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FLUORESCENCE LIFETIME AND QUENCHING STUDIES ON SOME INTERESTING DIPHENYLHEXATRIENE MEMBRANE PROBES

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The fluorescence lifetimes of a number of membrane probes based on the 1,6-diphenylhexatriene (DPH) chromophore have been measured in small unilamellar phospholipid vesicles and found to be multiphasic. These probes were quenched by sodium iodide with different efficiencies in vesicles and this has been attributed to the depth of the particular probe in the bilayer. The distribution of the probe between the outer and inner monolayer has been determined for those probes with fixed positions in the bilayer. The iodide ion permeability of the bilayer was found to be immeasurably small over a 3 h period.

Introduction

Although the receptor and transport activities of membrane-bound proteins are associated primarily with their protein structures, these functions can be effectively modulated by both lipid-protein interactions and by the properties of the bilayer matrix itself. In view of the increasing importance of obtaining a detailed picture of the molecular architecture of membrane systems in attempts to elucidate the mechanisms of membrane transport [1], molecular mechanisms of anaesthesia [2], etc. many physical methods have been employed as 'structural probes' (see, for example, Ref. 3). In this context 1,6-diphenyl-1,3,5-hexatriene (DPH) has been used as a fluorescent probe for both intact membranes and liposomes (used as synthetic model systems) [4–6].

In the current literature there is still some debate as to the detailed photophysics of the decay processes observed. For example, is the decay single exponential or does it obey a multiexponential law [7,8]? Despite these facts DPH and DPH derivatives have provided very powerful aids to the study of membrane systems [9]. In this paper we have attempted to characterise (via fluorescence and sodium iodide quenching studies) the properties of DPH probes which occupy very specific positions in bilayers of small unilamellar vesicles. The specific probes we have used are: 1,6-diphenylhexa-1,3,5-triene; 1(1-(trimethylamino)phenyl)-6-phenyl)hexa-1,3,5-triene; 1-palmitoyl-2-DPH-propanoylphosphatidylcholine; 1-DPH-propanoyl-2,3-dipalmitoylglycerol; and DPH-propanoyl cholesterol ester.

The iodide ion permeability of small unilamellar vesicles is also investigated.

Materials and Methods

L-α-Phosphatidylcholine, type V-E (PC) and L-α-dipalmitoylphosphatidylcholine (DPPC) were obtained from Sigma (London). Sodium iodide, tris(hydroxymethyl)methylamine (Tris), sodium thiosulphate, sodium chloride, and sodium perchlorate were all Analar grade and from BDH Chemicals Ltd., U.K. All other chemicals were

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analytical grade. All solvents used were spectroscopic grade except for ethanol which was AR grade and showed no detectable fluorescence at the wavelengths employed in this work.

1,6-Diphenylhexa-1,3,5-triene (DPH, Fig. 1 I) was obtained from Koch-Light Laboratories Ltd. (U.K.) and was scintillation grade. 1(4-(Trimethylamino)phenyl)-6-phenylhexa-1,3,5-triene (TMA-DPH, Fig. 1 II) was prepared as previously reported [11]. The synthesis of 1-palmitoyl-2-DPH-propanoylphosphatidylcholine (DPH-PC, Fig. 1 III), 1-DPH-propanoyl-2,3-dipalmitoylglycerol (DPH-DP, Fig. 1 IV), and DPH-propanoyl cholesterol ester (DPH-CH, Fig. 1 V) are described elsewhere [10,11].

Small unilamellar vesicles (SUV) were prepared by two methods: (i) By the method of Barenholz et al. [12], in which, phospholipids and probe were suspended in buffer and sonicated with a 150 W sonicator (M.S.E., U.K.) followed by centrifugation at 100 000 × g for 30 min and then at 159 000 × g for 3 h. The suspension of vesicles was then diluted to 0.3 mg/ml. (ii) By the ethanol injection technique [13] in which an ethanolic solution of phospholipids (approx. 20 mg/ml) plus probe was injected into buffer using a microlitre syringe whilst the buffer was vigorously mixed with a vortex mixer. EPC vesicles were then incubated at room temperature and DPPC were incubated at the phase transition temperature, 42°C (being prepared at 55°C), both for 0.75 h. The final concentration of phospholipid was 0.16 mg/ml and the ethanol was 1% v/v with buffer.

