CH_2=CHC_6H_4Me-*m*), 113509-11-8; **2** (olefin = CH_2=CHC_6H_4Cl-*m*), 113509-13-0; **2a**, 93612-05-6; **2b**, 79730-87-3; **2c**, 113509-07-2; **2d**, 95099-28-8; **2e**, 93712-13-1; **2f**, 95340-41-3; **3** (X = *m*-Me), 113509-14-1; **3** (X = *m*-Cl), 113509-15-2; **4** (X = *o*-Me), 113533-04-3; **4** (X = *o*-Cl), 113509-16-3; **6**, 113509-17-4; **7**, 31941-73-8; **8**, 113509-18-5; **9**, 113509-19-6; $Pt(C_4H_7)(Cl)(PPh_3)$, 35770-10-6.

Supplementary Material Available: ^1H NMR data for new complexes (Table S1), bond lengths (Table S2), bond angles (Table S3), equations of least-squares planes (Table S4), final atomic positional parameters (Table S5), anisotropic temperature factors (Table S6), and atomic parameters (Table S7) (29 pages); listing of observed and calculated structure factors (Table S8) (73 pages). Ordering information is given on any current masthead page.

Host–Guest Interactions: A Fluorescence Investigation of the Solubilization of Diphenylpolyene Solute Molecules in Lipid Bilayers

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Abstract: The photophysics of the chromophores 1,4-diphenyl-1,3-butadiene (DPB), 1,6-diphenyl-1,3,5-hexatriene (DPH), and their corresponding 4,4′-dialkyl-substituted derivative molecules, 4B4A and 4H4A, show large concentration effects in the ordered "gel" or "crystalline" phase below the phase transition temperature, T_c, of phospholipid vesicles. The phosphatidylcholine probe DPHpPC shows a similar dependence of its fluorescence intensity on concentration. The solubility of guest-impurity molecules in lipid bilayers is discussed in terms of possible conformational distortion of the chromophores and phase separation of solute within the bilayer, creating local "defect" sites in which the morphology of the bilayer is changed. Measurements of the steady-state anisotropy of these molecules do *not* reflect unusual solute/lipid interactions. Large limiting values of 0.28–0.30 were obtained for the anisotropy of both DPH and 4H4A at temperatures below T_c. At high temperatures above T_c, DPH experiences nearly isotropic rotation (r = 0.06) while that of 4H4A continues to be hindered (r = 0.14–0.16) in DPPC and DSPC vesicles. Fluorescence depolarization studies of DPB and 4B4A indicate that the fluidity of bilayer interiors decreases with vesicles formed from phospholipids of increasing chain length in the series DMPC, DPPC, and DSPC.

From a photophysical standpoint, *trans,trans,trans*-1,6-diphenyl-1,3,5-hexatriene (DPH) is scarcely the prototype polyene. Yet, DPH fluorescence is intense, relatively long-lived, and sensitive to its environment. At the same time, DPH is assumed to be relatively compatible with membranes due to its nonpolar hydrocarbon structure. These features and the long rodlike structure of DPH, similar to fatty acids and to the visual pigment *trans*-retinal, have prompted its wide use as a probe of membrane structure and dynamics. The fluorescence quantum yield, lifetime, and depolarization of the DPH chromophore are often suggested as parameters that may be measured and interpreted in terms of the physical characteristics of lipid bilayers.[1-6] However, there remains a controversy as to the specific details of its photophysics as well as the precise location of DPH in bilayers.[7-12] Critical to the use of DPH as a sampler of the bilayer microenvironment is confidence that it is solubilized in the hydrocarbon interior without causing significant perturbation.

This paper presents results obtained from steady-state absorption and fluorescence studies of DPH and 1,4-diphenyl-1,3-butadiene (DPB) and their surfactant derivatives 4H4A and 4B4A, respectively (see Chart I), when incorporated into phospholipid vesicles. In addition to these compounds we have also investigated the behavior of 2-(3-(diphenylhexatrienyl)propanoyl)-3-palmitoyl-L-α-phosphatidylcholine (DPHpPC), a DPH-containing phosphatidylcholine. We find that the fluorescence properties of these diphenylpolyene molecules in synthetic membranes are quite complex and show sensitivity to the relative concentrations of solute and lipd as well as to the phase of the bilayer. Although emission from these chromophores can be interpreted in terms of what is known about their solution photophysics, our results emphasize the dynamic nature of solute/lipid interactions and the caution with which any "probe" study must be approached.

To better understand the behavior of polyene chromophores, it is useful to review the salient features of the photophysics of the shortest member of the diphenylpolyene series, *trans*-stilbene (TS). It has been established that the excited singlet state behavior of TS is dominated by two processes.[13] Fluorescence decay from the first excited singlet state (of B_u symmetry) competes effectively with the activated twisting of the molecule into a perpendicular geometry (see Figure 1). This perpendicular excited state decays to an energy maximum on the ground-state potential surface from which either the cis or trans isomer is produced. There is a small energy barrier of 3.5 kcal/mol for forming the twisted "p" excited state.[14] This energy barrier is attributed to an avoided crossing of the ^1B_u* surface with a second excited ^1A_g* state as the molecule rotates out of its planar conformation. The height of the barrier to photoisomerization is a function of both the solvent polarity

