ANGLE-RESOLVED FLUORESCENCE DEPOLARIZATION OF MACROSCOPICALLY ORDERED BILAYERS OF UNSATURATED LIPIDS

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The orientational order and dynamics of diphenylhexatriene molecules incorporated in planar multibilayers of unsaturated lipids were studied by angle-resolved fluorescence depolarization at room temperature. Bilayer systems of the phosphatidylcholines soybean PC, dioleoyl PC and egg PC as well as the galactolipid digalactosyl diacylglycerol (DGDG) were used in the experiments. The depolarization ratios of diphenylhexatriene in these systems exhibited a weak angular dependence, in marked contrast to those observed from diphenylhexatriene in multibilayers of saturated phosphatidylcholines (Vos, M.H., Kooyman, R.P.H. and Levine, Y.K. (1983) Biochem. Biophys. Res. Commun. 116, 462–468). The results indicate that the diphenylhexatriene molecules possess a low degree of orientational order. However, the dynamics of the reorientational motions are inconsistent with the assumption that the diphenylhexatriene molecules possess effective cylindrical symmetry about their long axes. The fluorescence depolarization experiment is sensitive to the rotational motions about the long axes as the absorption and emission moments of diphenylhexatriene molecules are not mutually parallel. It is suggested that diphenylhexatriene molecules in bilayers of unsaturated lipids undergo slower reorientational motions around their long molecular axes than in bilayers of saturated phosphatidylcholines. A further conclusion of this study is that the 'wobbling-in-cone' model provides a poor description of the behaviour of diphenylhexatriene molecules in lipid bilayers.

Introduction

In recent years there has been a growing interest in the application of fluorescence depolarization experiments to studies of molecular order and dynamics in biological membranes and lipid bilayers. In these experiments fluorescent reporter molecules such as 1,6-diphenyl-1,3,5-hexatriene (DPH) are incorporated into the membranes and their fluorescence depolarization studied. The orientational distribution of the molecules in the membranes is commonly described in terms of the order parameters $\langle P_2 \rangle = \frac{1}{2} \langle 3 \cos^2 \beta - 1 \rangle$ and

$\langle P_4 \rangle = \frac{1}{8} \langle 35 \cos^4 \beta - 30 \cos^2 \beta + 3 \rangle$, where the angled brackets denote an ensemble average and P_L is the Legendre polynomial of order L. The dynamics of the probe molecules is characterized in terms of correlation times whose physical significance depends on the particular model of motion used in the analysis.

In two recent papers from our laboratory [1–3] we described a new type of steady-state fluorescence depolarization experiment suitable for studies of macroscopically ordered lipid bilayer systems. In this experiment the fluorescence depolarization ratios are determined as a function of two independent angles: (1) the angle of incidence of light measured relative to the normal to the lamellar surface and (2) the angle between the directions

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of incidence and observation.

We have shown both theoretically [1] and experimentally [2,3] that this type of angle-resolved fluorescence depolarization experiment (AFD) yields both the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ as well as information about molecular dynamics and the mutual orientation of the absorption and emission moments for molecules with effective cylindrical symmetry such as DPH. This method can thus be considered to be an alternative to time-resolved experiments on lipid vesicles [17].

AFD experiments on bilayers of saturated phosphatidylcholines, with or without cholesterol, have demonstrated the validity of this approach [2,3]. It was shown that a knowledge of both $\langle P_2 \rangle$ and $\langle P_4 \rangle$ is often necessary in order to describe adequately the ordering and preferential orientation of the DPH molecules in the membrane systems. An important finding was that the absorption and emission moments of DPH are not mutually parallel as is commonly assumed. Rather, the angle between the moments depends on the bilayer system in which the DPH molecules are embedded. The rotational correlation times of DPH in these bilayers were determined on the basis of the strong collision model [1,5–7].

Here we report an extension of these studies to bilayers of the unsaturated lipids egg phosphatidylcholine, soybean phosphatidylcholine, dioleoylphosphatidylcholine and digalactosyl diacylglycerol containing DPH molecules. It is shown that in these systems the angle dependence of the polarization ratios is significantly different from that observed in bilayers of saturated lipids and reported in Refs. 2 and 3. An analysis of the data reveals that the introduction of unsaturation into the lipid chains has a strong influence on the reorientational dynamics of the DPH molecules, but causes only qualitative changes in their static orientational order as reflected in the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$.

Firstly, we give a short recapitulation of the theory and pay particular attention to three models for the reorientational motion (strong collision, rotational diffusion and 'wobbling-in-cone') which are used in the interpretation of the experimental data. Secondly, experimental AFD results are presented and discussed in the light of our earlier results. Analysis of the data indicates that the 'wobbling-in-cone' model, in contrast to the strong collision and rotational diffusion models, yields a poor representation of the reorientational dynamics of DPH molecules in lipid bilayers. Finally, it is argued that DPH molecules are not particularly suitable as probes for monitoring order and dynamics in unsaturated lipid bilayers.

Theory

Consider a macroscopically ordered membrane system containing fluorescent 'reporter' molecules and subjected to continuous illumination with a well-defined wavelength and polarization direction. The geometrical arrangement of the experiment is shown in Fig. 1. We define an axis system (XYZ) such that X and Y are in the membrane plane and Z is parallel to the optical axis (director)
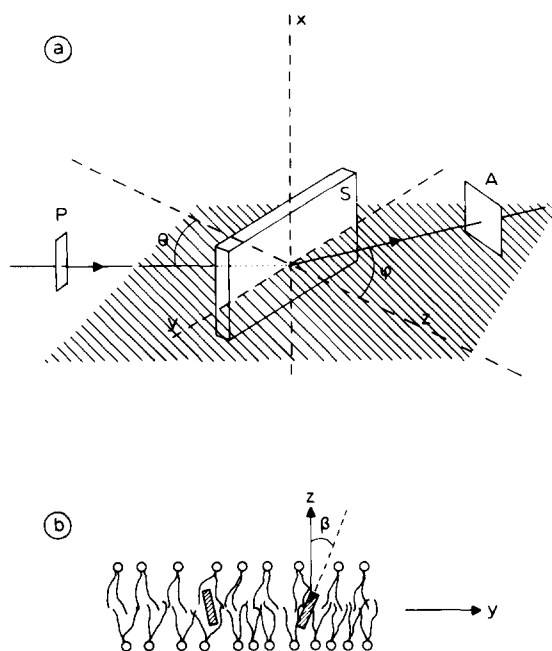

Fig. 1. (a) Experimental AFD geometry for a lamellar bilayer sample S lying in the XY plane. θ and ϕ are the angles in air between the macroscopic director Z and, respectively, the direction of excitation and observation of the fluorescence. The states of polarization, either parallel or perpendicular to the YZ plane are determined by the polarizer P and the analyzer A. The angles θ and ϕ (taken to be positive, as drawn in the figure) change sign on rotation of either P or A through the Z-axis. (b) A cylindrically symmetric probe molecule, embedded in one of the approx. 1000 stacked bilayers in sample S, has an instantaneous angle β relative to the director Z.

of the membrane system. The angle θ corresponds to the direction of the incident (exciting) beam relative to the director of the membrane system. The angle ϕ is defined as the angle under which the light emission is observed. The principal direction of the polarizer P can be set either perpendicular or parallel to the ZY plane.

