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THE USE OF *n*-(9-ANTHROYLOXY) FATTY ACIDS TO DETERMINE FLUIDITY AND POLARITY GRADIENTS IN PHOSPHOLIPID BILAYERS

KEITH R. THULBORN [a], LEANN M. TILLEY [a], WILLIAM H. SAWYER [a] and F. EDWARD TRELOAR [b]

[a] *Russell Grimwade School of Biochemistry, and* [b] *Department of Physical Chemistry, University of Melbourne, Parkville, Victoria, 3052 (Australia)*

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Summary

A set of *n*-(9-anthroyloxy) fatty acid probes (n = 2, 6, 9, 12) have been used to examine gradients in fluorescence polarization, lifetime (τ_F), relative quantum yield (ϕ_{rel}) and positions of emission maxima (λ_{max}) through bilayers composed of synthetic phospholipids. The fluorophores of these probes report the environment at a graded series of depths from the surface to the centre of the bilayer structure.

1. Polarizations decrease as the fluorophore is moved deeper into the bilayer indicating greater rotational motion of the fluorophore in the hydrocarbon core of the bilayer.

2. The different responses of the probe diphenylhexatriene and the anthroyloxy fatty acids to the action of cholesterol on lipid bilayers are discussed in terms of the orientation of these probes in the bilayer and the types of anisotropic rotational motions which result in depolarization of fluorescence.

3. Stearic acid derivatives which have the fluorophore in the 6-, 9- and 12 positions along the acyl chain have a similar response to solvent polarity as measured by values of λ_{max} and ϕ_{rel} in a variety of organic solvents.

4. The position of the emission maximum has little dependence on solvent viscosity, but viscosity does change the degree of vibrational structure seen in the emission spectrum. The vibrational structure itself may be used as an indication of the 'mciroviscosity' gradient in the transverse plane of the bilayer.

Abbreviations: 2-AP, 2-(9-anthroyloxy) palmitic acid; 6-, 9-, 12-AS, 6-, 9- and 12-(9-anthroyloxy) stearic acid.

5. Values of λ_{max}, τ_F and ϕ_{rel} indicate that a gradient of polarity exists from the surface to the centre of the bilayer. For dipalmitoyl phosphatidylcholine in the crystalline phase, cholesterol acts to make this polarity gradient shallower.

Introduction

Fluorescent probes have proved useful in monitoring structural changes and proximity relationships in biomembranes [1,2]. Although the choice of probe depends on the application involved, there are two universal requirements which must be fulfilled. Firstly, the location of the probe in the membrane must be known. When the fluorophore is covalently attached to a membrane component this position can be known with some certainty [3]. Secondly, the spectral characteristics of the probe must be fully documented in model systems so that data pertaining to natural membranes can be interpreted with confidence. We have recently described the properties of a set of *n*-(9-anthroyloxy) fatty acids (n = 2, 6, 9, 12) whose fluorophores locate at a graded series of positions in the transverse plane of the bilayer [4]. These probes are capable of sensing the 'fluidity' gradient through the bilayer leaflet [5]. The present study determines if these probes can accurately report changes in bilayer structure which result from the incorporation of cholesterol. In addition we explore the possibility that these probes can measure the gradient of polarity through the bilayer leaflet.

The effect of cholesterol on lipid bilayers has been extensively studied with respect to the specificity of the interaction and the structural changes which result (for review see Ref. 6). Several techniques have shown that cholesterol has opposite effects above and below the mesomorphic phase transition of liposomes composed of a pure phospholipid. NMR [7,8], ESR and Raman spectroscopy [9—13] measurements all indicate that cholesterol increases certain parameters which reflect the order of the liquid-crystalline phase and decreases these parameters for the crystalline phase. This interpretation has been extended to suggest that cholesterol increases the 'fluidity' of the crystalline phase [14]. While the above techniques measure the angular motion of the phospholipid chains, a more direct measure of membrane microviscosity is obtained by the use of fluorescent probes. Fluorescence polarization data for perylene [15] and for 12-(9-anthroyloxy) stearic acid [16] probes in dipalmitoyl phosphatidylcholine liposomes confirm that cholesterol increases the microviscosity of the fluid phase but suggest that the same is also true of the crystalline phase. We examine this anomaly with particular reference to the set of *n*-(9-anthroyloxy) fatty acid probes.