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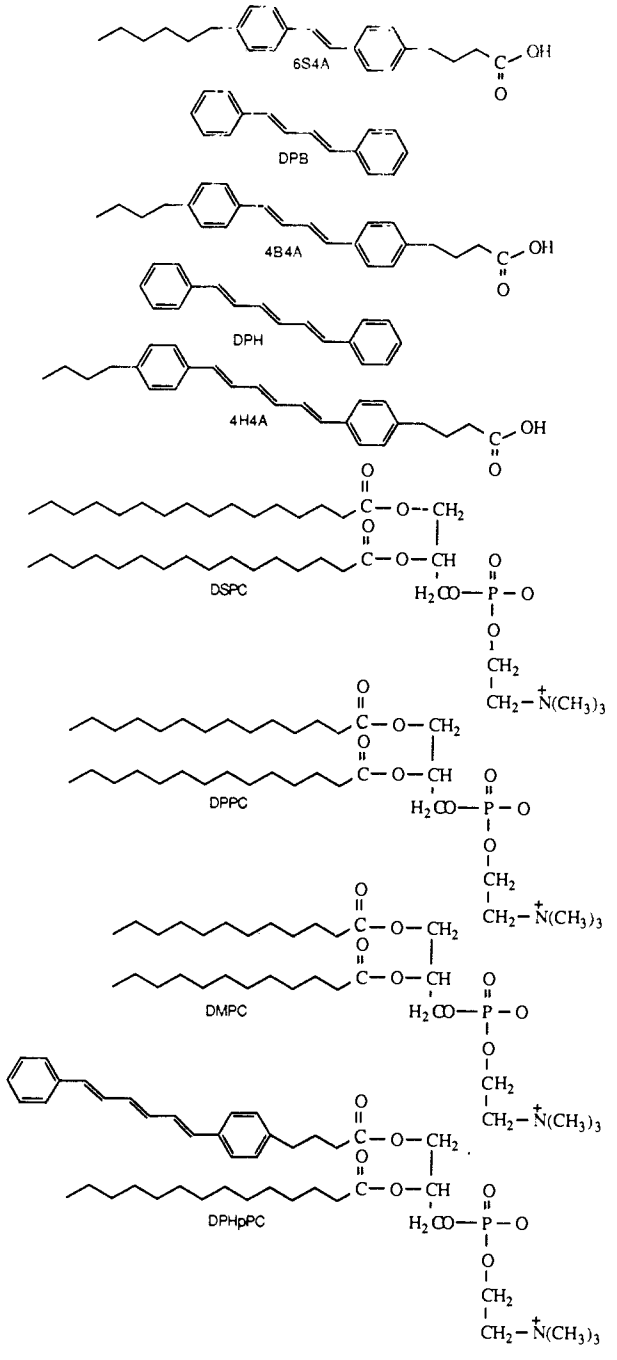
Chart I. Structures of Solute Molecules and Phosphatidylcholine Lipids Used in This Study



and viscosity.[15] This causes the rate of twisting in the excited state to be enhanced by increasing either the temperature or the solvent polarity and by decreasing the viscosity. The fluorescence quantum yield (0.05 in cyclohexane (CH) at 25 °C) has been found to increase almost to unity with a concomitant decrease in trans to cis isomerization efficiency upon incorporation into a rigid glass.[13] The corresponding fluorescence lifetime ranges from 60 ps in an isotropic hydrocarbon solvent to 1.7 ns in a rigid environment. This enhancement of fluorescence at the expense of photoisomerization provides a useful tool for probing the microviscosity of surfactant assemblies.[16]

In comparison with the behavior of *trans*-stilbene, that of DPH as a chromophore in homogeneous solution is seemingly anomalous.[17-20] Mirror image symmetry is not maintained between the

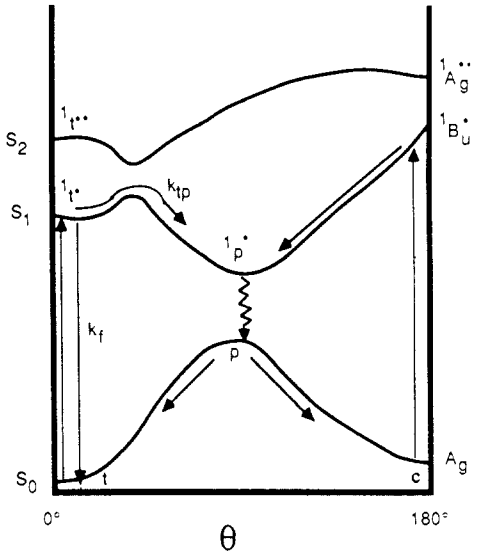


Figure 1. Potential energy surfaces of stilbene as a function of the angle of rotation about the C–C double bond.

absorption and fluorescence spectra of DPH. The absorption spectrum is slightly sensitive to solvent polarity while the fluorescence spectrum is not. However, both the fluorescence quantum yield and lifetime decrease with increasing polarity. The fluorescence lifetime is also significantly longer than that calculated from integrated absorption intensities: 12.4 ns as opposed to 1.56 ns. A collection of studies of substituent effects, picosecond spectroscopy, and theoretical calculations has culminated in an interpretation of these properties of DPH that is generally accepted.[21-28]

The intense absorption can be accounted for in terms of excitation from the ground state of DPH (^1A_g symmetry) to a second excited state of ^1B_u symmetry (an allowed $S_2 \leftarrow S_0$ transition). Rapid internal conversion from $^1B_u^*$ (S_2) to a lower energy $^1A_g^*$ (S_1) state is followed by relatively slow fluorescence due to the small transition moment of the symmetry-forbidden $S_1 \rightarrow S_0$ transition. This $^1A_g^* \rightarrow {}^1A_g$ transition derives oscillator strength through intensity borrowing from the $^1B_u^*$ state. As with *trans*-stilbene, there is for DPH an activated twisting of the molecule in the lowest excited singlet state that competes with fluorescence and is viscosity dependent.[29] However, for DPH the energy gap between the two lowest excited-state surfaces and thus their vibronic coupling is a function of both the solvent polarity and its polarizability. This may be due to both the relative stabilization of the $^1B_u^*$ state by polarizable solvents and of the twisted form of the $^1A_g^*$ state by polar solvents. Cehelnik et al. observed that in the polar solvents ethanol and acetonitrile there is evidence for temperature-dependent solvent-induced radiationless transitions that compete with fluorescence.[20] Furthermore, fluorescence studies of DPH may be complicated by $S_2 \rightarrow S_0$ transitions. Short-lived emission directly from S_2 has been observed as a contribution to the blue edge of the DPH fluorescence spectrum.[25,26] Increases in the solvent refractive index induced

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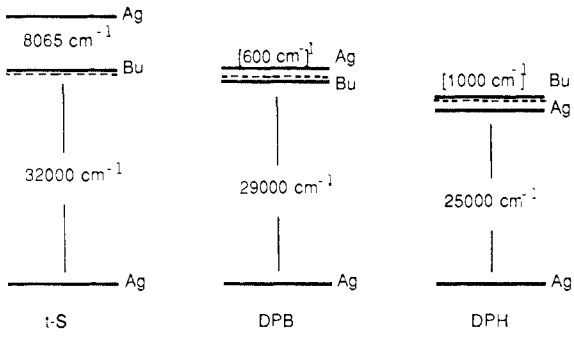

Figure 2. Relative energies of the first two excited singlet states of three *trans*-diphenylpolyenes. Approximate position of the $^1B_u^*$ state of the surfactant derivative (---).

by the use of moderately high hydrostatic pressures have demonstrated that emission from the $^1B_u^*$ state is dependent upon solvent polarizability.[27,28] Interestingly, Brey and co-workers have found that the DPH excited singlet nonradiative rate, k_{nr}, appears to be affected by increasing the pressure very differently than by increasing the viscosity through a lowering of temperature.[28] Both *trans*-stilbene and DPH show decreased nonradiative rates upon lowering the temperature. However, for the same increase in viscosity imposed by 10 kbar, the stilbene k_{nr} decreased by a factor of 4 while there was a factor of 10 increase observed for DPH. It appears that high pressures may open a new radiationless pathway in DPH. This may be attributable to an enhancement of Φ_{isc} under these conditions. Also, on the basis of solvent studies of the fluorescence quantum yields and lifetimes of DPH and several DPH derivatives over a range of temperatures, Alford and Palmer have recently proposed that delayed fluoresence due to thermal repopulation of S_2 from S_1 proceeds at a rate comparable to that of the radiative and nonradiative decay from S_1.[30] Such fluorescence would interfere not only with emission from S_1 but also with estimates of the energy gap between the two lowest excited state surfaces, S_2 and S_1.