The same holds for the emitted light where the analyzer A is placed in the path of the outgoing beam. Thus, four combinations of polarized fluorescence intensities I_{if} can be measured: I_{oo}, I_{oe}, I_{eo} and I_{ee} where the suffices o and e denote light polarized perpendicular to the Z-axis ('ordinary' beam) and light polarized in the ZY-plane ('extraordinary' beam), respectively. In order to eliminate the dependence of I_{if} on unknown quantities such as the incident light intensity, illuminated volume and absorption coefficients, only the depolarization ratios $R_o = I_{oe}/I_{oo}$ and $R_e = I_{eo}/I_{ee}$ are determined experimentally. It has been previously shown [1] that these polarization ratios are given by the relations:

$$R_o = R_o(\phi) = \left[1 - R_1 + (R_1 + R_2)\sin^2\phi\right]/(1 + R_1) \quad (1a)$$

$$R_e = R_e(\theta,\phi) = \left[1 - R_1 + (R_3 + R_1)\sin^2\theta\right]$$
$$\times \left[1 + R_1 + (R_2 - R_1)\sin^2\phi + (R_3 - R_1)\sin^2\theta\right.$$
$$\left. + R_5\sin^2\theta\sin^2\phi + R_4\sin 2\theta\cdot\sin 2\phi\right]^{-1} \quad (1b)$$

The only assumption underlying the derivation of Eqn. 1 is that the probe molecules are macroscopically distributed uniaxially about the normal to the surface of the sample. It can be shown further that Eqns. 1a and 1b are obtained even if several independent probe populations are present, provided only that the total macroscopic distribution is uniaxially symmetric.

The parameters $R_1,\ldots,R_5$ contain all the information about the orientational order and dynamics of the fluorescent 'reporter' molecules in the membrane system. These parameters can only be interpreted on the basis of a model for the reorientational motion of the molecules. In addition, the molecular geometry must be known. In the analysis we shall assume that even if several independent probe molecule populations are present, they can all be described by the same dynamic behaviour.

The DPH molecules used in this study will be considered to possess an effective cylindrical symmetry. Furthermore, the absorption transition moment, μ, will be taken to be parallel to the molecular symmetry axis. The emission transition moment, ν, will, however, be allowed to make an angle β_ν relative to that axis. The angle β_ν will be considered further to be independent of the orientational and dynamic behaviour of the molecules.

With these conditions Eqns. 16–19 of Ref. 1 can be written as

$$
\begin{array}{ll}
R_1 = 3g_2/A, & R_2 = 3(S_\nu - g_0)/A \\
R_3 = 3(S_\mu - g_0)/A, & R_4 = 3g_1/A \\
R_5 = 3(3g_0 + g_2)/A; & A = 1 - S_\mu - S_\nu + g_0
\end{array}
\quad (2)
$$

where the second rank order parameters for the absorption and emission transition moments S_μ and S_ν are defined by

$$S_\nu = \langle P_2 \rangle; \quad S_\nu = \langle P_2 \rangle P_2(\cos\beta_\nu) \quad (3)$$

Further

$$g_k = g(kk00)P_2(\cos\beta_\nu), \quad k = 0,1,2 \quad (4)$$

where

$$g(kk00) = \frac{1}{\tau_F}\int_0^\infty G(kk00t)\exp(-t/\tau_F)\mathrm{d}t \quad (5)$$

and the correlation functions $G(kk00t)$ are given by

$$G(kk00t) = \langle D_{k0}^2(\Omega_0)D_{k0}^{2*}(\Omega_t)\rangle \quad (6)$$

Here τ_F is the isotropic fluorescence decay constant (≈ 10 ns for DPH). $D_{m,n}^2$ are Wigner rotation matrix elements [4] and $\Omega_0 \equiv (\alpha_0\beta_0\gamma_0)$ denotes the set of three Euler angles that transform the laboratory frame into the molecular frame at time $t = 0$; Ω_t is defined analogously for time t. P_L is the Legendre polynomial of order L. The angled brackets denote an ensemble average over a macroscopic volume element. It is important to realize that the ensemble average entails an average over all the independent probe molecule populations in the sample.

We note further that, as a consequence of our assumption that every population can be described

by the same dynamic behaviour, the information about the populations will be contained only in the orientational order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$.

The order parameter $\langle P_2 \rangle$ can be obtained directly from the experimental data, as can the angle β_ν. However, $\langle P_4 \rangle$ and the information about the molecular dynamics is contained in the correlation functions $G(kk00t)$. The functions can only be evaluated in terms of a model for the reorientational motion of the molecule. We shall here consider only the strong collision [1,5-7] and the rotational diffusion [5-10] models.

(a)Strong collision model

The molecules are here assumed to undergo random rotational jumps, with a residence time τ_0 at any orientation [1,5-7]. The correlation functions $G(kk00t)$ can be shown to decay exponentially as

$$G(kk00t) = G(kk000)\exp(-t/\tau_0) + \langle P_2 \rangle^2$$

$$\times [1 - \exp(-t/\tau_0)]\delta_{k0} \tag{7}$$

Substitution of Eqn. 7 into Eqn. 5 leads to

$$g(kk00) = w_0 G(kk000) + \delta_{k0}\langle P_2 \rangle^2 (1 - w_0) \tag{8}$$

with

$$w_0 = \tau_0/(\tau_F + \tau_0) \tag{9}$$

The values of $G(kk000)$ have been tabulated in Refs. 5–7.

Within this model the system parameters $\langle P_2 \rangle$, $\langle P_4 \rangle$ and w_0 can be derived analytically from $R_1, \ldots, R_5$ [2,3].