The availability of this set of probes also presents an opportunity of studying the polarity gradient through the bilayer. The separation of the absorption and emission bands of a fluorophore is sensitive to the polarity of the solvent environment. The position of the emission maximum (λ_{max}) has usually been used as an index of solvent polarity although fluorescence lifetime and quantum yield may sometimes be employed [1,17,18]. This paper examines three requirements which must be met for the successful application of the anthroyloxy fatty acids to the determination of polarity in lipid bilayers.

Firstly, the position of the fluorophore along the acyl chain must not, in itself, influence the position of the emission maximum and lead to intrinsic differences between probes. Secondly, the probes must be equally sensitive to solvent polarity. Thirdly, the influence of solvent viscosity in restricting solvent rearrangement around the fluorophore between the Franck-Condon and the equilibrium excited states must be evaluated.

Materials and Methods

Dipalmitoyl phosphatidylcholine (Koch-Light Laboratories) migrated as a single spot on thin layer chromatography in several solvent systems. Cholesterol (BDH Chemicals) was recrystallized three times from ethanol. Paraffin oils calibrated for viscosity over a range of temperatures were the gift of Shell Chemicals (Shell Ondina oil 15(17) and 68(33)). Ethanol and methanol were of spectroscopic grade and all other solvents were of the highest grade available. Diphenylhexatriene and *N*-phenylnaphthylamine were from Koch-Light Laboratories. The properties and synthesis of the fluorescent fatty acids (2-AP, 6-, 9-, and 12-AS), and the preparation of sonicated and unsonicated dispersions of phospholipid have been described previously [4]. Dispersions contained 500 μM phospholipid. Probes were applied in small volumes of methanol (10 μl) to give a final concentration of 3 μM. Electron microscopy showed that the sonicated dispersions consisted predominantly of single bilayer vesicles of approximately 30 nm diameter. Vesicle preparations were annealed above the phase transition temperature for at least 30 min prior to probe addition [19]. Dispersions were prepared in water and had a final pH of 6.85.

Fluorescence measurements were made with a Hitachi-Perkin Elmer MPF3 spectrofluorometer; emission spectra were uncorrected.

The dependence of polarization on temperature was measured with a continuously recording instrument constructed in our laboratory [4].

Relative quantum yields were measured by the comparative method of Parker and Rees [20] using quinine sulphate in 0.1 N H_2SO_4 as standard (ϕ = 0.5 at 20°C [21]); absorbances (<0.08) of the probes in liposome suspensions could not be accurately measured due to the scattering of light and the absorbance of the same concentration of probe in ethanol was used.

Fluorescence lifetimes (τ_F) were measured by the method of single photon counting using 363 nm vertically polarized exciting light. A 420 nm cut off filter and a polarizer set at 54.7° to the vertical plane were used in the emission beam to eliminate the effects of polarized emission on the lifetime [22]. The fluorescence decay was deconvoluted from the lamp impulse function as a single exponential using a non-linear least squares fitting program. The error in the lifetime is less than ± 0.5 ns in all cases. In some experiments there was evidence of a small contribution to the decay from a long lifetime component suggesting that the location of the anthroyloxy fluorophore in the bilayer may not be unique.

Fluorescence polarization (p) is defined in terms of the intensities of the vertical (I_V) and horizontal (I_H) components of the emission as

$$p = (I_V - I_H)/(I_V + I_H) \quad (1)$$

The isotropic rotation of a spherical fluorophore is described by the Perrin equation

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

where p_0 is the limiting polarization in the absence of rotational motion, and ρ is the rotational relaxation time related to the solvent viscosity (η) by the equation, $\rho = 3V\eta/RT$, where V is the molar volume. The application of these equations to the anisotropic motion of a fluorophore in a lipid bilayer has been questioned [23,24] although 'apparent microviscosities' and 'apparent rotational relaxation times' so calculated are useful for comparative purposes for an homologous set of probes containing a common fluorophore.

Results

Fluorescence polarization

Fig. 1 shows the temperature dependence of fluorescence polarization for several chemically distinct probes in dipalmitoyl phosphatidylcholine multilayered liposomes in the presence (45 mol%) and absence of cholesterol. In the absence of cholesterol, all three probes (12-AS, *N*-phenylnaphthylamine and 1,6-diphenyl-1,3,5-hexatriene) sensed the pretransition at 34—35°C and the main transition at 41—42°C. In the presence of cholesterol, the polarization of 12-AS and *N*-phenylnaphthylamine increased in the crystalline (gel) phase (<34°C) and in the liquid-crystalline (fluid) phase (>42°C). However, cholesterol increased the polarization of diphenylhexatriene in the fluid phase but decreased it in the gel phase.

