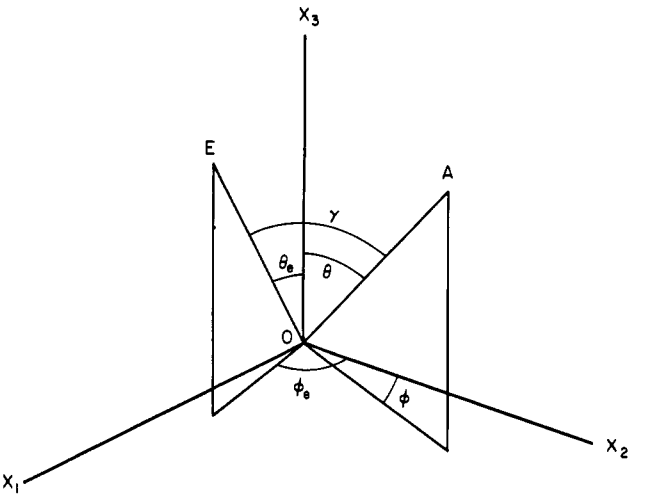


FIGURE 2: The Cartesian coordinate system ($OX_1X_2X_3$) used to define the angles θ, θ_e, γ, ϕ, and α_e. OA and OE represent the absorption and emission vectors.

Analysis of the case where a nonmobile fluorescent probe has a single, fixed linear oscillator for both absorption and emission has been carried out (Desper and Kimura, 1967). Biaxial (the X_1X_2, X_1X_3, and X_2X_3 planes are planes of symmetry for the distribution function) or uniaxial symmetry (cylindrical symmetry around a preferred axis, normally X_3) was assumed.

The present study extends the treatment for uniaxial models to nonideal situations and more complex distributions. Four sources of such behavior have been considered: (i) nonparallel absorption and emission dipoles, (ii) resonance energy transfer between nonparallel probes, (iii) slow motion of the probe during a probe's fluorescence lifetime, and (iv) fast but restricted motion on the same time scale.

To consider these cases two further angles are required in addition to the two (θ and ϕ) already used (Desper and Kimura, 1967). The angle between the absorption and emission vectors is γ, and δ the aximuthal angle of the emission vector around the absorption vector. This azimuthal angle is considered to be randomized in all the cases mentioned and will not be discussed further. For the rapidly moving cases, (iv), γ is considered to lie, with equal probability, at any position between 0° and a chosen maximum, γ_{max}.

The nine relative fluorescence intensities are easily calculated from trigonometrical relations, in terms of θ, ϕ, θ_e, and ϕ_e (Figure 2), remembering that the intensities are proportional to the squares of the appropriate vectors. In general the intensities are as shown where fences denote averaging over a chosen angular distribution.

$$I = \begin{vmatrix} \langle \sin^2\theta \sin^2\phi \sin^2\theta_e \sin^2\phi_e \rangle & \langle \sin^2\theta \sin^2\phi \sin^2\theta_e \cos^2\phi_e \rangle & \langle \sin^2\theta \sin^2\phi \cos^2\theta_e \rangle \\ \langle \sin^2\theta \cos^2\phi \sin^2\theta_e \sin^2\phi_e \rangle & \langle \sin^2\theta \cos^2\phi \sin^2\theta_e \cos^2\phi_e \rangle & \langle \sin^2\theta \cos^2\phi \cos^2\theta_e \rangle \\ \langle \cos^2\theta \sin^2\theta_e \sin^2\phi_e \rangle & \langle \cos^2\theta \sin^2\theta_e \cos^2\phi_e \rangle & \langle \cos^2\theta \cos^2\theta_e \rangle \end{vmatrix}$$