Critical to the understanding of the discrepancies between the photophysical properties of the diphenylpolyene chromophores is the reversal in the relative energies of the first two excited states ($^1A_g^*$ and $^1B_u^*$) on going from TS to DPH. Naturally, the nature of the excited state of the intermediate case, 1,4-diphenyl-1,3-butadiene (DPB), is of particular interest. Although the actual ordering of $^1A_g^*$ and $^1B_u^*$, the two lowest excited states of DPB, has not been adequately resolved, Fleming and co-workers have observed that its photophysical properties are more like those of *trans*-stilbene than DPH.[31,32] As is the case for stilbene, the radiative rate of DPB is relatively insensitive to solvent. The emitting state must, therefore, not be significantly different in character from the absorbing state. Their studies show that the $^1A_g^* - {}^1B_u^*$ energy gap is quite small, perhaps separated by only a few hundred wavenumbers. This results in pronounced solvent effects both on the rate of nonradiative decay and on the coupling between the two states. A value of 4.7 ± 0.5 kcal/mol is calculated for the internal barrier to isomerization. This is less than that observed in solution. The barrier height is found to decrease in more polar solvents and with decreasing solvent viscosity. The relative energies of the states that dominate the photophysics of the first three *trans*-diphenylpolyenes are depicted in Figure 2.

Despite the potential for complex emission, DPH is considered a useful probe for studying the models of biological membranes, synthetic bilayers. Phosphatidylcholines are zwitterionic lipids that constitute approximately 40% of most natural membranes. The bilayers that may be formed by dispersal of PC lipids in an aqueous medium have characteristic properties such as size and melting temperature (T_c) that are determined by the length of their alkyl chains. For example, vesicles formed from the lipids dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC) undergo melting at 25, 41, and 55 °C, respectively. At this temperature the phase of the bilayer changes from one of relatively high rigidity and order (the low-temperature "gel" phase) to one in which the alkyl chains have more freedom to twist and rotate (the high-temperature "fluid" or "liquid crystalline" state). The vesicle phase transition is accompanied by increased permeability of the bilayer as well as increased flexibility and lateral diffusion of the lipids. In addition to the obvious physiological interest in the properties of vesicles is their ability to solubilize guest molecules and therefore potential to act as a transporting or reaction medium. A topic of related interest is the specific properties imposed by the structure of the vesicles formed. Sonication of the lipid/aqueous solution produces a mixture of small unilamellar vesicles (SUV's) and larger multilamellar vesicles (MLV's) that contain many layers of bilayers separated from one another by a layer of water. These two types of structures may be separated by size on a gel filtration column. Large unilamellar vesicles (LUV's) can be formed by methods such as ether fusion, reverse phase evaporation, or calcium-induced fusion. The difference in curvature of the bilayer surface between the various types of vesicle structures has been shown to lead to significant differences in their physical properties.[33,34]

Fluorescence lifetimes, quenching, and energy-transfer studies of both natural membranes and synthetic bilayers using DPH have been used to obtain information about vesicle structure and about the density and location of molecules solubilized within them.[35-37] Most commonly, diphenylhexatriene itself has been used as a probe of the order of bilayer interiors in fluorescence depolarization studies.[38-41] Recently, however, surfactant derivatives of DPH have become commerically available and are being employed as membrane probes with increasing frequency.[42-47]

The competition between fluorescence and photoisomerization of diphenylpolyene chromophores can be exploited to probe the interiors of lipid bilayers or vesicles. Providing one is certain of the location of the solute, information about the microviscosity and order of synthetic membranes can be gained as well as an understanding of the solubilization process itself. Using surfactant derivatives of *trans*-stilbene, Suddaby et al. were able to make a distinction between "order" and "viscosity" within phospholipid vesicles.[16] In their experiments, *trans*-stilbene was covalently bound to fatty acids of varying lengths. These were attached via the para position to the acyl chain terminus or within the chain, forming an "intrachain" surfactant stilbene. This should provide for the specific placement of the chromophore at various locations within the bilayer interior. An important result from that work is the finding that the nonsurfactant parent molecule, *trans*-stilbene, locates primarily at the vesicle interface rather than in the bilayer interior as one might expect for a hydrophobic molecule. However, the limitations imposed by the short lifetime of the stilbene probes and their short-wavelength excitation have led us to try similar experiments using the longer polyenes DPB and

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Table I. Spectroscopic Characteristics of Diphenylpolyene Solute Molecules in Hydrocarbon Solvents

compd	λ_{abs}^{max}	λ_{fluor}^{max}	Φ_f	τ_f, ns	solvent
DPB	330	375	0.44[a]	0.6	CH
4B4A	318	365	0.3	0.6	CH
DPH	358	455	0.78[b]	13.1[c]	CH
4H4A	366	455	0.89	8.7[c]	MCH

[a] Taken from ref 17. [b] Taken from: Berlman, I. B. *Handbook of Fluorescence Spectra of Aromatic Molecules*; Academic: New York, 1965. [c] Argon degassed.

DPH and some surfactant probes derived from them. Our results address the concern over whether DPH and DPB can be confidently assumed to reside within the interior of the bilayer as well as the nature of fluorescence from diphenylpolyenes in membrane environments.

Experimental Section

Materials. DL-α-Dimyristoylphosphatidylcholine (DMPC), DL-α-dipalmitoylphosphatidylcholine (DPPC), and DL-α-distearoylphosphatidylcholine (DSPC) were purchased from Sigma and used as received. Sodium dodecyl sulfate (SDS, BioRad, electrophoresis grade) and dihydrocholesterol (3-β-cholestanol, Aldrich) were recrystallized from ethanol. Phosphate buffer was prepared from the Aldrich Gold Label grade reagents Na_2HPO_4 (0.64 mM), KH_2PO_4 (0.14 mM), NaCl (13.7 mM), and KCl (0.26 mM) and Millipore-filtered water.