To study this effect in greater detail we examined the effect of cholesterol on the polarization values of the four fatty acid probes in sonicated and vortexed dispersions of dipalmitoyl phosphatidylcholine at several tempera-

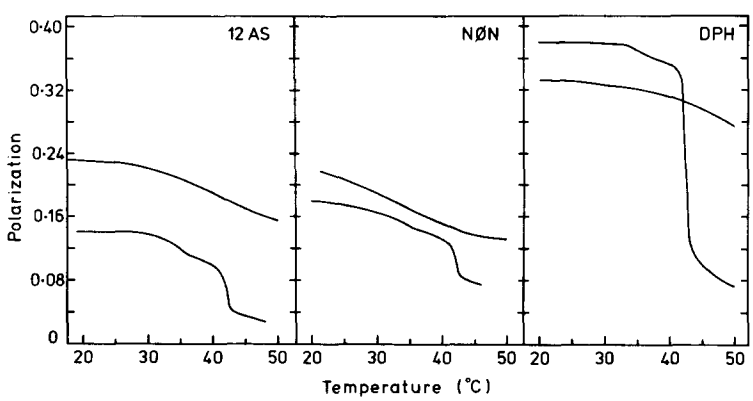

Fig. 1. The effect of cholesterol on the temperature dependence of polarization of vortexed dispersions of phosphatidylcholine as measured by different fluorescence probes. Left, 12-AS; centre, *N*-phenylnaphthalamine (NØN); right, diphenylhexatriene (DPH). The curves showing no sharp transitions are for liposomes containing 45 mol% cholesterol. In the absence of cholesterol all three probes register transitions at 36°C (pretransition) and 41°C (main transition).

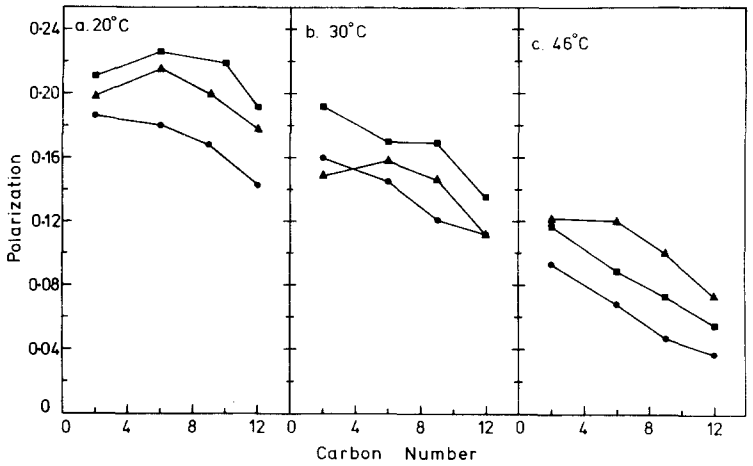

Fig. 2. Polarization gradients for sonicated dispersions of dipalmitoyl phosphatidylcholine. (1) 20°C; (b) 30°C; (c) 46°C. •, No cholesterol; ■, 20 mol% cholesterol; ▲, 50 mol% cholesterol. Excitation and emission wavelengths were 365 and 450 nm, respectively. Polarizations are measured to ±0.025.

tures. The polarization values for multilayered liposomes were always lower than for sonicated dispersions (vesicles) regardless of whether the lipid was in the crystalline or liquid-crystalline state. This relationship held true both in the presence and absence of cholesterol. However, cholesterol altered the shape of the polarization profile inducing a maximum near the C-6 position of the probe as shown for sonicated dispersions in Fig. 2. This maximum disappeared as the temperature was increased, higher cholesterol concentrations requiring higher temperatures to bring this about. In the crystalline phase the presence of cholesterol caused an increase in polarization followed by a decline at high cholesterol : phospholipid ratios. For example, polarizations at 45 mol% cholesterol were lower than for 20 mol% cholesterol (Figs. 2a and 2b). Separate experiments measuring polarization as a function of cholesterol concentration using 6-AS as the probe showed that the maximum polarization was attained at approx. 30 mol% cholesterol. Such behaviour was not observed above the phase transition temperature (Fig. 2c).