(b) Rotational diffusion model

The molecule is assumed to undergo stochastic rotational diffusion subject to an anisotropic orienting potential $U(\beta)$, where β is the angle between the local director (the normal to the bilayer surface) and the long molecular axis. The equation for the orientational conditional probability is [5-8]

$$\frac{\partial}{\partial t} P(\Omega_0|\Omega t) = D_\perp \Gamma_\Omega P(\Omega_0|\Omega t) \tag{10}$$

where $D_\perp$ is the perpendicular component of the rotational diffusion tensor D which is assumed to have cylindrical symmetry about the molecular axis. The stochastic operator Γ_Ω is given by

$$\Gamma_\Omega P(\Omega_0|\Omega t) = \nabla_\Omega^2 P(\Omega_0|\Omega t)$$

$$+ (\sin \beta)^{-1} \cdot \frac{\partial}{\partial \beta}\left[\sin \beta P(\Omega_0|\Omega t)\frac{d}{d\beta} U(\beta)\right]$$

$$\tag{11}$$

and ∇_Ω^2 is the Laplacian operator written in terms of the Euler angles describing the orientation of the molecule with respect to the laboratory frame. The initial boundary condition is $P(\Omega_0|\Omega 0) = \delta(\Omega - \Omega_0)$. We shall here assume that $U(\beta)$ is cylindrically symmetric and is given by

$$U(\beta) = \{\lambda_2 P_2(\cos \beta) + \lambda_4 P_4(\cos \beta)\}kT \tag{12}$$

Expansion of $P(\Omega_0|\Omega t)$ in a basis set of Wigner rotation matrices transforms the differential equation into a matrix equation which is solved numerically.

The correlation functions $G(kk00t)$ can now be shown [5-8] to be described as a sum of exponential decays:

$$G(kk00t) = \sum_M b_M^k \exp\left(-D_\perp \alpha_M^k t\right) \tag{13}$$

where the decay amplitudes b_M^k and the decay rates α_M^k are complex functions of λ_2 and λ_4 in Eqn. 14 and are obtained from the numerical solutions of the equation of motion. Within this model we finally obtain

$$g(kk00) = \sum_M \frac{b_M^k}{\left(1 + D_\perp \tau_F \alpha_M^k\right)} \tag{14}$$

In order to apply this model a non-linear least-squares procedure is used to fit the experimental parameters $R_1, \ldots, R_5$. Here λ_2, λ_4, $D_\perp \tau_F$ and $P_2(\cos \beta_\nu)$ are used as free model parameters. The order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are determined by the coefficients λ_2 and λ_4. We note here that our choice of the orienting potential, Eqn. 12 spans the entire $\langle P_2 \rangle$, $\langle P_4 \rangle$ plane, Fig. 2. This plane is defined by the inequalities [1-3]:

$$-1/2 \leqslant \langle P_2 \rangle \leqslant 1$$

$$1 \geqslant \langle P_4 \rangle \geqslant \left(35\langle P_2 \rangle^2 - 10\langle P_2 \rangle - 7\right)/18$$

$$-3/7 \leqslant \langle P_4 \rangle \leqslant \left(5\langle P_2 \rangle + 7\right)/12 \qquad (15)$$

The 'Wobbling-in-cone' model developed by Kinosita et al. [9] and discussed by Lipari and Szabo [10] is a special case of the diffusion model. Here the orienting potential $U(\beta)$ is assumed to have the form

$$U(\beta) = 0 \qquad 0 \leqslant \beta < \beta_0$$

$$U(\beta) = \infty \qquad |\beta| \geqslant \beta_0 \qquad (16)$$

With this form of the orienting potential the values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are uniquely determined by the value of β_0 [10]. This relation defines a line in the $\langle P_2 \rangle$, $\langle P_4 \rangle$ plane, Fig. 2. It can be seen that the model predicts negative values for $\langle P_4 \rangle$ if $0 \leqslant \langle P_2 \rangle \leqslant 0.55$. We shall show below that this behaviour is inconsistent with our experiments. The relation between $\langle P_2 \rangle$ and $\langle P_4 \rangle$ for the Gaussian model proposed by Kinosita et al. [11] is also shown in Fig. 2. This model is equivalent to the one described above but with $\lambda_4 = 0$ in Eqn. 12.

Materials

Dioleoylphosphatidylcholine (DOPC) and egg phosphatidylcholine were obtained from Sigma
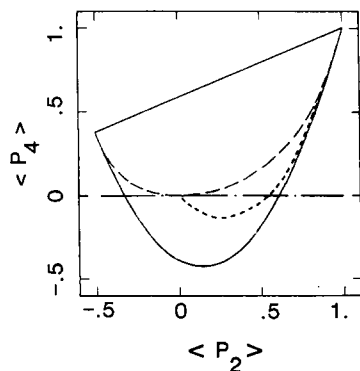
Fig. 2. The relation between $\langle P_2 \rangle$ and $\langle P_4 \rangle$. The continuous lines give the physical boundaries (see text). ---, the relation obtained from a gaussian orientational distribution function; ------, the relation defined by the 'wobbling-in-cone' model; $-\cdot-$, is the $\langle P_4 \rangle = 0$ line.

and used without further purification after chromatographical checks. Soybean phosphatidylcholine and digalactosyl diacylglycerol (DGDG) were prepared from soybeans [12] and spinach leaves [13], respectively, as described below. 1,6-Diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich. Ethanol was obtained from Baker (AR). Doubly distilled water was used throughout.

All the preparative steps were carried out strictly under a nitrogen atmosphere. The lipids came into contact with air for a short time only during the alignment procedure, see below. The formation of peroxides was monitored following [20] by recording absorption spectra in the 200–300 nm region.

Purification of soybean lecithin. Crude soybean lecithin was bought from V.N.R. Reformproducten B.V., Ede, The Netherlands and prepurified by column chromatography on aluminium oxide 90 (Merck, Darmstadt, F.R.G.) using chloroform with increasing proportions of methanol as the eluent. The soybean lecithin fractions obtained were purified further by preparative high pressure liquid chromatography(HPLC) with the HPLC-equipment described in Ref. 12 or with home-built HPLC-equipment. As preparative chromatographic columns were used: (1) 500 × 50 mm stainless steel (S.S.), packed with Polygosil 60-1525 Machery Nagel and Co, Düren, F.R.G.; (b) 250 × 22.7 mm Lichrosorb Si-60-7 (S.S.) (Chrompack Nederland, Middelburg, The Netherlands). The columns were eluted with chloroform/methanol (70 : 30, v/v). The fractions collected were analysed for purity on Silica gel 60 plates (10 × 10 cm, for nano-TLC, art 5633, Merck, Darmstadt, F.R.G.). Chloroform/methanol/water (65 : 25 : 4, v/v) was used as the eluting mixture. The fractions containing pure soybean phosphatidylcholine were dried and stored dry under N_2 at $-20\,°C$.