In order to put these expressions into a form suitable for averaging by integration, the following two relationships were used: (1) $\cos\theta_e = \cos\theta\cos\gamma + \sin\theta\sin\gamma\cos\delta$ and (2) $\sin\theta_e\cos\phi_e = \cos\gamma\sin\theta\cos\phi - \sin\gamma\cos\delta\cos\theta\cos\phi - \sin\gamma\sin\delta\sin\phi$. Using these expressions I_{33} becomes, for example, $\cos^4\theta\cos^2\gamma + \cos^2\theta\sin^2\theta\sin^2\gamma\cos^2\gamma + 2\cos\gamma\cos^3\theta\sin\gamma\cos\delta\sin\theta$. This expression can then be integrated over the chosen distribution. Symmetry conditions show that the hemispherical calculation is applicable to the total array. The models which have been calculated are listed in Table II, and the results are shown in Figures 3 and 4.

Examination of the calculations shows some useful general results. (1) Ideal behavior is characterized by two conditions: $I_{21}/I_{22} = 1/3$ (equivalent to $p = 0.5$ in normal fluorescence polarization); $I_{ij} = I_{ji}$ ($i \neq j$). (2) For nonideal behavior of types i–iii both conditions fail. Here the oscillators are considered to have moved through a fixed angle, γ, during the fluorescence lifetime (models 4 and 5). (3) For nonideal behavior of type iv, only the first condition fails. $I_{21}/I_{22} > 1/3$. Here the rotational diffusion of the oscillators is considered to be rapid compared to the lifetime, but restricted in angular range; the absorption and emission oscillators are randomized within the defined solid angle. γ takes any value between 0° and γ_{max} (models 1–3).

That nonparallelism of absorption and emission vectors or energy transfer are not responsible for any observed increase in I_{21}/I_{22} from 1/3 can be tested by determining I_{21}/I_{22} in a dilute viscous solution of the probe and by diluting the dye in the lipid layers, respectively. Rapid and slow motion can then be distinguished by comparing I_{ij} with I_{ji}($i \neq j$).

Mention should be made of the experimental difficulties in making the type of measurement described above. Certain intensities required a "straight-through" geometry which is of course impossible. However, by using quartz blocks, or by matching the refractive indices of lipid and surrounding medium, the critical angle for reflection was reduced to the order of 10°. In critical cases, an empirical extrapolation back to 0° was carried out using the calibrated movable detector. Also, sufficiently thin films are needed so that very little scattering can occur. This is particularly so for films of charged lipids which swell upon hydration.

Reproducibility of measurement depended on film stability, and the particular intensity being measured and was better within than between films. I_{12}/I_{22}, I_{21}/I_{22}, and I_{11}/I_{22} were reproducible ±5% within and between films. The remaining intensities were generally reproducible within ±15%. However by repeating measurements on many films (~10) differences of 5% could be detected. In cases where I_{13}/I_{22}, I_{23}/I_{22}, I_{31}/I_{22}, and I_{32}/I_{22} appeared equal within the limits outlined above, the average of all the values is shown in Table I to illustrate the symmetries of the grid.