Changes in polarization may be caused either by a change in the excited-state lifetime of the fluorophore, or by a change in membrane microviscosity affecting the rotational relaxation time (Eqn. 2). Fluorescence lifetimes were therefore measured in the presence and absence of cholesterol to determine if polarizations were a true reflection of differences in microviscosity (Fig. 3). The changes in the fluorescence lifetimes on incorporation of cholesterol into the bilayer are relatively small and only make a small contribution to the observed changes in polarization. A 10% decrease in lifetime would cause a 7% increase in polarization for a fluorophore with a rotational relaxation time of 20 ns and $p_0 = 0.29$, whereas increases in polarization observed experimentally in the presence of cholesterol are as high as 30%. Thus, rotational relaxation times which are a more direct measure of membrane microviscosity follow similar trends to values of fluorescence polarization.

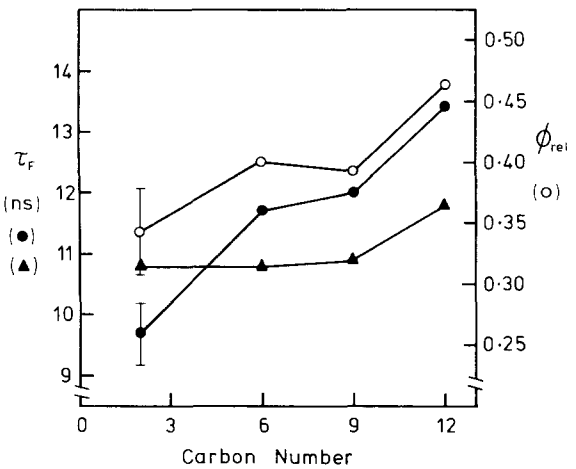

Fig. 3. The dependence of the fluorescence lifetime (τ_F) and relative quantum yield (ϕ) on the position of the anthroyloxy fluorophore in sonicated dispersions of dipalmitoyl phosphatidylcholine at 20° C. ○, ϕ_{rel} in absence of cholesterol, ●, τ_F in absence of cholesterol; ▲, τ_F in presence of 45 mol% cholesterol. Error bars refer to all ϕ or τ_F points.

Polarity parameters

Emission spectra in low viscosity organic solvents. The response of 2-AP and 12-AS to solvent polarity is shown in Fig. 4 where the quantum yield and a measure of the transition energy ($\upsilon_F = 1/\lambda_{max}$) are plotted against the empirical measure of solvent polarity devised by Kosower [25]. The most sensitive region of response is at relatively high Z-values (>76). Both probes show specific

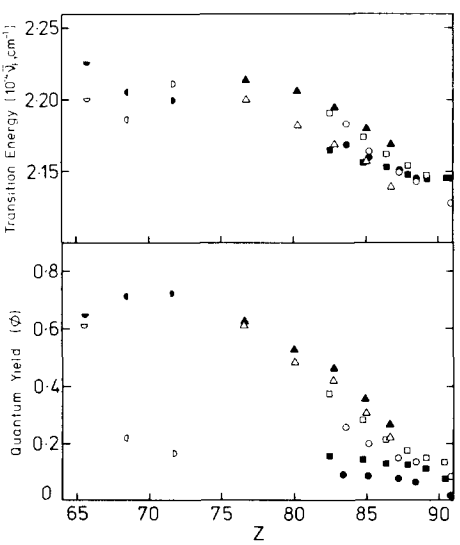

Fig. 4. The relative quantum yield (ϕ) and a measure of the transition energy ($\upsilon_F = 1/\lambda_{max}$) plotted against the Z parameter of Kosower [25] for 2-AP (open symbols) and 12-AS (filled symbols). ●,○, methanol/water; ■,□, ethanol/water; ▲,△, dioxane/water; ◗,◑, dimethylsulphoxide; ◖,◐, dimethylformimide; ◒,◓, acetone.