Purification of DGDG from spinach. Total lipid extraction from spinach chloroplasts and subsequent fractionation in lipid classes was performed as described in Ref. 13. The acetone fraction obtained, containing mainly galactolipids, was purified further by using preparative HPLC as described above. A Lichrosorb Si-60-7 column (S.S., 250 × 22.7 mm) was used. The column was eluted with chloroform/methanol (85 : 15, v/v). Analysis for purity was done as described above. We also

used DGDG bought from Lipid Products (South Nutfield, U.K.). Analysis for purity was done as described. Only DGDG containing no contaminants by these criteria was used for our investigations.

Preparation of DPH-containing bilayers. 20 mg lipid in ethanol solution were mixed with a $5 \cdot 10^{-4}$ M solution of DPH in tetrahydrofuran. The final lipid to DPH molar ratio was 250 : 1. The samples were dried in the dark by flushing with nitrogen gas. Water was added to the dry material to form a 30% (w/w) mixture. The mixture was then allowed to equilibrate for 5 h at 35°C under a humidified nitrogen atmosphere.

Macroscopically oriented multibilayers were prepared by gently rubbing the hydrated lipid mixture between two microscope glass coverslips (thickness $175 \pm 5 \mu$m). The alignment was carried out at room temperature (25°C) and monitored by optical microscopy following Asher and Pershan [14]. On using this preparation method about 20% of the samples of DOPC and soybean phosphatidylcholine exhibited excellent alignment over areas larger than 1 cm². These areas were selected for further experimental work. In marked contrast, well-aligned samples of DGDG and egg PC were produced consistently.

Well-aligned samples were sealed with a two component epoxy resin along their four rims in order to prevent dehydration. The contribution of scattered excitation or fluorescence light was minimized by completely blackening the samples except for a 5×5 mm wide square area. The thicknesses of the samples, measured by dark field microscopy, were between 12 and 40 μm.

Experimental

Angle-resolved fluorescence depolarization (AFD) experiments were carried out at room temperature (25°C) on a home-built fluorimeter. It was equipped with a water-cooled light-stabilized 1500 W Xe arc (Osram) and a Peltier cooled EMI 9816A or RCA 31034 PM tube, operating at approximately -25°C and a cathode voltage of -1200 V. The angles θ and ϕ were set to within 1° by two goniometers on which the sample holder and the PM tube assembly were independently mounted.

The necessary precautions to minimize systematic errors in the AFD experiments have been discussed in detail previously [2,3].

DPH was excited at 365 nm with an FWHM (full width at half maximum) bandwidth of 8 nm. The fluorescence was observed by means of interference filters, FWHM 12 nm, at 432 nm for all lipids and additionally at 448 and 494 nm for egg phosphatidylcholine in order to check a possible dependence of the depolarization ratios on the emission wavelength of DPH. The intensity of the exciting light was kept as low as possible in order to avoid bleaching of the DPH molecules, yet at the same time maintaining a good signal-to-noise ratio. The intensity of the DPH fluorescence, measured at the same scattering geometry, remained constant during the experiment. Control experiments using well-aligned samples of pure lipids showed that the intrinsic fluorescence signals from the lipids and the coverslips amounted to less than 1% of the DPH fluorescence intensity.

The refractive indices of the membrane samples were determined to within 1% with a thermostated ABBE refractometer (Bleeker). These were required for applying optical corrections [2,3].

Results and Discussion

(i) Determination of R_1-R_5

Angle-resolved fluorescence depolarization ratios were measured for DPH molecules embedded in bilayers of egg PC, soybean PC, DOPC and DGDG. The same values of the depolarization ratios, R_0 and R_e, within the experimental error of 2% were obtained on rotating the samples by an arbitrary angle about the normal to their surfaces. This indicates that the macroscopic distribution of DPH molecules is uniaxially symmetric. Furthermore, different samples of the same lipid system yielded reproducible results within experimental error.

The results obtained from egg PC bilayers were similar to those found earlier in our laboratory for bilayers of saturated phosphatidylcholines above the phase transition of the chains [2,3]. However, in marked contrast, a quite different angular dependence of R_o and R_e were observed in the other bilayer systems which contained predominantly unsaturated lipid molecules.

The experimental results, corrected for refraction and transmission losses at the air/glass and glass/ sample interfaces [1–3], were analysed following the procedure described by us previously [2,3]. This can be summarised as follows:

(1) R_1 and R_2 are determined from the linear dependence of R_o on $\sin^2 \theta$, Eqn. 1a.

(2) R_1 and R_2 are also determined from the 'extraordinary' experiment with $\theta = 0$. Eqn. 1b then yields

$$1/R_e = (1 + R_1)/(1 - R_1) + [(R_2 - R_1)/(1 - R_1)]\sin^2 \phi$$

(18a)

$1/R_e$ is now linear in $\sin^2 \phi$.

(3)R_1 and R_3 are determined from the 'extraordinary' experiment on setting $\phi = 0$. Eqn. 1b can now be written as

$$\frac{1}{(1/R_e) - 1} = \frac{R_3 + 1}{2R_1} \tan^2 \theta + \frac{1 - R_1}{2R_1}$$

(18b)

This experiment is particularly useful if the angle between the absorption and emission moments within the probe molecule is not known: the difference $R_2 - R_3$ is a measure of this angle. Only if $R_2 = R_3$ are the absorption and emission moments parallel.

(4)R_4 and R_5 are obtained from the 'extraordinary' experiment on varying both θ and ϕ. In the general case Eqn. 1b can be cast as

$$Y(\theta,\phi) \equiv a_1(\theta)/R_e - a_2(\theta)$$

$$= A(\theta)\sin^2 \phi + B(\theta)\sin 2\phi$$

(18c)

with

$$a_1(\theta) = 1 - R_1 + (R_3 + R_1)\sin^2 \theta$$

$$a_2(\theta) = 1 + R_1 + (R_3 - R_1)\sin^2 \theta$$

and

$$A(\theta) = R_2 - R_1 + R_5 \sin^2 \theta$$

(18d)

$$B(\theta) = R_4 \sin 2\theta$$

As R_1, R_2, R_3 are known from the previous analysis, the coefficients a_1 and a_2 can be evaluated

at any angle θ_0. The coefficients $A(\theta_0)$ and $B(\theta_0)$ are then obtained from a least-squares fit to Eqn. 18c. By repeating this procedure for all the values of θ_i, a set of numbers $A(\theta_i)$ and $B(\theta_i)$ is obtained. Finally, values of R_4 and R_5 are extracted from the linear dependence of $A(\theta_i)$ and $B(\theta_i)$ on $\sin^2 \theta_i$ and $\sin 2\theta_i$, respectively (Eqns. 18d).