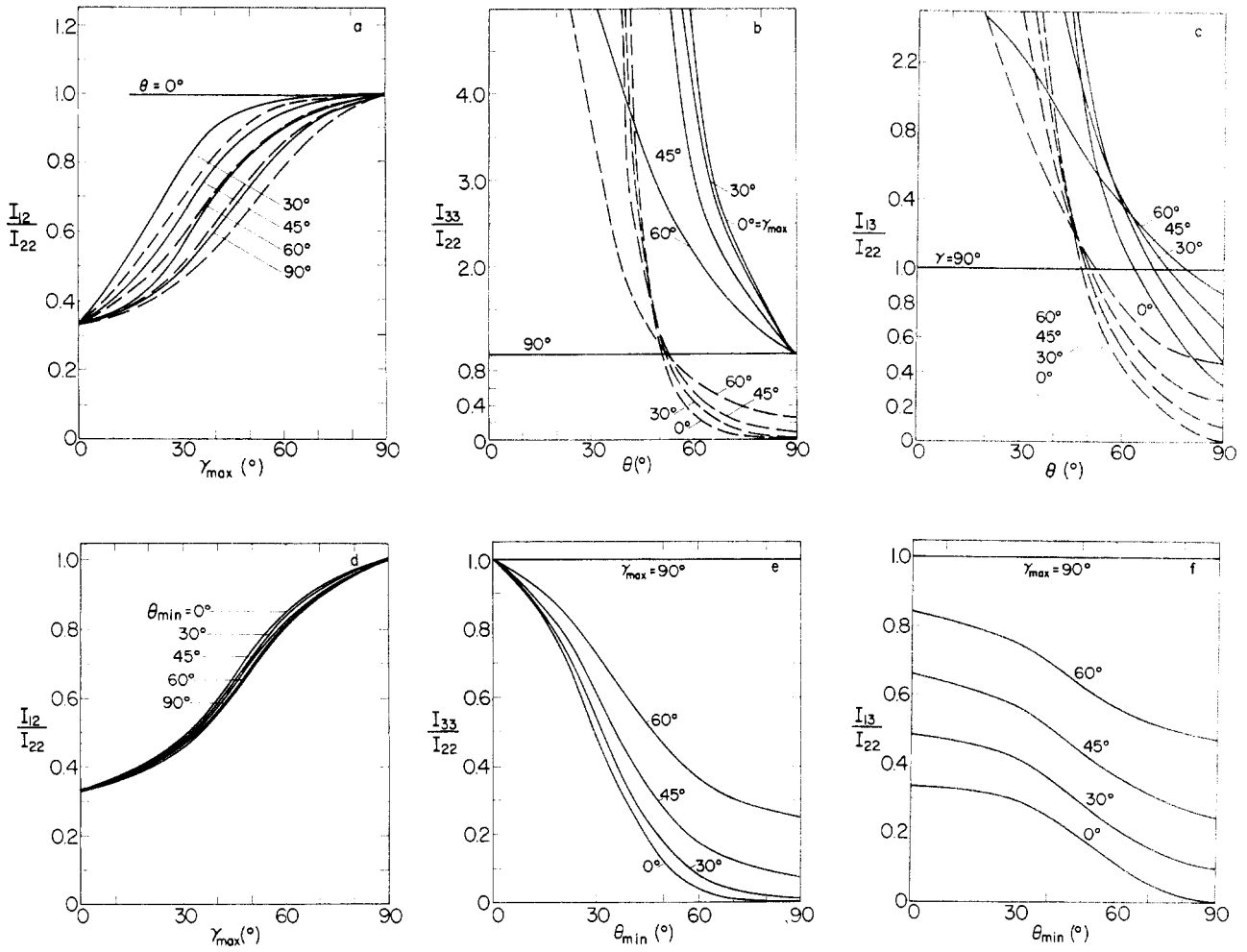


FIGURE 3: Calculated fluorescence intensities for models 1 (a, b, and c; —), 3 (a, b, and c; – – –) and 2 (d, e, and f).

Results

X-Ray Diffraction. Well-defined Bragg reflections were obtained with all specimens examined. From 6 to 11 orders were noted. Such reflections demonstrate that all or part of the specimen was organized as a multibilayer system with parallel equally spaced lamellae. It was noted that the appearance of the film, often apparently pitted, did not affect the ability to cause strong X-ray reflections.

Fluorescence of the Probes in Lipid Multibilayers. Fluorescence matrices for the probes in egg lecithin and egg lecithin–cholesterol (1:1) multibilayers hydrated with 50 mM NaCl (pH 6) are shown in Table I together with the excitation wavelength, probe concentrations, filters, type of film holder, and the probe lifetimes. Also included are values of θ and γ estimated from the indicated models. The significance (2–15°) of differences in these angles depends on the part of a particular model being used, *e.g.*, compare the left and right hand sides of Figure 4h. In all cases it was possible to estimate the cause of the observed nonideal behavior. For all probes, except DPE, a rapid restricted motion had to be invoked together with a varying degree of nonparallelism of abosrption and fluorescence vectors. It was found that a model with a distribution of θ and γ was the only one which would fit the results in some cases (notably VPBO) and therefore this type of model was also used in cases where either a fixed value or a distribution of θ would fit the results. An additional reason for choosing this model is that it is difficult to see how a molecule apparently undergoing rapid motions (variation in γ) could maintain a constant value of θ at the same time. The DPE consistently appeared to fit better a model using a slow motion of the probe, since $I_{13} \neq I_{31}$, but the difference was marginal due to the wide distribution of θ. To obtain an idea of this distribution model 2 was used.