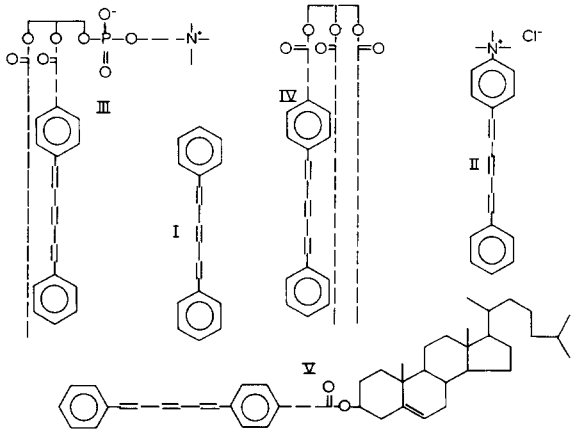

Fig. 1. Structures of DPH and analogues: I, DPH; II, TMA-DPH; III, DPH-PC; IV, DPH-DP; V, DPH-CH.

The buffer used in all cases was Tris/HCl (10 mM at pH 8.2) plus NaCl to a concentration of 0.1 mol/l. Sonication was not used to prepare vesicles containing DPH-DP, and DPH-PC, or DPH-CH to avoid possible degradation of the probe molecules. The molar ratio of probe to phospholipid was 1 in 500 for DPH, TMA-DPH and DPH-PC; 1 in 750 for DPH-DP; and 1 in 1000 for DPH-CH, to avoid possible phase separation of the probe molecules.

Vesicle suspensions containing sodium iodide were made up in the following manner: A stock suspension of vesicles in buffer (containing 10^{-3} mol/l sodium thiosulphate, to avoid the formation of I_2 or I_3^-) was added to graduated flasks containing the sodium iodide. It was found to be essential that all glassware and optical cells were coated with a silating agent prior to use to avoid the deposition of vesicles thereon. In control experiments, vesicle suspensions were made up with sodium chloride and sodium perchlorate instead of sodium iodide so that any effects on the fluorescence intensity or fluorescence lifetime due to the high ionic strengths used could be measured.

All absorption measurements were performed on a Perkin-Elmer Lambda 5 Spectrophotometer at 25°C. Fluorescence spectra were recorded on a Schoeffel RRS 1000 spectrofluorimeter coupled to an Apple II microcomputer which facilitated spectral correction for emission response and determined the areas of the emission spectral envelopes. The latter were used to compare relative quantum yields in fluorescence quenching experiments. All DPH samples were illuminated using narrow slits and for the shortest time possible so that decreases in fluorescence intensity due to the formation of 'dark' isomers of DPH [14] were avoided. A correction was also made to the fluorescence to account for the dilution of the probe/vesicle suspension at high concentrations of salt. In this case, the quantity of vesicle suspension required to make the salt solution up to 5 ml is measured accurately. From this the concentration of probe can be determined and thus a correction made. All fluorescence measurements were performed at 25°C unless otherwise stated.

In all iodide quenching experiments, solutions and vesicle suspensions were free of any yellow

colouration which would indicate the formation of I^- or I_3^-. No fluorescence was observed when TMA-DPH was ethanol injected into buffer alone.

Single photon counting measurements were performed on an Edinburgh Instruments 199 Fluorescence Spectrometer using a hyperbaric nitrogen filled flash lamp, which gave an instrumental response profile with a full width at half maximum height of approx. 1.5 ns. A polariser orientated at the magic angle (54.6°) was placed between the sample and the emission monochromator to avoid the interference of time dependent anisotropies in the lifetime data. Computer programs used were those supplied with the instrument and fitted mono- and biexponential decay kinetics to the lifetime data. 'Goodness of fit' was judged by reduced χ^2 and residual analysis of a non-linear least-squares fitting routine.