This procedure suffers from the drawback that the accuracy of the values of R_4 and R_5 is largely determined by errors in R_1, R_2 and R_3. In order to avoid this problem we have also used a non-linear optimalization procedure for the determination of $R_1, \ldots, R_5$ from Eqn. 1b.

It must be emphasized that the angles θ and ϕ appearing in Eqns. 1 and 18 are both angles measured within the sample.

The experimental results for soybean PC and egg PC are shown in Figs. 3 and 4 and in Figs. 5 and 6, respectively. The values of $R_1, \ldots, R_5$ for all the systems studied are summarized in Table I under A. Figs. 3b, c, d and 5 b, c, d show plots of $Y(\theta,\phi)$, Eqn. 18c, as a function of ϕ for a number of values of θ. It is important to note that the $Y(\theta,\phi)$ axes in the figures are scaled differently for the various values of θ, as given in the legends. Consequently, the experimental errors (2%) shown, appear larger for the lower values of θ. Figs. 4 and 6 show that the derived values of $A(\theta)$ and $B(\theta)$, Eqn. 18d closely follow the predicted angular de-
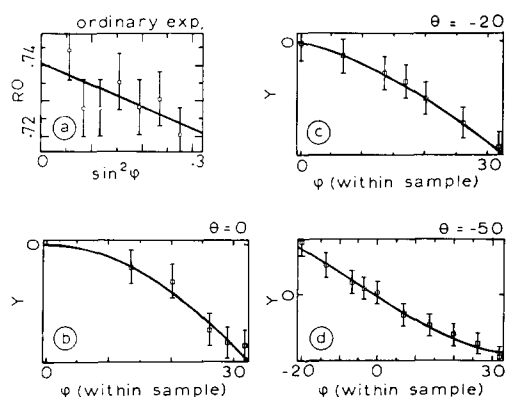

Fig. 3. Theoretical fits of AFD results for a soybean PC/water 70:30 (w/w) bilayer system with $4\cdot10^{-3}$ M DPH at 25°C. □: experimental values, relative accuracy 2%. Solid lines indicate least-squares fits according to Eqns. 1a and 18. (a) Ordinary experiment; (b), (c), (d) extraordinary experiment. Vertical scales $\theta = 0°$: 0.10; $\theta = -20°$: 0.14 and $\theta = -50°$: 0.30.
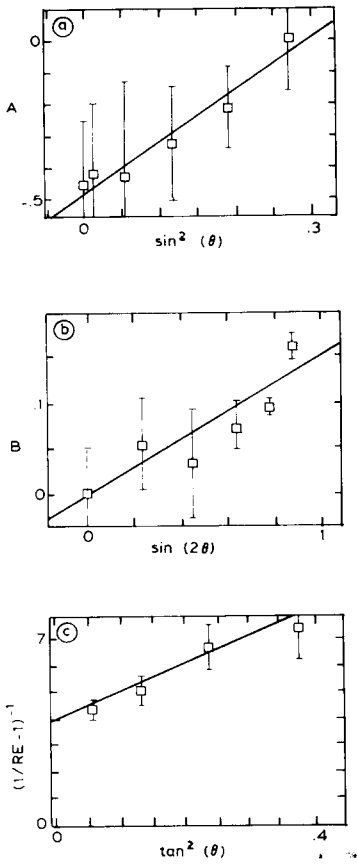
Fig. 4. AFD results for soybean PC. (a) $A(\theta_i)$ obtained from extraordinary experiments, as a function of $\sin^2\theta_i$, Eqn. 18d. (b) $B(\theta_i)$ as a function of $\sin 2\theta_i$, Eqn. 18d. (c) The determination of R_3 from Eqn. 18b. Note that if $R_3 = R_2$ the slope would be three times smaller than that obtained.
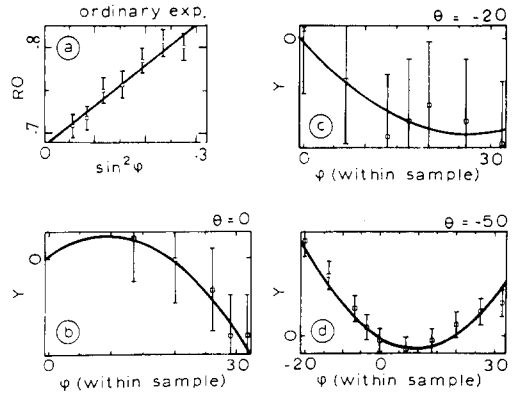

Fig. 5. Theoretical fits of AFD results for an egg PC/water 70:30 (w/w) bilayer system with $4 \cdot 10^{-3}$ M DPH at 25°C. ⊡: experimental values, relative accuracy 2%. Solid lines indicate least-squares fits according to Eqns. 1a and 18. (a) Ordinary experiment, (b), (c), (d) extraordinary experiments. Vertical scales $\theta = 0°$: 0.054; $\theta = -20°$: 0.045 and $\theta = -50°$: 0.27.

pendence. Furthermore, the results, Figs. 4 and 6, show unequivocally that $R_2 \neq R_3$, thus indicating that the emission moment of DPH is tilted relative to the molecular symmetry axis. A similar behaviour had been observed by us in systems of saturated lipids, with or without cholesterol [2,3].

The values of R_1 and R_2 obtained from the 'ordinary' and 'extraordinary' experiments were found to be in good agreement within experimental error.

(ii) $\langle P_2 \rangle$ and β_ν

The order parameters S_μ and S_ν of the absorption and emission moments, respectively, can be obtained directly from $R_1, \ldots, R_5$, as can the values of g_k, $k = 0, 1, 2$ [1–3]
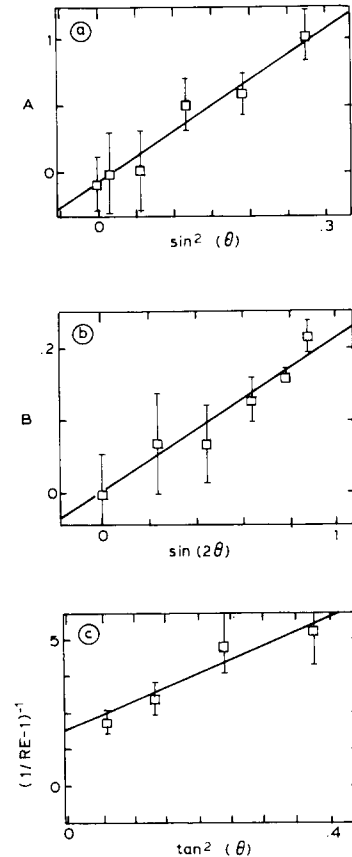
Fig. 6. AFD results for egg PC. (a) $A(\theta_i)$ obtained from extraordinary experiments, as a function of $\sin^2\theta_i$, Eqn. 18d. (b) $B(\theta_i)$ as a function of $\sin 2\theta_i$, Eqn. 18d. (c) The determination of R_3 from Eqn. 18b. Note that if $R_3 = R_2$, the slope of the line would be half that obtained.