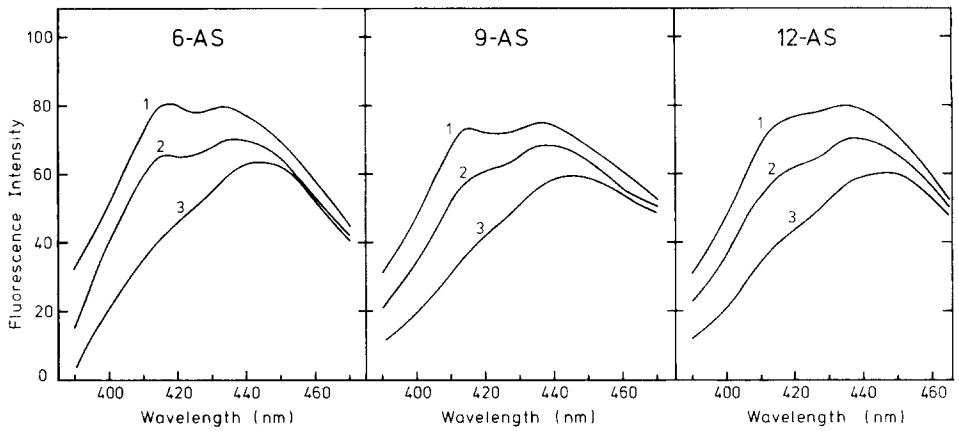

Fig. 5. The effect of temperature on the emission spectra of 6-AS, 9-AS and 12-AS in vortexed dispersions of dipalmitoyl phosphatidylcholine. (1) 25°C, (2) 35°C, (3) 45°C. Excitation was at 365 nm. Excitation and emission band passes were 8 nm and 3 nm respectively.

solvent effects but these tend to be more pronounced in the measurement of quantum yield. All fatty acid probes showed a single broad emission band in such low viscosity solvents although the emission in hexane did show some structure (see below). In a single solvent (e.g. methanol/water (70 : 100, v/v), dioxane/water (60 : 90, v/v), hexane, acetone), 6-, 9- and 12-AS had the same emission maximum indicating that the position of conjugation to the acyl chain had no effect on the λ_{max} of these probes. In the case of 2-AP small differences were observed as indicated in Fig. 4.

Emission spectra in high viscosity organic solvents. A comparison was made of the emission spectra of 9-AS in methanol, hexane and in two liquid paraffins of different viscosity. In contrast to the diffuse emission observed in methanol, vibronic structure was seen in hexane and in the paraffins, with two major contributions at 436 and 448 nm and a shoulder near 418 nm. The ratio but not the position of the major contributions depended on the solvent viscosity. In the liquid paraffin of high viscosity the contribution at 436 nm as well as the shoulder at 418 nm became more pronounced. This was confirmed by measuring spectra in each liquid paraffin over the temperature range —1—70°C covering a viscosity range 0.07 to 11.0 poise (1 P = 0.1 kg · m^{-1} · s^{-2}).

Emission spectra in lipid bilayers. Vibronic structure in the emission spectrum is even more apparent when the probes are incorporated into lipid bilayers. Fig. 5 shows the effect of temperature on the spectra of 6-AS, 9-AS and 12-AS in vortexed dispersions of dipalmitoyl phosphatidylcholine. Partially resolved bands occur at about 418 and 438 nm. The proportion of these two emission bands changes with increasing temperature and as the fluorophore is moved deeper into the bilayer. In both cases, there is an increase in the proportion of the 438 nm peak. Since solvent viscosity affects vibronic structure (see above), this behaviour is consistent with the decrease in membrane 'microviscosity' which occurs through the phase transition as well as with the decrease in 'microviscosity' which occurs from the surface to the centre

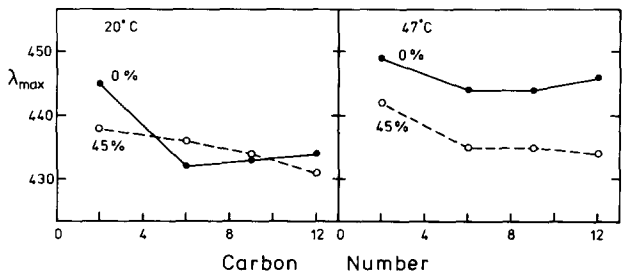

Fig. 6. Emission maxima of *n*-(9-anthroyloxy) fatty acids in vortexed dispersions of dipalmitoyl phosphatidylcholine. Left, crystalline phase, 20°C; right, liquid-crystalline phase, 47°C. Figures refer to the mol percent of cholesterol. Excitation was at 365 nm, and the excitation and emission band passes were 8 nm and 5 nm, respectively.

of the bilayer. Additional experiments indicated that the incorporation of cholesterol into a fluid bilayer also increased the proportion of the 418 nm band due to the decrease in fluidity.