Chromophores. 1,6-Diphenyl-1,3,5-hexatriene (DPH, Aldrich) was recrystallized twice from acetone. 1,4-Diphenyl-1,3-butadiene (DPB, Riedel-de Haen) was recrystallized three times from benzene/alcohol. 4H4A and 4B4A were prepared as reported previously.[48] 2-(3-Diphenylhexatrienyl)propanoyl)-3-palmitoyl-L-α-phosphatidylcholine (DPHpPC) was purchased from Molecular Probes and used as received.

Vesicle Solutions. The appropriate volume of a chloroform (Baker, spectrophotometric grade) stock solution of solute was added to a flask. The solvent was then evaporated under a nitrogen stream. To this a weighed quantity of lipid was added. Buffer (10.0 mL) was added and the resulting Suspension sonicated with a Heat Systems Model 220F sonication microtip at a power setting of 4 for 40 min at temperatures 20 °C above the vesicle phase transition temperature, T_c. The vesicle solutions were then allowed to cool to room temperature and centrifuged for 20 min at medium speed to remove unsuspended surfactant and titanium particles. The concentrations of the vesicle solutions were 2×10^{-6} to 1×10^{-5} M in solute and 2×10^{-4} to 2×10^{-3} M in lipid, yielding solute:lipid ratios from 1:50 to 1:1000.

Fluorescence Quantum Yields. A reference solution with a known quantum yield was prepared with the same optical density as the solute for each solution studied. The references DPB/CH and DPH/CH at 25 °C have quantum yields of 0.44 and 0.78, respectively. The integrated intensity of the emission band was compared for the solute-containing solution to that of the corresponding reference over the same wavelength region with constant excitation wavelength.

Anisotropy. Fluorescence depolarization measurements were made by using a polarization filter after the excitation and before the emission monochromator. A polarization ratio, *Pr*, was determined by taking measurements of the fluorescence intensity with the polarizing filters placed in all combinations of the horizontal (H) and vertical (V) orientations; $Pr=(VV/VH) \times (HH/HV)$. From this, the polarization, *P*, and anisotropy, *r*, can be calculated according to the relations $P = (Pr - 1)/(Pr + 1)$ and $r = 2P/(3 - P)$.

Instrumentation. Ultraviolet spectra were recorded on either a Hewlett-Packard 8450A or an IBM 9430 UV-visible spectrophotometer. Fluorescence spectra were recorded on a SPEX 111CM spectrofluorimeter. Fluorescence lifetime measurements were obtained by a single-photon-counting method using a PRA fluorescence lifetime instrument, interfaced to a PDP 11/23 microcomputer. Temperatures were controlled by a Haake A81 temperature bath and monitored by a thermistor probe, accurate to ±0.2 °C. The hydrodynamic diameters of vesicles were determined by right-angle light scattering measurements using a Malvern particle sizer equipped with a 632.8-nm He–Ne laser.

Results

The structures of the DPB and DPH are compared in Chart I to those of their surfactant derivatives, denoted 4B4A and 4H4A. Also shown is the commerically available probe molecule DPHpPC in which the DPH chromophore is covalently attached to one of the alkyl chains of a phosphatidylcholine lipid molecule. To give an indication of relative lengths, the structures of the surfactants used in this study are also shown. Thus, 4H4A should be slightly shorter than a DSPC lipid while of comparable length to a DPPC molecule. Table I summarizes the absorption and emission maxima of each of the solute molecules used in this study in homogeneous hydrocarbon (cyclohexane (CH) or methylcyclohexane (MCH)) solutions. As noted previously for DPH, both the absorption and fluorescence spectra of each probe are relatively insensitive to changes in solvent polarity, shifting a few nanometers at most. For example, the DPH absorption maximum occurs at 351 nm in ethanol and at 358 nm in cyclohexane, a shift of 557 cm^{-1}. The fluorescence quantum yields and lifetimes obtained in hydrocarbon solvents are also listed. The fluorescence decay obtained by deconvolution of the data in MCH and in CH is fit well by a single exponential for each probe. No temperature- or concentration-dependent aggregation was observed by either absorption spectral changes or unusual lifetime components. As would be expected, the lifetimes of DPH and 4H4A are considerably longer than those of the DPB probes due to the forbidden nature of the $S_1 \rightarrow S_0$ transition.

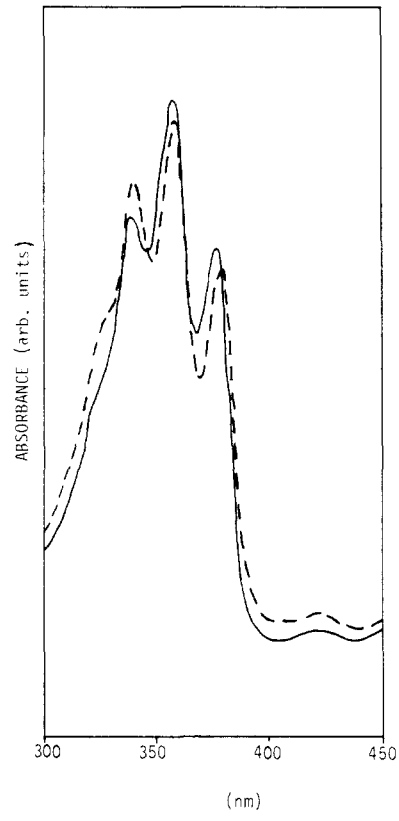

Figure 3. Absorption spectrum of 1:80 DPH/DPPC at 25 °C (---) and at 41 °C (—).

Our goal has been to examine the behavior of diphenylpolyenes and their surfactant derivatives when incorporated into phospholipid vesicles. The vesicle solutions used in these studies were prepared by probe sonication and therefore contain a mixture of MLV and SUV structures. Right-angle light scattering measurements made on both types of vesicles reveal their average hydrodynamic diameters. For MLV and SUV particles prepared from the lipid DPPC the average diameters are 1250–1300 and 250 Å, respectively. A range of solute:lipid ratios (1:50–1:1000) was used in these experiments. The specific ratio used in each experiment is noted.