TABLE I

EVALUATION OF $R_1 - R_5$ FOR ORIENTED LIPID MEMBRANE SYSTEMS

(A) Values obtained from a non-linear least-squares analysis of the experimental data. (B) Best fit values obtained from the rotational diffusion model. (C) Best fit values obtained from the 'wobbling-in-cone' model.

70:30 (w/w) lipid/water mixture with $4 \cdot 10^{-3}$ M DPH in		R_1	R_2	R_3	R_4	R_5
Soybean PC	A	0.111	−0.333	0.920	0.143	1.17
	B	0.12	−0.13	0.88	0.11	1.2
	C	0.12	0.04	0.77	0.26	0.68
DGDG	A	0.230	−0.339	0.451	0.306	1.81
	B	0.21	−0.19	0.43	0.23	1.9
	C	0.25	−0.03	0.28	0.47	1.3
DOPC	A	0.181	−0.441	0.382	0.154	1.72
	B	0.19	−0.25	0.36	0.12	1.8
	C	0.20	−0.07	0.24	0.33	0.94
Egg PC $\lambda_{em} = 432$ nm	A	0.165	0.138	1.43	0.202	3.46
	B	0.147	0.138	1.42	0.209	3.47
	C	0.141	0.221	1.52	0.502	1.41
$\lambda_{em} = 448$ nm	A	0.178	−0.0014	1.39	0.181	3.47
	B	0.161	−0.0014	1.38	0.186	3.47
	C	0.203	0.0016	0.44	0.408	1.10
$\lambda_{em} = 494$ nm	A	0.168	0.071	1.04	0.196	3.28
	B	0.164	0.071	1.04	0.197	3.28
	C	0.170	0.126	0.953	0.465	1.22

In all the systems studied here $S_\mu > S_\nu$, so that $\beta_\nu > 0°$ (see Eqn. 3), and thus the absorption and emission moments are not mutually parallel. This is in agreement with earlier results [2,3]. The values of $\langle P_2 \rangle = S_\mu$ are given in Table II.

Values for β_ν were obtained from S_ν and $\langle P_2 \rangle$, Eqn. 3. The angle of $\approx 36°$ calculated for DPH in egg PC is comparable to those found earlier [2,3] for DPH in bilayers of saturated lipids. In contrast, the low values of S_ν obtained for the other bilayer systems yield $\beta_\nu \approx 50°$. Such an angle, however, would yield much lower values for the g_k's, Eqn. 4, than are in fact observed. The calculations presented below indicate that an angle of $\approx 40°$ is necessary to describe the experimental results. This inconsistency could well arise if the assumption of effective molecular cylindrical sym-

TABLE II

THE SECOND AND FOURTH RANK ORIENTATIONAL ORDER PARAMETERS $\langle P_2 \rangle$ AND $\langle P_4 \rangle$, w_0 AND THE ROTATIONAL CORRELATION TIME τ_0 AS OBTAINED FROM THE STRONG COLLISION MODEL

70:30 (w/w) lipid/water mixture with $4 \cdot 10^{-3}$ M DPH in	$\langle P_2 \rangle$	$\langle P_4 \rangle$	w_0	τ_0 [a] (ns)
Soybean PC	0.32	0.24	> 0.95	≫ 10
DGDG	0.23	0.12	> 0.95	≫ 10
DOPC	0.26	0.31	> 0.95	≫ 10
Egg PC				
$\lambda_{em} = 432$ nm	0.45	0.39	0.56	13
$\lambda_{em} = 448$ nm	0.45	0.45	0.68	21
$\lambda_{em} = 494$ nm	0.40	0.36	0.53	11

[a] Here we have assumed $\tau_F = 10$ ns.

metry is no longer valid as a consequence of a lower rate of reorientation about the long axis of the molecule. A description of the experiment in terms of non-cylindrically symmetric molecules may thus be needed. This requires the introduction of extra order parameters and correlation times into the calculations [1,15], so that more than five model parameters must now be derived from the values of $R_1, \ldots, R_5$. In view of this we have not attempted such an analysis of the experimental data.

(iii) $\langle P_4 \rangle$ and reorientational dynamics

The information about $\langle P_4 \rangle$ and the reorientational motion of the molecules is contained in the three functions g_0, g_1 and g_2. As discussed above these have been interpreted on the basis of three models for the reorientational motion of the molecules.

The results obtained from the strong collision model, using Eqns. 2–9 are given in Table II. We note here again that in the theoretical derivation of Eqns. 1–5, β_ν was taken to be a molecular property independent of the orientational dynamics of the probes. It can be seen that the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are of the same magnitude for all the bilayer systems. However, significant differences are found in the values of w_0. This parameter must lie in the range $0 \leqslant w_0 \leqslant 1$. The values of w_0 for egg PC bilayers lie within this range and on taking $\tau_F = 10$ ns [16], yield rotational correlation times of 10–20 ns for the DPH molecules. These values, again, are similar to those obtained for

DPH in bilayers of saturated lipids [2,3].

On the other hand, the results for soybean PC, DGDG and DOPC show that $w_0 > 0.95$ and thus that $\tau_0 \gg \tau_F$. Consequently these systems are effectively frozen on the fluorescence time scale and the values of the correlation functions at $t = 0$, $G(kk000)$, determine the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$. Furthermore, we find that the experimental values of the functions g_0, g_1 and g_2 are only consistent with $\beta_\nu \approx 35$–$45°$, in contrast with the values of β_ν obtained from S_ν. These values of β_ν are comparable with those of [2,3] and with that of $\approx 36°$ obtained for egg phosphatidylcholine. The results again suggest that the assumption of effective cylindrical symmetry for the DPH molecules breaks down in the unsaturated systems.