Results

Absorption and corrected fluorescence spectra of DPH and derivatives are shown in Fig. 2. The absorption spectra were obtained free of interfering scattered light by using a suspension of vesicles, without probe, of the same concentration in the reference beam of the spectrophotometer. All derivatives show small red shifts in absorption and fluorescence when compared with DPH.

Typical values for the fluorescence lifetimes of the DPH probes are listed in Table I. It should be noted that DPH in deoxygenated hexane shows a reasonable single exponential fit ($\tau = 16.8$ ns). All measurements made on probe/vesicle systems gave poor fits with the single exponential decay function and rather better fits with the biexponential decay function. There was a small variation of lifetimes obtained from preparation to preparation and we will suggest the reason for this later. The fluorescence quenching of the TMA-DPH emission in vesicles with sodium iodide is shown in Fig. 3. The area of each emission envelope was computed and these were taken to be proportional to the relative quantum yields. The areas were used to calculate the Stern-Volmer quenching constant, K_{SV}, by plotting F_0/F against C from the equation [15]:

$$F_0/F = K_{SV}C + 1$$

where F_0 is the unquenched emission; F is the quenched emission; and C is the concentration of quencher. The Stern-Volmer plots for the iodide ion quenching of DPH, TMA-DPH, DPH-DP, and DPH-CH in vesicles are shown in Fig. 4.

Fig. 5 shows the changes in the fluorescence decay profiles of the DPH probes in vesicles with increasing concentration of iodide ion. These again were best fitted with a biexponential decay function. The weighted mean lifetime [16] was calculated from the lifetimes and the pre-exponentials values and this value was used in the Stern-Volmer plots (Fig. 5). The gradients, $K_{SV\tau}$ and $K_{SV\Phi}$, for TMA-DPH, DPH, DPH-DP, and DPH-CH probes in vesicles are collected in Table II. The changes in the two component lifetimes and the proportion of the pre-exponential of the first lifetime from the double exponential analysis of the probe in vesicles, alone and in the presence of 3.78 mol/l sodium iodide are shown in Table III.

Electron micrographs of the vesicle suspensions showed a diminution of the internal volume of individual vesicles as well as aggregation at high ionic strength (> 1 mol/l). This must add further complexity to any model based on simple Stern-Volmer style kinetics (for example in micelles, see Ref. 17) and so the values of K_{SV} are used only for purpose of comparison. Sodium chloride and sodium perchlorate up to a con-

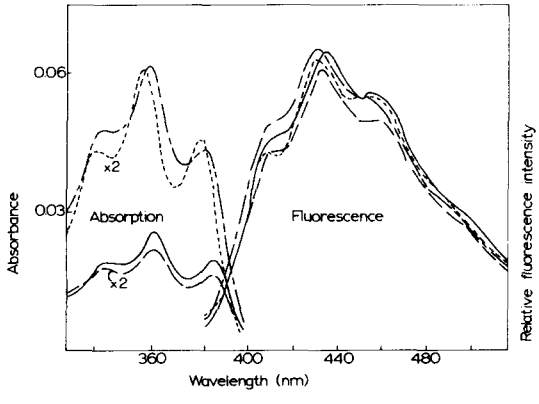

Fig. 2. Absorption and corrected fluorescence emission spectra of DPH and some derivatives in small unilamellar type V-E PC vesicles: ------, DPH; - — - — -, TMA-DPH; ———, DPH-CH; and — — —, DPH-DP. Fluorescence spectra are normalised.