Absorbance. At the concentration levels used in these experiments (5×10^{-6} to 1×10^{-5} M solute and 1×10^{-4} to 2×10^{-3} M lipid) all absorption and fluorescence spectra appear to be monomeric. However, Figure 3 shows that as the temperature of a DPH/DPPC (1:80) system is raised from 25 to 41 °C (T_c for DPPC vesicles), there is a change in the relative intensities

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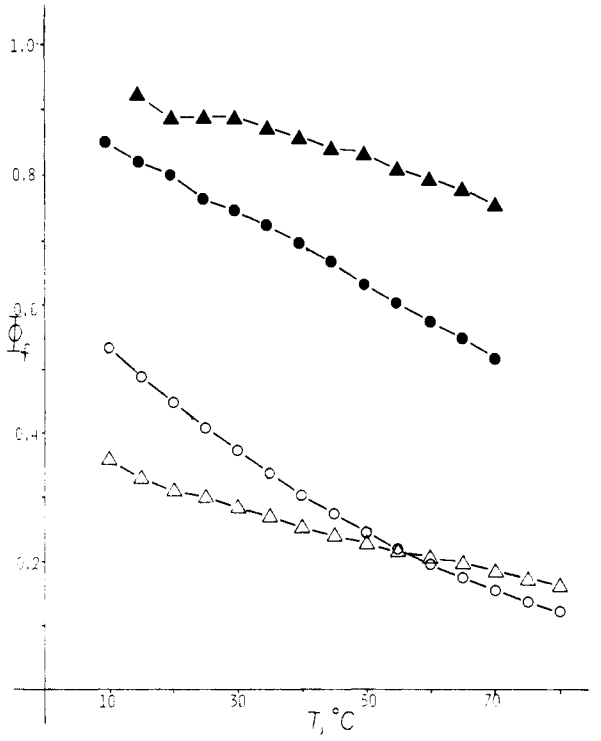

Figure 4. Fluorescence quantum yield vs temperature of polyenes in hydrocarbon solvents: (●) DPH/CH; (▲) 4H4A/MCH; (○) DPB/CH; (△) 4B4A/CH.

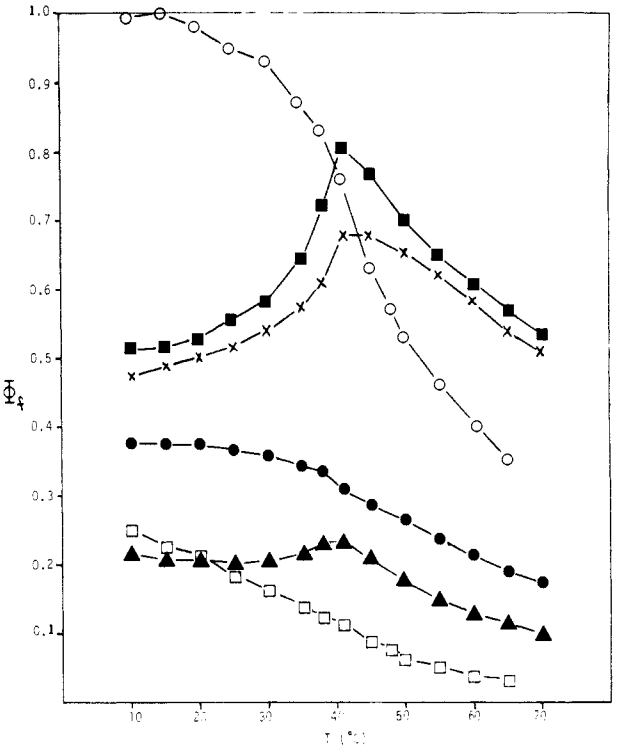

Figure 5. Fluorescence quantum yield as a function of temperature for the following solutes in DPPC vesicles: (■) DPH; (×) 4H4A; (●) 4B4A; (▲) DPB. Data for 6S4A (○) and TS (□) taken from ref 16.

of the different vibrational peaks in the first ($S_2 \leftarrow S_0$) absorption band. The intensity of the high-energy peak at 341 nm decreases with respect to the first two, lower energy, peaks at 358 and 376 nm as T_c is approached. Light scattered by the vesicles in this wavelength range, which could otherwise distort the absorption spectrum, has been subtracted from these spectra at each temperature. A change in vibronic structure with temperature is not observed for preparations in which the ratio of lipid:solute is 400:1 or greater.

Fluorescence. The competition between fluorescence and rotation away from a planar geometry in the excited state of diphenylpolyene molecules might be expected to lead to a temperature dependence of the fluorescence quantum yield similar to that of *trans*-stilbene. As can be seen in Figure 4 the quantum yield of fluorescence of each probe increases with decreasing temperature in homogeneous solution throughout the temperature range 10–60 °C. The fluorescence yields of the DPH probes not only are significantly greater than those of the DPB probes but also decrease linearly with temperature. In Figure 5 the relative fluorescence quantum yield of each solute solubilized in DPPC vesicles (T_c = 41 °C) as a function of temperature is plotted. The ratio of solute to lipid used in these experiments is between 1:50 and 1:100. The unexpected decrease in Φ_f as the temperature is lowered below T_c can be contrasted to the results obtained by Suddaby et al. in which all of the surfactant *trans*-stilbenes show an increase in Φ_f with decreasing temperature throughout the temperature range 10–70 °C.[16] In their experiments the solute:lipid ratio was maintained at approximately 1:100. However, in their studies a discontinuity *was* observed at the phase transition temperature of the specific vesicle system used. In those cases there is a sharp increase in the slope in the region of the phase transition. In contrast, there is an effective *quenching* of the polyene fluorescence in the low-temperature phase of the vesicles. This is more pronounced for the DPH probes than for the DPB probes. It also appears that the fluorescence of the surfactant derivative 4H4A is less dramatically reduced below T_c than that of its parent molecule DPH. Similarly, while DPB shows a small decrease in Φ_f at low temperatures, 4B4A does not experience any decrease in fluorescence quantum yield at all as the temperature is lowered. In DSPC vesicles each of the solutes exhibits even