In order to indicate the procedure involved in analyzing a fluorescence matrix an example is given by using the result for CY in lecithin (Table I). Firstly, $3I_{12} > I_{22}$. The possible causes, i–iv, of this depolarization are outlined in the previous section. By comparing I_{13} and I_{31} types i–iii are eliminated leaving some form of rapid but restricted motion as the depolarizing factor. Since I_{33}/I_{22} is <1, a majority of the probe vectors must be lying towards the bilayer plane and models 2 and 3 are appropriate. By using I_{12}/I_{22} a possible range of γ_{max} is obtained from Figure 3a,d. I_{33}/I_{22} and I_{13}/I_{22} are used together with the ranges of γ_{max} to obtain a range of θ or θ_{min} (Figure 3b,c,e,f). Repeating this cycle by putting the θ values back into Figure 3a,c provides more specific values of both γ_{max} and θ or θ_{min}. Since agreement of the θ or θ_{min} values obtained from I_{33}/I_{22} and I_{13}/I_{22} is better for model 2, this is the one used.

Most of the probes listed in Table II do not have parallel absorption and emission vectors. The deviation was estimated by measuring the polarization of the fluorescence of each dye dissolved in glycerol at a concentration where no energy

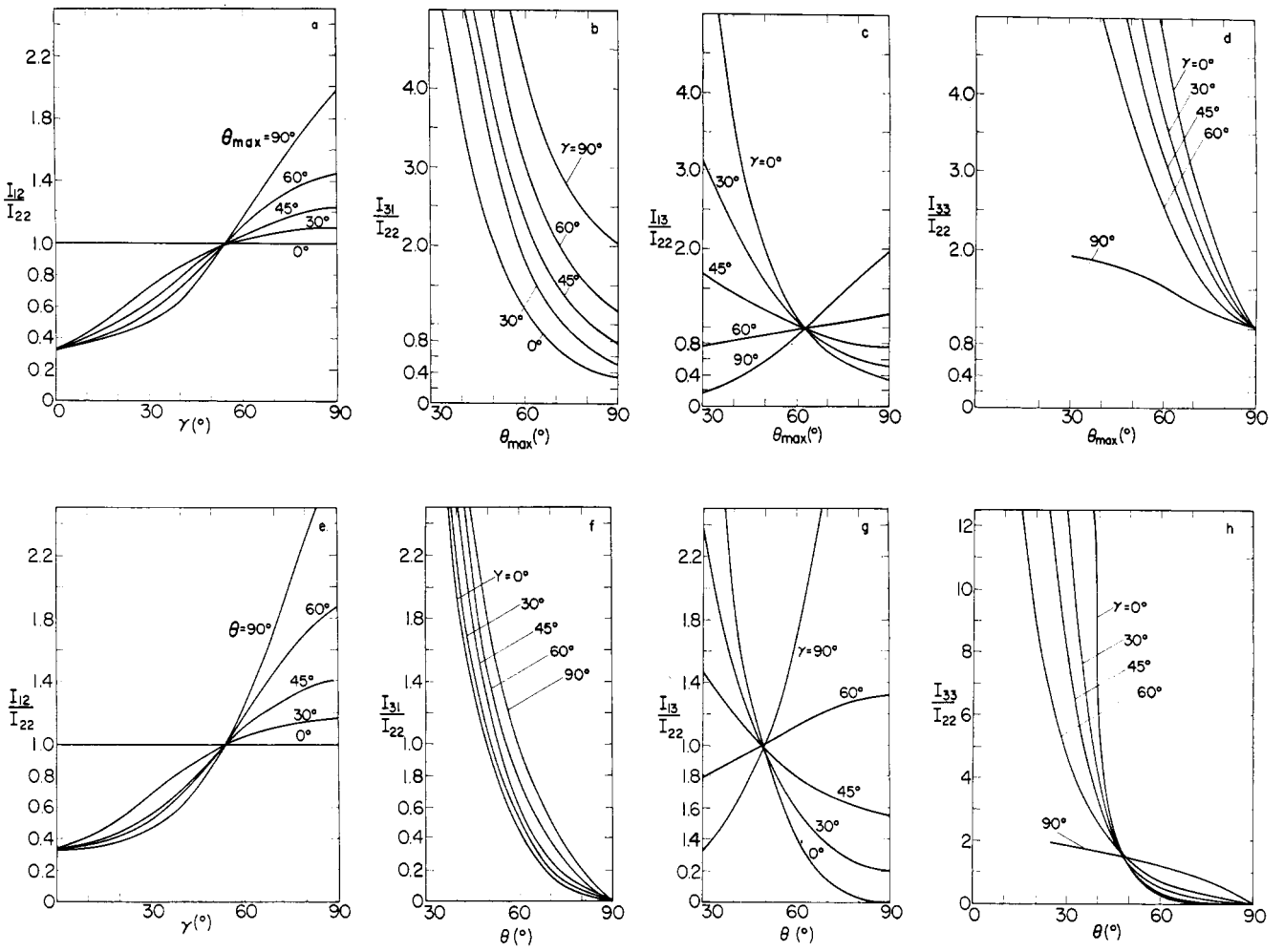


FIGURE 4: Calculated fluorescence intensities for models 4 (a, b, c, and d) and 5 (e, f, g, and h).

transfer could occur. The fluorescence spectra of the probes in glycerol and in the lipid films were similar. Application of Jablonski's (1935) equation yields the appropriate angle which is indicated on Table I. This angle is implicitly included in the values for γ derived from the matrices. Subtraction from γ or γ_{max} yields the actual motion to a first approximation. A "chromophore long axis" has been arbitrarily assigned to each probe (see Table I) to facilitate discussion of its orientation distribution.