We now direct attention to the position of the dominant band near 438 nm. The position of this band has been examined in vortexed dispersions of dipalmitoyl phosphatidylcholine both above and below the phase transition and in the absence and presence of 45 mol% cholesterol. The results are summarized in Fig. 6. We make the following points. (1) In the gel phase at 20°C there is a large blue shift in λ_{max} in going from the C-2 to the C-6 positioned fluorophore. This is followed by a small red shift at deeper levels in the membrane. The presence of cholesterol decreases the difference in λ_{max} between the C-2 and C-6 positions and evens out the gradient. (2) In the fluid phase at 47°C all probes show a red shift in λ_{max} compared to 20°C and the gradient in λ_{max} between C-2 and C-12 positions is shallow. In the fluid phase the presence of cholesterol causes a significant blue shift in λ_{max} at all levels in the membrane. The effect of the phase transition on λ_{max} is also evident in the spectra presented in Fig. 5.

Fluorescence lifetimes and quantum yields. The existence of a polarity gradient through the lipid bilayer should also be evident from measurements of two other polarity sensitive parameters of anthroic acid esters, namely fluorescence lifetimes and quantum yield [26]. Measurements were carried out on sonicated dispersions of dipalmitoyl phosphatidylcholine to minimize interference by scatttered light. The results in Fig. 3 show that in the gel phase at 20°C the fluorescence lifetime increases as the fluorophore is moved deeper into the bilayer. The presence of cholesterol makes the gradient shallower. Following the trend in lifetime, the relative quantum yield also increases from the C-2 to the C-12 position. The fluorescence lifetime gradient into the bilayer centre should be compared with published values of τ_F of 1.9 ± 1 ns in 50% ethanol/water and 1.7 ± 1 ns in methanol [26] through to 12.1 ns in hexane [4] for methyl-9-anthroate.

The fluorescence lifetime of 6-AS in the two liquid paraffins of viscosity 34 and 210 cP was 9.7 ± 0.5 and 10.9 ± 0.5 ns, respectively. The solvent viscosity seems to have little effect on the lifetime of the probe.

Discussion

There is considerable evidence indicating that the 'fluidity' of a membrane can be understood in terms of the rate and amplitude of the anisotropic motion of the phospholipid acyl chains. In using fluorescent probes to report such motion it must be recognized that the type of motion sensed by the probe is determined by its orientation in the lipid bilayer and by the direction of its emission transition moment. A nonspherical fluorophore will have several relaxation modes corresponding to the axes about which rotation occurs. Depolarization is due to those rotations which change the orientation of the emission transition moment. In the case of the anthroyloxy fatty acid probes excited at 365 nm the emission moment is in the plane of the anthracene ring and at 30° to the short molecular axis (Z axis) as depicted in Fig. 7 *. Thus the main depolarizing rotations are those about the X and Y axes. When the fluorescent fatty acid is incorporated into a lipid bilayer with the long (Y) axis of the anthracene ring parallel to the acyl chains [29], there will be little rotation about the X axis and the main depolarizing rotation about the Y axis will be induced by the motions of the acyl chains themselves. ^{13}C NMR studies indicate that there is little independent motion of the anthracene ring relative to the acyl chain to which it is attached [30].

In the case of diphenylhexatriene, the long axis of the molecule is preferentially but not perfectly aligned parallel to the phospholipid acyl chains [31,32]. The emission transition moment is parallel to the long axis of the probe [33] and is therefore normal to the bilayer surface. Thus the main depolarizing rotations sensed by diphenylhexatriene must occur about the X and Z axes (Fig. 7).

The different responses of 12-AS and diphenylhexatriene to the presence of cholesterol when the bilayer is in the gel phase (Fig. 1) cannot be explained by changes in fluorescence lifetime but may be due to a limitation in the motion of 12-AS about the Y axis and to an increase in the motion of diphenylhexatriene about the X and/or Z axes (Fig. 7). In addition, there are likely to be several depolarizing rotational modes available to any fluorophore and the number and type of these modes may be influenced by the presence of cholesterol. This latter possibility has been examined by Badley et al. [29] whose studies on oriented bilayers showed that cholesterol restricts the range of orientations assumed by the probe 2,2′-(vinylenedi-*p*-phenylene)-bisbenzoxazole. However, these authors report that the orientation of 12-AS in egg phosphatidylcholine, which is in a fluid state at room temperature, is little affected by the presence of cholesterol. They conclude that the effect of cholesterol on probe orientation varies widely amongst different probes.