TABLE I

FLUORESCENCE LIFETIME DATA FOR VARIOUS DPH PROBES IN TYPE V-E PC AND DPPC UNILAMELLAR VESICLES

Probe	Lipid	Method of vesicle formation	Single exponential lifetime τ	Reduced χ^2	Double exponential lifetimes τ	Reduced χ^2	Weighted mean lifetime $\langle\tau\rangle$
DPH	PC	Ethanol injection	8.4±0.02	1.94	4.2 ±0.5 9.0 ±0.04	1.19	8.4±0.2
DPH	PC	Sonicated	8.74±0.03	2.1	4.8 ±0.6 9.4 ±0.06	1.07	8.7±0.3
DPH-PC	PC	Ethanol injection	6.4±0.07	5.4	2.1 ±0.2 8.05±0.07	1.3	6.5±0.2
DPH-PC	DPPC	Ethanol injection	6.8±0.06	2.7	3.0 ±0.3 7.6 ±0.07	1.15	6.4±0.2
TMA-DPH	PC	Ethanol injection	3.9±0.03	3.87	1.9 ±0.2 4.4 ±0.03	1.09	3.9±0.1
TMA-DPH	DPPC	Ethanol injection	8.6±0.03	2.9	4.2 ±0.5 9.5 ±0.05	1.3	8.7±0.2
TMA-DPH	PC	Sonicated	3.9±0.02	2.8	1.2 ±0.2 4.2 ±0.02	1.31	3.7±0.1
DPH-DP	PC	Ethanol injection	7.3±0.02	1.91	5.2 ±0.45 8.2 ±0.1	1.3	7.3±0.3
DPH-CH	PC	Ethanol injection	6.4±0.02	2.0	4.8 ±0.3 7.6 ±0.1	1.2	6.2±0.3
DPH	Hexane		16.8±0.02	1.15			

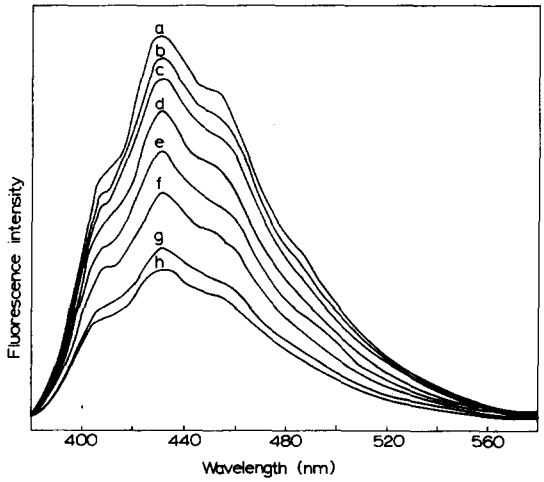

Fig. 3. Uncorrected fluorescence spectra of TMA-DPH in small unilamellar type V-E PC vesicles, a; and with 0.12 M NaI, b; 0.28 M NaI, c; 0.725 M NaI, d; 1.375 M NaI, e; 1.8 M NaI, f; 2.61 M NaI, g; 3.78 M NaI, h.

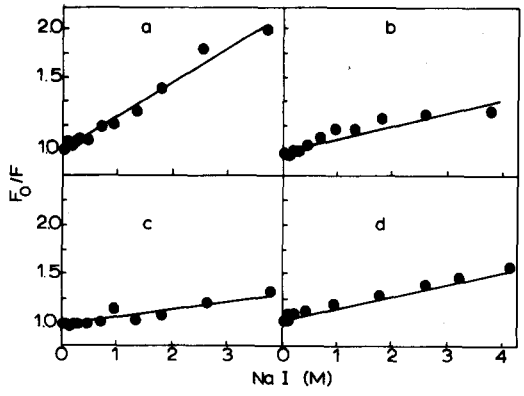

Fig. 4. Stern-Volmer plots, from integrated fluorescence intensity data of the quenching in small type V-E PC vesicles of a, TMA-DPH; b, DPH-DP; c, DPH-CH; and d, DPH.

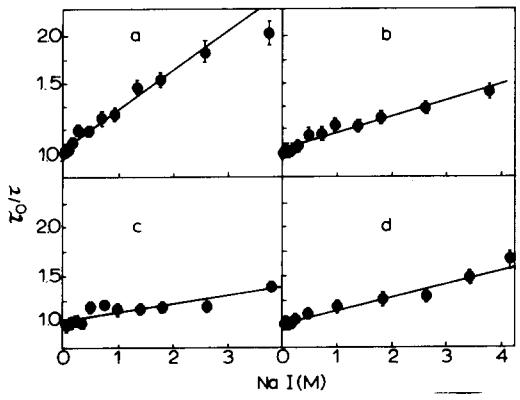

Fig. 5. Stern-Volmer plots, from fluorescence lifetime data of the quenching of TMA-DPH, a; DPH-DP, b; DPH-CH, c; and DPH, d; in small unilamellar type V-E PC vesicles with NaI.