Table III shows the behavior of VPBO in several different lipid systems, all of which yielded strong X-ray diffraction patterns indicative of the presence of plane, parallel bilayers. The values of θ_{max} and γ_{max} are derived from model 1. The effect of temperature is also presented for VPBO in dipalmitoyllecithin films. Model 1 was again used.

Discussion

Three types of orientation distribution were noted. Firstly there is the very restricted distribution of CY with the long axis of the chromophore lying in the plane of the bilayer. Secondly there is a distribution of probe long axes centered on the normal to the bilayer plane and including all angles from zero to some maximum (VPBO, AS, ONS, EQ). Thirdly there is a distribution centered on the plane of the bilayer and extending away from this to some minimum value of θ (DPE, ANS). An original prediction (Badley *et al.*, 1971) that rigid molecules with the large axial ratio will have a narrower distribution than those without does not necessarily appear to be valid (compare EQ and ONS, VPBO and AS). The large distributions are thought to reflect slow motions of the probes on the nanosecond time scale rather than fixed arrays. This would correlate with known microsecond motions of the fatty acid chains of phospholipids as determined by nuclear magnetic resonance (Chan *et al.*, 1972).

The bilayer structural changes caused by cholesterol (Butler *et al.*, 1970) can have differing effects on the various probes.

TABLE III: VPBO in Various Lipid Multibilayers.[a]

Lipid (at 20°)	θ_{max}	λ_{max}
Egg lecithin	80	30
Egg lecithin + cholesterol (1:1)	67	28
Dipalmitoyllecithin	62	28
Dipalmitoyllecithin + cholesterol (1:1)	71	30
Dipalmitoyllecithin (45°C)	80	33
Sphingomyelin	82	30
Sphingomyelin + cholesterol (2:1)	73	33
Phosphatidylserine	71	25

[a] Angles estimated from model 1.