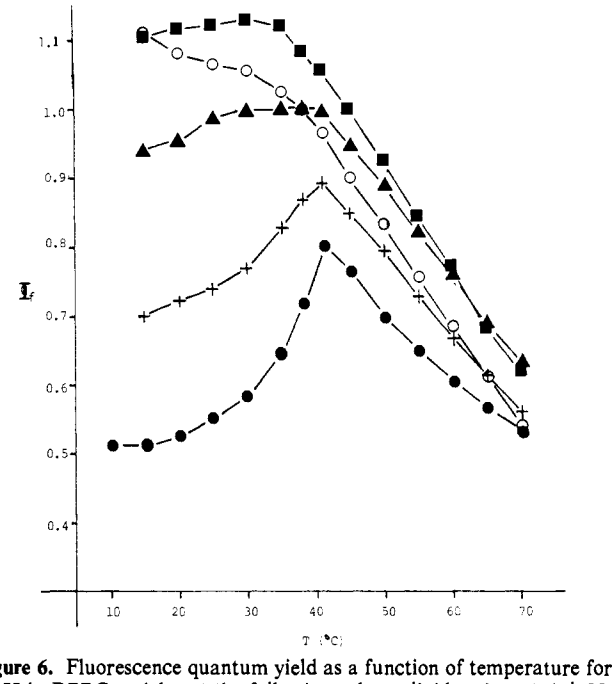

Figure 6. Fluorescence quantum yield as a function of temperature for DPH in DPPC vesicles at the following solute: lipid ratios: (●) 1:55; (+) 1:105; (▲) 1:224; (■) 1:510; (○) 1:1024.

more pronounced decreases in Φ_f in the low-temperature phase than in vesicles formed from DPPC. This effect on the fluorescence of DPH and DPB was found for both MLV and SUV vesicle preparations.

The results of a study of the effect of lipid concentration on the fluorescence behavior of the DPH/DPPC system are shown in Figure 6. As the solute:lipid ratio is decreased (by increasing the concentration of lipid), the fluorescence behavior as a function of temperature changes markedly. Rather than decrease as the temperature is lowered below T_c, the Φ_f of systems in which the lipid concentration is at least 500 times that of the solute increases with decreasing temperature throughout the temperature range.

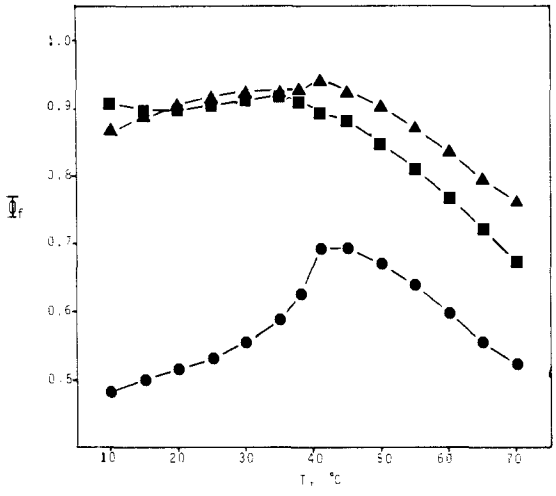

Figure 7. Fluorescence quantum yield vs temperature of 4H4A/DPPC at (●) 1:50, (▲) 1:270, and (■) 1:610.

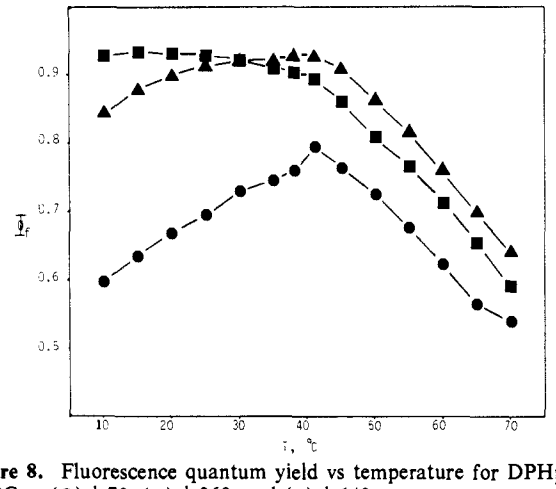

Figure 8. Fluorescence quantum yield vs temperature for DPHpPc/DPPC at (●) 1:70, (▲) 1:260, and (■) 1:640.

This change in fluorescence yield is not accompanied by any shift in or change in the shape of the emission envelope. The results obtained from studies of the 4H4A/DPPC system at both high (1:610) and low (1:50) concentration of lipid are plotted in Figure 7. While the fluorescence behavior of DPPC vesicles is similar to that observed for its nonsurfactant parent compound, the quantum yield of 4H4A does not change with temperature as dramatically as it does in the case of DPH. A similar concentration dependence was also observed (see Figure 8) for the fluorescence intensities of the lipid probe DPHpPC in DPPC vesicles.

When solubilized in DSPC vesicles (T_c = 55 °C) both DPH and 4H4A again show decreases in Φ_f as the temperature is lowered below T_c for systems doped with relatively high concentration of solute. When the lipid:solute ratio is increased to 400, the DPH/DSPC fluorescence no longer decreases with decreasing temperature but merely levels off at a value of about 0.75. This can be observed in Figure 9.

A concentration study was also carried out for the DPB probes in DSPC vesicles, in which the behavior observed was more pronounced than in DPPC vesicles. The results of that study are depicted in Figure 10. As the lipid:solute ratio is increased above 200, the nature of the quantum yield curve becomes more like those of the surfactant stilbenes studied by Suddaby et al.[16] Although the 4B4A/DSPC system also shows a concentration dependence, the small decrease in Φ_f at the lower temperatures is never completely lost, even at the highest lipid concentrations. This is shown in Figure 11. In Figure 12 a comparison of the fluorescence behavior of the DPB probes in DPPC vesicles of both low and high concentration of lipid is shown. Again, the nature of the quantum yield curves at high lipid concentration (or low

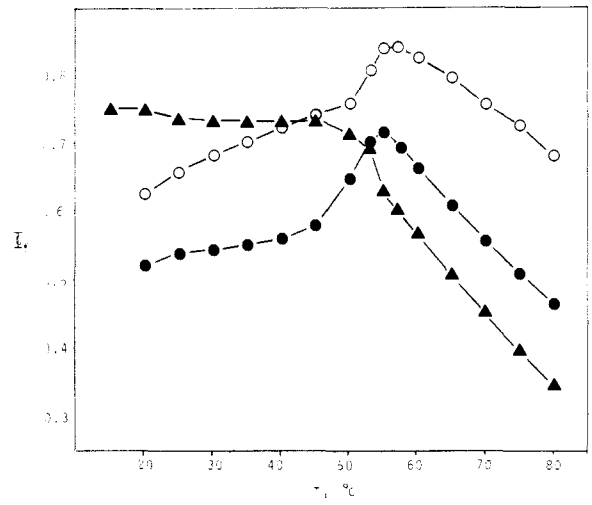

Figure 9. Fluorescence quantum yield of the following solutes in DSPC vesicles: (●) DPH at 1:70; (○) 4H4A at 1:100; (▲) DPH at 1:400.