TABLE II

SLOPES OF THE STERN-VOLMER PLOTS OBTAINED FROM FIGURES 4 AND 5 USING SMALL PC VESICLES

Probe	Method of formation	$K_{SV\Phi}$ ($l \cdot mol^{-1}$)	$K_{SV\langle\tau\rangle}$ ($l \cdot mol^{-1}$)
DPH	Sonicated	0.19	0.165
DPH	Ethanol injection	0.13	0.14
TMA-DPH	Sonicated	-	0.39
TMA-DPH	Ethanol injection	0.37	0.4
DPH-DP	Ethanol injection	0.14	0.17
DPH-CH	Ethanol injection	0.08	0.088

centration of 4 mol/l in place of sodium iodide had only a small effect on the fluorescence intensity and lifetime of probe-vesicle suspensions (both increased by approx. 5% at 4 mol/l). It was important to monitor the absorption whilst undertaking the measurement of fluorescence emission, as aggregation at high salt concentrations can sometimes lead to an increase in the scattering power of the suspension, thus decreasing the true absorption. This effect was reduced by vortex mixing the vesicles at regular intervals during equilibration. Even so, changes in absorption between vesicle suspensions must introduce errors in fluorescence measurements.

Table IV shows the difference between the quenching of the TMA-DPH and DPH-DP probes when the buffer contained iodide before ethanol injection and the quenching when the iodide was added after vesicle formation. In the former preparation both the inner and outer monolayers were exposed to iodide ions. The ionic strengths were equivalent at each stage in these preparations using sodium chloride in place of sodium iodide for the controls. It was not appropriate to take comparative fluorescence intensity measurements on these preparations, because of their different absorbances and refractive indices. Although, the buffer trapped inside the vesicle is hypotonic (on the addition of salt to the suspension) to the buffer outside the vesicle, electronmicrographs show intact vesicles with a slightly diminished trapped volume. This illustrates the semi-permeable nature of the bilayer [18]. The addition of sodium chlo-

TABLE III

COMPARISON OF THE BIEXPONENTIALLY FITTED LIFETIME DATA OF DPH PROBES IN PC VESICLES WITH AND WITHOUT SODIUM IODIDE

Probe	NaI (mol/l)	τ_1 (ns)	τ_2 (ns)	Ratio of the prexponential term, B_1, to $(B_1 + B_2)$ [a]
TMA-DPH	0	1.5+0.16	4.2 +0.03	0.45
TMA-DPH	3.78	1.0+0.1	2.5 +0.04	0.7
DPH-DP	0	4.9+0.4	8.5 +0.1	0.48
DPH-DP	3.78	1.8+0.14	5.7 +0.03	0.44
DPH	0	4.3+0.5	9.0 +0.04	0.23
DPH	4.1	1.5+0.2	5.3 +0.03	0.36
DPH-CH	0	3.5+0.2	7.5 +0.06	0.52
DPH-CH	3.78	1.9+0.1	5.45+0.04	0.59

[a] From the biexponential decay equation: $F(I) = A + B_1{}^*\text{Exp}(-I/\tau_1) + B_2{}^*\text{Exp}(-I/\tau_2)$.

TABLE IV

EFFECT OF THE QUENCHING OF TMA-DPH AND DPH-DP PROBES IN SMALL UNILAMELLAR VESICLES PREPARED UNDER VARIOUS CONDITIONS

n.a., measurement not attempted.

Probe	Lipid	Conditions of formation of the vesicles	Percentage decrease in lifetime	Percentage decrease in fluorescence
DPH-DP	PC	Iodide added to 0.75 mol/l after formation	7	n.a.
EPH-DP	PC	Formed in the presence of 0.75 mol/l iodide	12	n.a.
TMA-DPH	PC	Iodide added to 0.5 mol/l after formation	27	n.a.
TMA-DPH	PC	Formed in the presence of 0.5 mol/l iodide	27	n.a.
TMA-DPH	PC	Probe added after vesicle formation then iodide added to 3.78 mol/l	56	48
TMA-DPH	PC	Probe added to lipid before formation then iodide added to 3.78 mol/l	56	50.6
TMA-DPH	DPPC	Vesicles at 53°C in the presence of 3.78 mol/l iodide	n.a.	56
TMA-DPH	DPPC	Vesicles at 20°C in the presence of 3.78 mol/l iodide	n.a.	52.6

ride to a concentration of 1.5 mol/l to vesicles containing the TMA-DPH or the DPH-DP probes did not significantly change their fluorescence lifetime.