The distribution for θ of one interior probe, VPBO, is narrowed (solid angle decreased by one-quarter) while that for another, AS, is hardly affected. Even more variability occurs with surface probes. There was no effect on ANS or EQ. However, DPE tended to become randomly distributed while the range for ONS decreased slightly. These differences in response show that the probe can influence its own distribution. Nevertheless, probes can be sensitive to lipid structural parameters, as illustrated by the variation in θ_{max} of VPBO in bilayers of differing composition and temperature (Table III). Systems such as egg lecithin plus cholesterol or dipalmitoyllecithin at 20° yielded a narrower distribution compared to those which may be considered less ordered, egg lecithin and spingomyelin (Oldfield and Chapman, 1971b). In addition, differences due to the well characterized phase changes of dipalmitoyllecithin bilayers at approximately 40° (Chapman *et al.*, 1967) and to the effects of cholesterol (Oldfield and Chapman, 1971b) could be detected. Comparison of the effects of cholesterol on egg and dipalmitoyllecithin at 20° shows that its addition causes opposite effects on the distribution of θ. This correlates with the spin label results of Oldfield and Chapman (1971b). In general the results showed that the range of θ of a probe tended to increase whenever lipid chain mobility increased as measured by other techniques such as nuclear magnetic resonance (Darke *et al.*, 1971; Lee *et al.*, 1972), electron spin resonance (Hubbell and McConnell, 1971; Butler *et al.*, 1970; Oldfield and Chapman, 1971b) or permeability of bilayers (Demel *et al.*, 1972).

All the probes, except DPE, show the apparent rapid restricted motion in lecithin multibilayers. The extent varies from 10° to 30° (CY, EQ). A mobile linkage between chromophore and lipid anchor results in considerable motion (AS, ONS) whereas the rigid probes can exhibit either little (CY, VPBO) or a good deal of motion (EQ). Addition of cholesterol to egg lecithin caused varying effects on γ, depending on the probe, as found also for θ. AS showed a small decrease while VPBO, the other interior probe, was unaffected. Two of the surface probes (ONS and EQ) were unaffected by cholesterol while ANS showed a 30% decrease in γ_{max}, and the rapid motion of CY was apparently stopped altogether. Changing lipid composition or temperature also had little effect on γ_{max} for VPBO. In general, however, the range of γ, like that of θ, tended to increase when the other techniques (nuclear magnetic resonance, electron spin resonance and permeability) indicated increased chain mobility. Even for the largest amplitudes of γ, the rotational freedom of the probes was still severely restricted relative to that expected in hydrocarbon solution.

The apparent rapid oscillations of many of the probes is attributed to the existence of holes or free space within lipid bilayers. The existence of such free space of the volume of one or two methylene groups has been postulated to account for the diffusion of water and other nonelectrolytes through lipid bilayers by Stein (Lieb and Stein, 1969) and Träuble (1971). Such holes are thought to arise from imperfections in the packing of fatty acid residues caused by twisting around single C–C bonds or the presence of C=C bonds. The introduction of lipid-soluble molecules, such as the fluorescent probes, which have structures differing from that of the surrounding molecules would also cause further imperfections in packing, and effectively produce sufficient room for the observed mobility. Such perturbation raises the question of the possible "impurity" nature of the probes. This same question has recently been discussed in relation to spin labels by Henry and Keith (1971) and by Chan *et al.* (1972). In seems probable that the aromatic molecules studied here perturb their local environment to the extent that considerable freedom of movement is shown without disturbing the overall geometry of the bilayer as detected by X-ray diffraction or calorimetry. Further, such molecules would still be expected to be sensitive to changes in lipid organization and motion since the energies of activation for both rapid (yielding γ) and slow (yielding θ) probe motion would be affected by these lipid properties. The fact that both the distribution and motion of the probes can vary with lipid composition or temperature is in accord with this view.