The higher polarization values found for sonicated dispersions compared

* The value of 30° (σ) is determined using $\cos^2\sigma = (1 + 3p_0)/(3 - p_0)$ [27]. An alternative rationalization of the fact that p_0 is less than the theoretical value of 0.5 supposes that the emission and absorption moments are parallel but that fast torsional vibrations of the excited state reduce p_0 [28].

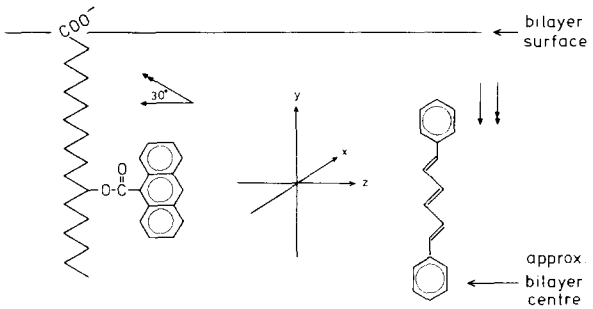

Fig. 7. The orientation of 12-AS (left) and diphenylhexatriene (right) in the lipid bilayer. The single and double-headed arrows indicate the directions of the absorption and emission transition moments respectively. The diagram is based on the work of Badley et al. [29] on 12-AS and of Andrich and Vanderkooi [31] and Cehelnik et al. [33] on diphenylhexatriene.

with unsonicated dispersions cannot be attributed to changes in lifetimes. The lifetimes for sonicated dispersions have been determined previously [34]. The result suggests that the depolarizing motion of the anthroyloxy probes is more restricted in vesicles than in multilayered liposomes. Results of NMR and of laser Raman experiments show that the acyl chains in vesicles are substantially less ordered than in multilayers [12,35,36]. The Raman experiments further indicate that the addition of cholesterol to multilayers below the phase transition increases the number of *gauche* conformers whereas little effect is seen for vesicles [12]. A simple explanation of the polarization data in terms of the motion of the phospholipid acyl chains is not tenable, and the anisotropic motion of the probes must be an important determinant of polarization. With respect to the fluorescent fatty acids, we are examining this phenomenon further by studying the kinetics of anisotropy decay. It is noteworthy that the effect of cholesterol in changing the rotational anisotropy of diphenylhexatriene has been described recently by Veatch and Stryer [37]. These authors find that the emission anisotropy curve may be fitted to the sum of two components (τ_1 = 6 ns, τ_2 = 50 ns); cholesterol increases the contribution of the slow component probably by restricting the angular range of rotational motion available to the diphenylhexatriene molecule in the bilayer. Recent experiments using differential polarized phase fluorometry have shown that even in the absence of cholesterol the motion of diphenylhexatriene is severely hindered by the anisotropic environment in the lipid bilayer [38].

Comparison of fluorescence polarization with other techniques as discussed above must also take into account the inherent differences in the time scales involved. ^{13}C NMR spin-lattice relaxation time measurements are primarily sensitive to the *trans-gauche* isomerizations about the carbon-carbon bonds due to the short time scale of measurement ($\approx 10^{-10}$—10^{-11} s), whereas ^{1}H and ^{2}H NMR order parameters have contributions from both chain isomerizations and reorientations ($\approx 10^{-7}$ s), the latter involving fluctuations in the tilt of the chains about the bilayer normal [36]. Fluorescence polarization data yield rotational relaxation times for the probes in bilayers in the 10^{-8} s range and therefore chain reorientation may be the dominant motion being measured.

Associated with these effects may be the problem of perturbation of the

lipid bilayer by the bulky fluorophore. Such perturbation has been observed at high probe/lipid ratios [39]. Although we have failed to detect any perturbation in terms of a decrease in the phase transition temperature of dipalmitoyl phosphatidylcholine at low probe/lipid ratios (1 : 150—1 : 500), perturbation is apparent when electrical properties of the bilayer are measured (Thulborn et al., unpublished work).