We have also analysed the experimental results in terms of the rotational diffusion model with $D_\perp \tau_F$, λ_2, λ_4 and $P_2(\cos \beta_\nu)$ as free model parameters (see above). Eqns. 4 and 14 show that in this case products of the form

$$P_2(\cos \beta_\nu) \sum_M b_M^k / \left(1 + D_\perp \tau_F \alpha_M^k\right)$$

appear in the expressions for the functions g_k. The best-fit values for $R_1, \ldots, R_5$ are given in Table I under B and the values of the corresponding model parameters λ_2, λ_4, $D_\perp \tau_F$ and $P_2(\cos \beta_\nu)$ are shown in Table III. It can be seen from Table I that the fits for egg PC bilayers are in excellent agreement with the experimental values. The agreement for the other systems is less satisfactory and in partic-

TABLE III

EVALUATION OF THE PARAMETERS, λ_2, λ_4, $D_\perp \tau_F$ AND $P_2(\cos \beta_\nu)$ OBTAINED FOR THE ROTATIONAL DIFFUSION MODEL

70:30 (w/w) lipid/water mixture with $4 \cdot 10^{-3}$ M DPH in	λ_2	λ_4	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$D_\perp \tau_F$	$P_2(\cos \beta_\nu)$
Soybean PC	-0.57	-2.1	0.30	0.33	$\ll 1$	0.20
DGDG	-0.70	-1.4	0.26	0.23	$\ll 1$	0.39
DOPC	-0.20	-2.7	0.24	0.37	$\ll 1$	0.32
EGG PC						
$\lambda_{em} = 432$ nm	-1.4	-1.2	0.45	0.28	0.12	0.49
$\lambda_{em} = 448$ nm	-1.2	-1.7	0.45	0.34	0.08	0.44
$\lambda_{em} = 494$ nm	-1.2	-1.3	0.40	0.28	0.12	0.54

ular significantly different values are obtained for R_2. This is again a consequence of the low values of S_ν in these systems.

We have been unable to obtain satisfactory fits for any of the bilayer systems by setting $\lambda_4 = 0$ in the model calculations. The introduction of an extra term $P_6(\cos \beta)$ into the orienting potential, Eqn. 14, produced no significant improvement in the fits and the low values of $|\lambda_6| \leqslant 0.05$ indicated that this term is not in fact important. The discrepancies between the experimental and best fit values for $R_1, \ldots, R_5$ for bilayers of soybean PC, DOPC and DGDG may well be alleviated by the introduction of non-cylindrically symmetric terms into the orienting potential. As a check of the model calculations we note that the values of $\langle P_2 \rangle$ are in excellent agreement with those of S_μ for all the systems as are the values of the product $\langle P_2 \rangle P_2(\cos \beta_\nu)$ and S_ν for egg PC, Eqn. 3.

The calculations indicate further that the experimental results for the other bilayer systems can be well described if β_ν lies in the range $35^\circ - 45^\circ$. This is in good agreement with the values obtained from the strong collision model.

The order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ obtained from the rotational diffusion model indicate that significant though qualitative differences exist in the orientational statistics of the DPH molecules in the systems studied here. The same conclusion is also reached on the basis of the strong collision model. A comparison of Tables II and III shows further that similar values of $\langle P_4 \rangle$ are obtained from both models.

Although the DPH molecules appear to have similar static orientational properties in all the bilayer systems studied here, they exhibit marked differences in their reorientational dynamics. The calculations indicate that in bilayers of soybean PC, DOPC and DGDG the DPH molecules are effectively stationary on the time scale of the fluorescence decay as $D_\perp \tau_F \ll 1$. On the other hand, the DPH molecules undergo a fairly rapid reorientational diffusion with $D_\perp \sim (1-2) \cdot 10^7 \text{ s}^{-1}$ in egg PC bilayers. This result is in agreement with the conclusion drawn from the strong collision model.

The agreement in the results obtained from the Strong Collision and Rotational Diffusion models is gratifying and the reason can be found in the time dependence of the correlation functions $G(kk00t)$. Inspection of Eqns. 7 and 13 shows that since $w_0 > 0.95$ and $D_\perp \tau_F \ll 1$, for bilayers of soybean PC, DGDG and DOPC, the correlation functions do not appreciably decay on the time scale defined by the fluorescence life time of DPH. Thus, the values of the correlation functions at time $t = 0$ effectively determine the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$. As the correlation functions at $t = 0$ are strictly model independent [1-3,5-7], both models should then yield the same order parameters. The observed differences between the results obtained with the two models simply reflect the difference in the numerical procedures used to evaluate the model parameters. The calculations for egg PC bilayers using the Rotational Diffusion model show that the decay of each correlation function $G(kk00t)$, $k = 0, 1, 2$, is dominated by a single exponential component. This is in agreement with the theoretical considerations of Zannoni [5-7]. Thus, in this case, the differences between our two models are to be found only in the values of the correlation times assigned to the correlation functions. For the strong collision model we find $\tau_0 \approx 13$ ns, while the three correlation times yielded by the Rotational Diffusion model, Eqn. 13, are $1/(D_\perp \alpha^0) \approx 28$ ns, $1/(D_\perp \alpha^1) \approx 9$ ns and $1/(D_\perp \alpha^2) \approx 13$ ns. Here we have taken $\tau_F \approx 10$ ns. Thus, since both models yield correlation times of the same order of magnitude, they also yield similar values for the order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$.

We have also analysed our experiment with the 'wobbling-in-cone' model using the analytical expressions obtained by Lipari and Szabo [10]. Here we have taken $\cos \beta_0$, $D_w \tau_F$ and $P_2(\cos \beta_\nu)$ as free model parameters. The best-fit values for $R_1, \ldots, R_5$ are given in Table I under C and the values of the corresponding parameters are shown in Table IV. It can be seen from Table I that the fits are much less satisfactory than those obtained with the Rotational Diffusion model. In particular, no satisfactory fits could be obtained for egg PC bilayers with the 'wobbling-in-cone' model, in contrast to the other two models used here. We have also attempted to fit the experimental data directly with this model. However, the values for the model parameters obtained were the same as those from the fitting of the R's. Table IV shows further that the values of $\langle P_2 \rangle$, $\langle P_4 \rangle$ and $D_w \tau_F$ yielded by this

TABLE IV

EVALUATION OF THE PARAMETERS $\langle P_2 \rangle$, $\langle P_4 \rangle$, $D_w \tau_F$ AND $P_2(\cos \beta_r)$ OBTAINED FOR THE 'WOBBLING-IN-CONE' MODEL

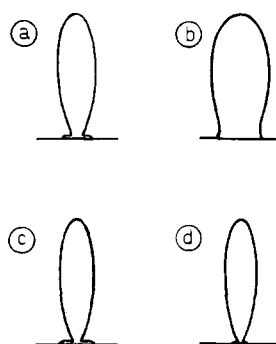| 70:30 (w/w) lipid/water mixture with $4 \cdot 10^{-3}$ M DPH in	β_0	$\langle P_2 \rangle$	$\langle P_4 \rangle$	$D_w \tau_F$	$P_2(\cos \beta_r)$
Soybean PC	69.2°	0.24	−0.13	≪1	0.23
DGDG	73.7°	0.18	−0.11	≪1	0.49
DOPC	76.8°	0.14	−0.09	≪1	0.38
Egg PC					
$\lambda_{em} = 432$ nm	59.7°	0.38	−0.12	≪1	0.33
$\lambda_{em} = 448$ nm	72.1°	0.20	−0.12	≪1	0.41
$\lambda_{em} = 494$ nm	65.7°	0.29	−0.13	≪1	0.37