It is obvious that lipid orientations and motions cannot be determined directly with probes producing perturbations in packing and probe behavior will only reflect to a greater or lesser extent the actual situation. Differences in the probes' molecular structures affect the nature of the information obtained, as would be expected, since the packing imperfections caused by the probes will depend on their size, rigidity, and shape. For example, two of the surface probes ONS and ANS show opposite effects upon addition of cholesterol to egg lecithin. In the former case the distribution of θ widens while γ_{max} is unchanged but in the latter case γ_{max} decreases and θ_{max} remains constant. Obtaining the most direct correlation between probe and lipid properties would seem to require that the probes pack isomorphously. To obtain information about lipid orientations the probe would also have to be rigid in the region of interest, highly asymmetrical, with one polar end, and have both transition moments parallel to the long axis. On the other hand, to study lipid motions the chromophore should be as small as possible but rigidly attached to the section of interest. When, as normally occurs, the condition of isomorphous packing is not met, probes can be expected to be of greatest use in looking at the more dynamic aspects of membrane behavior where the focus is on change rather than on absolute values of some membrance parameter. Using fluorophores does allow both very low concentrations and very fast measurements.

One way to avoid the difficulty of probe-induced perturbations is to utilize intrinisic probes, *i.e.*, fluorescent lipids occurring naturally in a membrane. The detection of fluorescent molecules, possibly steroids, in brain lipid extracts (Khan and Hess, 1971) is noteworthy in this respect. The mathematical formalism developed for the bilayer is general and can be applied to any planar system. Thus, non-lipid constituents of natural or model membranes can be used as intrinsic probes, *e.g.*, the tryptophan residues of peptides and proteins, antibiotics, or the flavine component of membrane-bound enzymes.

As has been shown elsewhere it is also possible to look at single bilayers (Yguerabide and Stryer, 1971) and it does not seem unreasonable to suppose that study of parts of membranes of single cells could be undertaken using appropriate fluorescence microscopic techniques, with either intrinsic or extrinsic probes.

In the multibilayer system laid down on the glass or silica surfaces there is the possibility that other phases may be present. This would not easily be detected by X-ray experiments. In the present experiments it is thought that this is not the case because a comparison can be made to a single bilayer. Polarized fluorescence intensities from such a system have been measured using a slightly different technique (Yguerabide and Stryer, 1971) for CY and AS as well as 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene scintillator (which structurally and spectrally is almost identical with VPBO). Oxidized cholesterol in octane was used to form the bilayer. The intensi-

ties obtained yield very similar probe distributions and motions compared to the same molecules in lecithin multibilayers, after similar processing. The presence of other phases would tend to randomize the distribution, and particularly in the case of CY this seems not to occur. Additional evidence that only a lamellar phase was present with the lamellar planes parallel to the substrate surface is provided by comparison of the results obtained for samples with and without compression. The plane of cleavage of a lamellar phase lies along the lamellar plane and crushing of liquid crystalline structures between two flat surfaces would be expected to cause orientation of these planes parallel to the surfaces. Identical results were obtained by this compression method and by evaporation of solvent from the lipid. In addition spin-label data on lipid films prepared in similar as well as slightly different ways (Butler *et al.*, 1970; Libertini *et al.*, 1969; Seelig, 1970) are all consistent with almost all of the lipids being in a plane, parallel array.

In summary it has been shown that it is possible to measure the fluorescence matrix, or grid, of a suitable fluorescent sample and to obtain orientation and motion information therefrom. The rapid restricted motion exhibited by a number of fluorescent probes together with the presence of probe specific effects suggests that the probes could be acting as perturbants. Therefore such molecules would seem to be more useful in studying changes in bilayer properties, rather than for making absolute measurements. Application of the treatment presented to intrinsic and extrinsic probes in natural and model membranes has been pointed out.

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