We now examine the use of the fluorescent fatty acids to measure the gradient of polarity through lipid bilayers. We wish to set this discussion in the context of the detailed studies of the fluorescence of anthroic acid esters made by Werner and his associates [26,40]. The anthroic acid esters have a broad structureless emission in organic solvents compared to the characteristic vibrational structure seen for anthracene. The diffuse emission of the esters is due to resonance interaction between the ester carboxyl group and the peri-hydrogens of the anthracene ring. This interaction arises on rotation of the carboxyl group from a position normal to the anthracene ring in the ground state to a position coplanar with the ring in the lowest excited singlet state. The carboxyl group can then engage in intramolecular hydrogen bonding with the peri-hydrogens resulting in the formation of two six-membered rings. A more polar excited state results from this charge transfer interaction and thus the excited state is more sensitive to solvent polarity than the ground state. If, however, the ester group is 'locked' into its ground state configuration by an increase in solvent viscosity, then the ground state vibronic levels typical of anthracene are retained.

The broad diffuse emission seen for the anthroyloxy fatty acids in low viscosity organic solvents suggests that in such solvents the excited state rotation can readily occur. Standardization of the response of λ_{max} and quantum yield to solvent polarity in terms of the Kosower Z parameter showed that there are no intrinsic differences in the behaviour of 6-, 9- and 12-AS originating from the position of the anthroyloxy group along the acyl chain. The small differences found for 2-AP (Fig. 4) are probably due to the influence of the fatty acid carboxyl group on the 9-anthroyloxy fluorescence.

In applying these probes to the measurement of polarity in lipid bilayers account must be taken of the influence of solvent viscosity since some fluorophores (e.g. *N*-phenylnaphthylamine, *N*-arylnaphthalenesulphonates) show a blue shift in λ_{max} with increasing solvent viscosity due to restriction of solute-solvent relaxation between the Franck-Condon and equilibrium excited states [41,42]. The spectra in liquid paraffins of similar dielectric constant indicate that for the anthroyloxy fatty acids λ_{max} is relatively insensitive to viscosity. However, the viscosity is sufficiently high that the beginnings of virbational structure are seen in the spectra and it is clear that the proportion of the two major vibrational contributions to the emission may change with viscosity. In lipid bilayers the vibrational structure is even more pronounced, the extent depending on the bilayer fluidity (Fig. 5). The vibrational structure decreases as the fluorophore is moved to more fluid regions deeper in the bilayer, and is enhanced by any process which decreases the fluidity of the bilayer, e.g. fluid → gel phase transition or the incorporation of cholesterol above the phase transition.

The presence of vibrational structure in the emission spectra of the probes in

bilayers creates difficulty in the use of λ_{max} as a polarity index since a change in the proportion of the emission peaks may itself cause apparent changes in peak positions. Nevertheless, the considerable red shift seen through the phase transition is in agreement with the model proposed by Träuble [43] whereby the formation of 2 gl kinks in the phospholipid acyl chains creates pockets large enough to accommodate water molecules and to facilitate the transport of water across the bilayer. Reduction in the number of *gauche* isomers either by decreasing the temperature or by the incorporation of cholesterol reduces the number of pockets of free volume available. The blue shift found on the addition of cholesterol to the fluid phase (Fig. 6) is in accord with this interpretation.

Below the phase transition the interpretation is clearer since the λ_{max} data are supported by the results of fluorescence lifetime and quantum yield measurements. Values of λ_{max}, τ_F and ϕ_{rel} all indicate that a gradient of polarity exists through the bilayer although the shape is somewhat different in each case. Moreover, the data for λ_{max} and τ_F show that cholesterol acts to even out this gradient. There are differences in the precise mechanism by which solvent polarity affects λ_{max}, τ_F and ϕ_{rel}. For example, such differences probably result in ϕ_{rel} being more sensitive to solvent effects than λ_{max} (Fig. 4). Thus, the shape of the apparent polarity gradient through the lipid bilayer will depend on the particular polarity sensitive parameter chosen for the measurement. For the anthroyloxy fatty acids, τ_F and ϕ_{rel} are the parameters of choice since the appearance of vibrational structure in the emission spectrum makes the use of λ_{max} difficult.

Finally, the interpretations provided here with respect to the cholesterol effects assume that the presence of cholesterol does not markedly change the position of the fluorophores in the bilayer. This assumption was checked by determining the accessibility of the fluorophores in multilayers of dipalmitoyl phosphatidylcholine to a water soluble quencher namely, Cu(II) [4]. The relative quenching efficiencies, and therefore the relative positions, were in the expected order (2-AP > 6-AS > 9-AS > 12-AS) although differences in the absolute magnitude of the quenching efficiencies compared to cholesterol-free multilayers were noted [44].

Acknowledgements

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