Vesicle preparations, in which an attempt was made to label only the outside monolayer of the bilayer with TMA-DPH probe, by adding the probe after the vesicles had been formed and equilibrated, were no more efficiently quenched with added iodide ion than preparations in which both probe and phospholipid were ethanol injected together (thus allow probe to distribute between both monolayers) (Table IV). The weighted mean lifetime of this preparation was 3.1 ns; this value was lower than when probe and phospholipid were injected together (Table I). When vesicles were made up in 1 mol/l sodium iodide by ethanol injecting phospholipids alone, and this preparation then dialysed against 1 mol/l sodium chloride for 3 h (thus exposing the inner monolayer to iodide ions and the outer monolayer to chloride ions), TMA-DPH added; there was no change in fluorescence after 3 h. This shows that there is little or no diffusion of the TMA-DPH probe from the outer monolayer to the inner monolayer over this time scale.

Experiments at temperatures above and below the main phase transition temperature of DPPC (i.e., 52°C and 22°C) showed only minor differences in quenching of the TMA-DPH probe in the presence of 3.78 mol/l sodium iodide.

Discussion

The absorption and fluorescence spectra of DPH and derivatives (in PC small unilamellar vesicles, Fig. 2) are typical of spectra reported elsewhere [19]. The derivatives show small red shifts in absorption and fluorescence relative to DPH. Generally, these spectra show a characteristic diminution of emission fine structure compared with their spectra in solvent systems [20] due to the vibronic coupling of the excited probe with the matrix of the bilayer.

Iodide ion is a very efficient quencher of excited state species (by a spin-orbit coupling mechanism), highly soluble in aqueous environments, and much less soluble in the apolar environment of the bilayer. Also, iodide has a low effectiveness (Schulze-Hardy rule and Hofmeister series) in causing flocculation of phospholipid vesicles, because of its small hydrated radius. The efficiency of quenching is used here to assess the depth of each particular probe in the bilayer. In all cases

quenching is inefficient requiring molar concentrations of iodide ion to effectively decrease the fluorescence yield and lifetime, implying that few ions penetrate beneath the surface of the bilayer. The TMA-DPH probe proved to be the most highly quenched and the DPH-CH probe the least (Figs. 4 and 5), indicating that the position of the TMA-DPH probe is closer to the surface, as expected because of its polar nature. Whilst we stress the semi-empirical nature of the Stern-Volmer plots at such high concentrations of salt, and we are aware of the complexities of quenching processes in the small unilamallar vesicles, reasonable approximations to Stern-Volmer type kinetics are indicated. The least-squares gradients of these plots, the Stern-Volmer quenching constant (K_{SV}), are listed for each probe, for comparison, in Table II. The lifetime data for these quenching experiments show reasonable agreement with the fluorescence emission data.

Inspection of the Stern-Volmer constants (Table II) enables clear distinctions to be made between the positions of the DPH chromophore of the various probes. Since it is known from the work of Engel and Prendergast [21] that DPH molecules occupy the region near the centre of the bilayer, which is highly disordered, we can assume that the probe DPH-DP occupies a similar position in the bilayer. In contrast to this similarity, it is obvious that the probes TMA-DPH and DPH-CH are in very different environments. It should be noted that unlike the TMA-DPH probe DPH is not 'anchored' in the bilayer. Although the uncharged DPH-DP resembles a triacylglycerol (a two carbon spacer separating the chromophore from the glycerol moiety) there must remain some doubt as to its exact location. In view of the 750 : 1 phospholipid to probe ratios used in this work we would reasonably expect no phase separation to occur and the DPH-DP sited in a 'normal' membrane configuration. For DPH-CH, the extremely small values (approx. 0.08) obtained for the quenching constants are consistent with the probe being situated at the centre of the bilayer.