Fig. 7. Orientational distribution functions $f(\beta)$ for DPH molecules in (a) soybean PC, (b) DGDG, (c) DOPC and (d) egg PC. The lipid to water ratio was 70:30 (w/w) for all samples.

model are inconsistent with the results obtained from the other models discussed above. The reason for the poor fits can be traced back to an unsatisfactory feature of the 'wobbling-in-cone' model. As can be seen from Fig. 2, the model constrains $\langle P_4 \rangle$ to take negative values in the range of $\langle P_2 \rangle$ involved here. Note that the value of $\langle P_2 \rangle$ is strictly model independent, Eqns. 3–5. In the lipid systems studied by us thus far $\langle P_4 \rangle$ has always been found to take on positive values. It is interesting that this model yields satisfactory fits in systems with a high degree of orientational order [2,3] where it predicts positive values for $\langle P_4 \rangle$. We are therefore, forced to conclude that the 'wobbling-in-cone' model is unsuitable for the analysis of the reorientational dynamics of DPH molecules in our bilayer systems.

(iv) Interpretation of the order parameters

The orientational ordering of the DPH molecules in lipid bilayers can be fully described in terms of an orientational distribution function $f(\beta)$ in which β is the angle between the bilayer and the molecular symmetry axes [1–3,5–7,15]. In its turn $f(\beta)$ is characterized by an infinite set of order parameters $\langle P_2 \rangle$, $\langle P_4 \rangle$, ...,$\langle P_L \rangle$, L even, which are the averages of Legendre polynomials of order L over the distribution function. Consequently, the interpretation of the order parameters will depend on the assumed functional form of $f(\beta)$. We have previously argued [1–3] that a useful estimate of $f(\beta)$ can be obtained if both $\langle P_2 \rangle$ and $\langle P_4 \rangle$ are

known and if $f(\beta)$ is reconstructed on the basis of an information-theoretic approach. The essential point here is that given $\langle P_2 \rangle$ and $\langle P_4 \rangle$ the most probable values of $\langle P_L \rangle$'s, $L \geqslant 6$, are calculated under the constraint that the informational entropy of $f(\beta)$ is a maximum. The resulting distribution function has the form [18]

$$f(\beta) = A \exp\{ \lambda_2 P_2(\cos \beta) + \lambda_4 P_4(\cos \beta) \} \quad (19)$$

where A is a normalization constant and λ_2 and λ_4 are determined from the known values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$. This distribution function corresponds to a Boltzmann distribution with the angle-dependent orienting potential of Eqn. 12.

Fig. 7 shows the distribution functions for soybean PC, DGDG, DOPC and egg PC reconstructed using Eqn. 19 with the values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ given in Table II. It can be seen that most of the DPH molecules are preferentially aligned perpendicular to the bilayer surface. Nevertheless, in soybean PC, DGDG and DOPC bilayers a small, but significant, fraction of the molecules lie parallel to the plane of the bilayers. Similar distribution functions for DPH have been obtained by us in systems of saturated lipids above the phase transition [2,3].

(v) Conclusions

The results presented above show that although the DPH molecules appear to have similar static orientational properties in all the bilayer systems studied here, they exhibit marked differences in

their reorientational dynamics. The introduction of unsaturation into the lipid chains seems to cause a slowing down in the dynamics of the DPH molecules. This conclusion is based on an analysis of the experimental data in which the DPH molecules are assumed to possess an effective cylindrical symmetry.

We have encountered internal inconsistencies in analysing the results for soybean PC, DOPC and DGDG bilayers on taking the DPH molecules to be cylindrically symmetric. On the other hand, this model can be successfully used in analyzing angle-resolved experiments on DPH embedded in bilayers of egg PC and saturated phosphatidylcholines with or without cholesterol [2,3]. Our experiments strongly suggest that DPH molecules in bilayers of unsaturated lipids lose their effective cylindrical symmetry as a result of a lower rate of reorientation around their long axes. The fluorescence depolarization experiment senses this mode of motion as the absorption and emission transition moments are not mutually parallel. This conclusion is based on the observation that $R_2 \neq R_3$, so that $S_\mu > S_\nu$ as discussed above. Unfortunately, a description of the data in terms of non-cylindrically symmetric molecules requires more model parameters than the five quantities yielded by our steady-state experiments.

Corroborative evidence for our conclusion can, however, be found in an earlier study of DPH molecules in vesicles of unsaturated lecithins using time-resolved techniques [17] This study revealed that the time-dependent fluorescence anisotropy, $r(t)$, decays appreciably on the time scale of the fluorescence life time τ_F. Yet the analysis of our experimental data in terms of cylindrically symmetric molecules leads to the opposite conclusion. The discrepancy can be resolved simply if the DPH molecules no longer possess and effective cylindrical symmetry. Furthermore the experimental results obtained from vesicle systems seem to be poorly described by a model based on the assumption of cylindrically symmetric DPH molecules. An examination of Fig. 1 of Ref. 17 reveals large and systematic discrepancies between the experimental and theoretical curves for $r(t)$; the differences are masked to some extent by the use of a logarithmic scale. Nevertheless we note that the parameters derived from our experiments re-

produce the values for the steady-state anisotropy r^s, Eqn. 5 of Ref. 17 and the cone angles quoted for the same systems.

Our suggestion that DPH molecules in unsaturated lipid bilayer systems cannot be treated as having effective cylindrical symmetry is contrary to the assumptions made in Ref. 17. We emphasize, however, that our angle-resolved experiments on macroscopically oriented samples are more discriminating than experiments on vesicles as they afford the direct determination of the time-averaged correlation functions g_0, g_1 and g_2 as well as the order parameters of the absorption and emission dipole moments. Such details cannot be obtained from experiments on macroscopically isotropic vesicle systems.

The results presented above are at odds with the generally accepted picture that unsaturation in the lipid chains causes the rates of reorientational motion to increase. However, studies in our laboratory of the motion of cholestane spin-label molecules incorporated into DGDG bilayers [19] are in agreement with the fluorescence depolarization experiments.

One conclusion to be drawn from our work is simply that DPH molecules are not suitable probes for monitoring bilayers of unsaturated lipids.

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