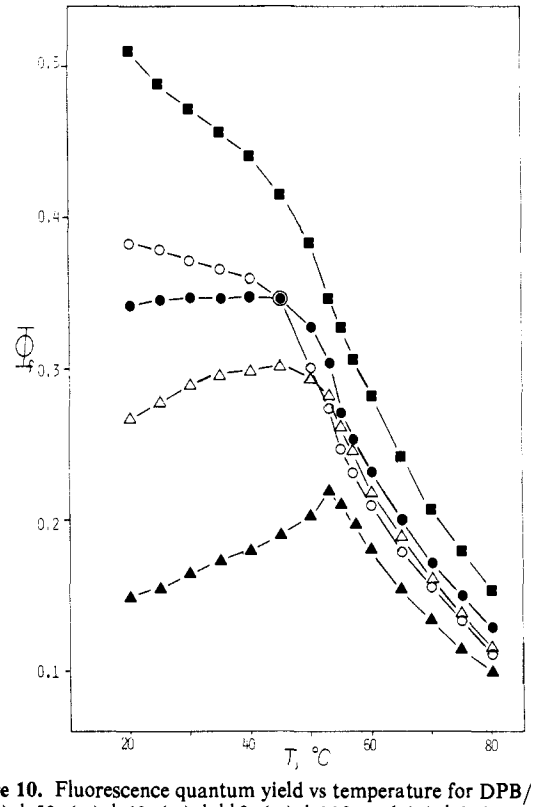

Figure 10. Fluorescence quantum yield vs temperature for DPB/DSPC at (▲) 1:50, (△) 1:60, (●) 1:110, (○) 1:220, and (■) 1:370.

probe:lipid ratio) is quite similar to that of the surfactant stilbenes.

Lifetimes. The fluorescence lifetimes for several of these solute/lipid systems were obtained by deconvolution of the fluorescence decay. The resulting lifetime data are potentially richer in information than the quantum yield measurements discussed above. A measure of the total fluorescence intensity emitted by a sample of fluorophores gives no indication of the number of solute species or different sites that exist for a given population. Rather, only an average environment type may be inferred. Measurement of the fluorescence lifetimes, on the other hand, may reveal the relative proportions of different emitting species within a sample.

Our data reveal that diphenylpolyene molecules solubilized within phosphatidylcholine vesicles yield fluorescence decays that are most often best fit by a double exponential. Occasionally, at temperatures above T_c, a single-exponential fit is observed or a triple exponential at the lowest temperatures in DSPC vesicles. In general, though, two lifetimes are obtained. The relative contributions of each to the total intensity and the absolute values

Table II. Fluorescence Lifetime Data for DPH/DPPC

1:55				1:220				1:510			
temp, °C	τ^a	%	τ_{av}	temp, °C	τ	%	τ_{av}	temp, °C	τ	%	τ_{av}
15	3.6	18.6	8.1	15	5.2	16.6	9.3	15	4.8	13.5	9.6
	9.2	81.4			10.1	83.4			10.3	86.5	
25	4	19.1	8.4	25	5.9	18.3	9.7	25	4.1	10.1	9.8
	9.4	80.9			10.5	81.7			10.4	89.9	
35	4.5	18	8.6	35	5.1	13.1	9.6	35	5.4	13.2	9.8
	9.5	82			10.2	86.9			10.5	86.8	
41	5.9	37.8	7.95	41	6.5	27.1	8.8	41	5.8	12	8.8
	9.1	62.2			9.7	72.9			9.2	88	
45	3	10	6.9	45	4.5	4.7	8	45	8.2	100	8.2
	7.3	90			8.2	95.3					
55	3.1	12.4	5.9	55	7.1	100	7.1	55	7.2	100	7.2
	6.3	87.6									
65	2.9	15.3	4.8	65	6.1	100	6.1	65	2.9	5.3	6.3
	5.2	84.7							6.5	94.7	

[a] Lifetimes are reported in nanoseconds; ±0.2 ns.

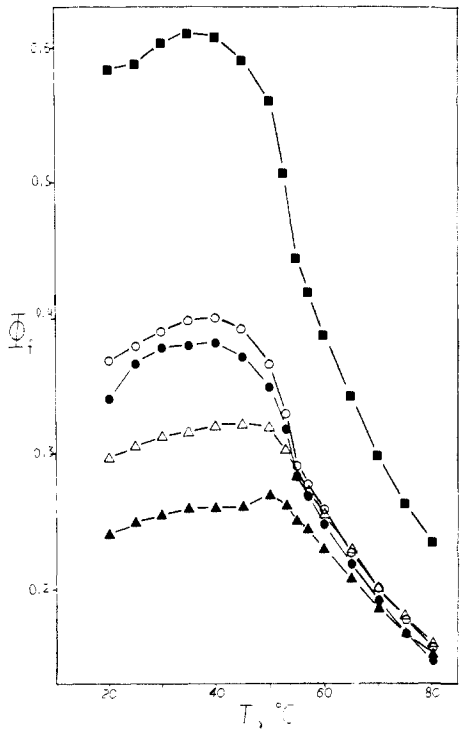

Figure 11. Fluorescence quantum yield vs temperature for 4B4A/DSPC at (▲) 1:40, (△) 1:80, (●) 1:100, (○) 1:160, and (■) 1:250.

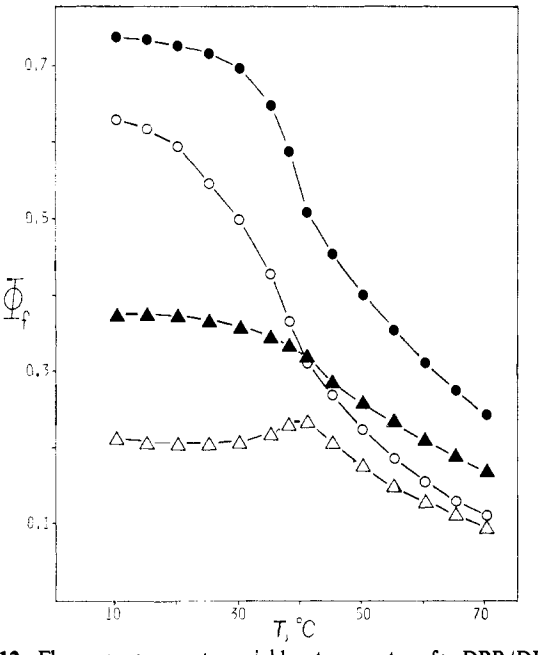

Figure 12. Fluorescence quantum yield vs temperature for DPB/DPPC at (△) 1:70 and (○) 1:700 and for 4B4A/DPPC at (▲) 1:110 and (●) 1:820.

of the lifetimes change with solute, vesicle, solute:lipid ratio, and temperature. The trends in the distinctions between the lifetime studies will be outlined.