In all of the DPH probes used in this study, analysis of the fluorescence decay data best fitted the double exponential law. Two possible explanations for the multiexponential decay of DPH in phospholipid vesicles have been offered by Chen et al. [7]. Firstly, that the DPH probe lies in two or more environments as a result of the constraints of molecular packing on the probe and due to the radius of curvature of small vesicles [22], and secondly, that the proposed biexponentiality arises in some unspecified way, from excited state conformational changes of DPH [14]. The latter explanation seems unlikely as using our single photon counting apparatus DPH in hexane fits well to the single exponential decay law (Table I). There are other examples of biphasic fluorescence decays in phospholipid vesicles, for example, thulborn and Beddard [8] have reported probable biexponential decays for *N*-(9-anthroyloxy) fatty acid probes in vesicles, where *trans-cis* isomerism of the probe molecule is unlikely to occur.

There is a dramatic increase in the fluorescence lifetime of the TMA-DPH probe in the gel phase of unilamellar vesicles compared with the lifetime of the probe incorporated in vesicles which are in the liquid-crystalline state (e.g. TMA-DPH in the liquid-crystalline PC vesicles and gel phase DPPC vesicles, Table I). Differences in the molecular packing around the probe appear to be responsible for this. We can only speculate on the photophysical reasons for such a difference in lifetime. It appears that the lifetime 'shortening' effect of the trimethylamino group on the DPH moiety is eliminated in the more ordered gel phase. There is no dramatic difference in the iodide quenching of this probe when it is incorporated into DPPC vesicles in the gel phase and the liquid-crystalline phase (Table IV) and so we predict little difference in the position of the probe. Probe molecules which are incorporated in the inner monolayer would be more highly constrained than those in the outer layer, yielding a biexponential fluorescence decay. We would expect the shorter lifetime to originate from the outer monolayer, and this lifetime to be more highly quenched by iodide ions. When iodide is added (to a concentration of 3.78 mol/l) to DPH-DP in EPC vesicles there is a 63% decrease in the shorter lifetime τ_1 and only a 33% decrease in the longer lifetime τ_2 (Table III). (At this concentration of salt, electron micrographs show that the vesicles have virtually collapsed and have no internal volume, so that quenching of the probe situated in the inner monolayer can only occur if iodide ions diffuse in

through the outer monolayer and this is unlikely to occur.) From quenching experiments on DPH-DP in EPC vesicles (Table IV), 60% of the probe is distributed in the outer monolayer and 40% in the inner monolayer. This distribution had not altered after 5 h. There is virtually no quenching of the DPH-CH probe in a similar experiment, showing the bilayers impermeability to iodide ions. DPH and the DPH-CH probe also have short component lifetimes which are more efficiently quenched by iodide ions, showing that τ_1 in these cases is associated, to a large part, with the probe in the outer monolayer.

Although the distribution of probe between the inner and outer monolayer may contribute largely in the above cases to non-monoexponential decays, it appears from Table IV that most of the TMA-DPH probe is distributed in the outer monolayer of small unilamellar vesicles (probably because the benzene ring cannot intrude into the densely packed inner surface headgroup region of the small unilamellar vesicles). In this case τ_2, the long component of the biexponential fit is more highly quenched with iodide than τ_1 (Table III) (the proportion of short component also increases significantly on quenching). The longer lifetime fluorescence is mostly associated with probe molecules which are positioned close to the surface of the outer monolayer of the vesicle, whilst the shorter lifetime is associated with probe positioned normal to the surface of the membrane lying alongside the fatty acid chains, their long axis directed towards the middle of the bilayer. Probe molecules with the longer fluorescence lifetime will be constrained by the interaction of the probe with the first few ordered CH_2 groups of the alkyl chains. Differences in the proportion of probe taking-up these different positions will account for the differences in lifetime between individual methods of preparation. The membrane, having a dynamic character, will allow the unbound end of the TMA-DPH molecule to move in and out of the more ordered regions of the liquid-crystalline phase bilayer.

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