The fluorescence lifetimes obtained for DPH/DPPC at a range of solute:lipid ratios are listed in Table II. Several trends must be noted. First, below T_c the longer lifetime component accounts for 80–90% of the total fluorescence intensity. The relative contribution of this component increases slightly as the lipid:solute ratio increases. For example, at 41 °C this component accounts for 62, 73, and 88% of the fluorescence as the ratio is changed from 55 to 220 to 510. The absolute value of the long-lifetime component also increases slightly upon going to systems that are progressively more concentrated in lipid. As the temperature is increased to T_c and above, the values of both the long- and short-lifetime components decrease. The relative contributions of the two lifetimes fluctuate in a seemingly random manner, and at some of the higher temperatures (at least for the systems of higher lipid:solute ratio) the decays are best fit by a single lifetime. The average fluorescence lifetimes, calculated from the integrated intensities, show in each case a small increase as T_c is approached and then a large decrease as the temperature is increased above T_c. Again, as the lipid:solute ratio is increased the average lifetimes increase at all temperatures.

Table III. Fluorescence Lifetime Data for DPH/DSPC

1:180				1:390			
temp, °C	τ^a	%	τ_{av}	temp, °C	τ	%	τ_{av}
15	4.6	30.5	8.0	15	4.4	23.3	8.3
	9.6	69.5			9.5	76.7	
25	4.4	17.6	8.8	25	4.9	19.9	9.1
	9.7	82.4			10.2	80.1	
35	5.7	28.4	9.3	35	4.6	12	9.7
	10.8	71.6			10.3	88	
45	4.9	17.5	9.6	45	6.3	22.3	10
	10.7	82.5			11.1	77.7	
50	3.8	11.3	9.6	50	5.2	13.8	9.9
	10.3	88.7			10.6	86.2	
55	4.6	10.6	8.4	55	7.1	35.3	8.4
	8.3	89.4			9.1	64.7	
65	6.0	62.2	6.8	65	6.9	65.2	8.3
	8.2	37.8			10.9	34.8	
75	3.9	13.7	5.8	75	2.9	4.9	5.8
	6.1	86.3			6.4	95.1	

[a] Lifetimes are reported in nanoseconds; ±0.2 ns.

Some of the same trends are observed for DPH in DSPC vesicles. As can be seen in Table III, there is an increase in the fraction of long-lifetime component with increasing relative concentration of lipid as well as an increase in that fraction with increasing temperature (8.0–9.6 ns from 15 to 45 °C in DSPC

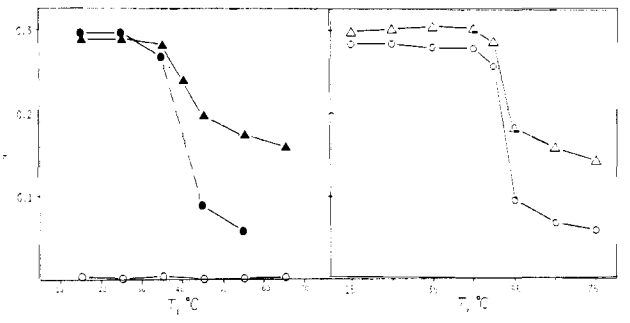

Figure 13. Anisotropy vs temperature: (a) (○) DPH/CH, (●) DPH/DPPC, and (▲) 4H4A/DPPC; (b) (○) DPH/DSPC and (△) 4H4A/DSPC.

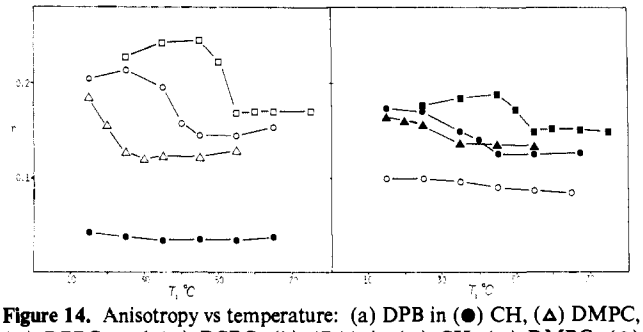

Figure 14. Anisotropy vs temperature: (a) DPB in (●) CH, (△) DMPC, (○) DPPC, and (□) DSPC; (b) 4B4A in (○) CH, (▲) DMPC, (●) DPPC, and (■) DSPC.

vs 8.1–8.6 ns in DPPC). However, both the proportion of the long-lived species and the average lifetimes calculated in DSPC show larger increases as the temperature approaches T_c than do those in DPPC vesicles. It is interesting to note that while 81–87% of the DPH fluorescence in DPPC vesicles can be attributed to a longer lifetime, this represents only 70–77% of the total fluorescence in DSPC vesicles at 15 °C. The lifetime data for 4H4A and for the DPB probes (not shown) also result in an increase in the average lifetime value with increasing lipid:solute ratio followed by a decrease at higher temperatures as T_c is approached. However, the changes with either temperature or lipid:solute ratio are not nearly as pronounced for the surfactant derivatives as are the changes in lifetime observed for the parent compounds.

Anisotropy. The concentration effects noted above may arise from differential solubility of the solute that is dependent upon the phase, and therefore the temperature, of the bilayer. The temperature dependence of the fluorescence anisotropy was measured in an attempt to determine whether the environment of the solute is more or less ordered at lower temperatures relative to above T_c. The results from that study are plotted in Figure 13 for the DPH probes. It is clear that as the bilayer is cooled, the anisotropy of both DPH and 4H4A rapidly increases, approaching the limiting value of 0.4 at the lowest temperatures. The surfactant probe, 4H4A, which might be expected to align more specifically along the acyl chains of the lipid molecules, does maintain a certain degree of anisotropy ($r = 0.15$) at the higher temperatures while DPH does not ($r = 0.06$). Figure 14 clearly demarcates the increasing order of vesicle with lipid chain length in the series DMPC, DPPC, and DSPC as experienced by DPB. This can be contrasted with the parallel study for 4B4A, in which the differences in order reported by the probe are not nearly as great.

Discussion

There are distinct differences between the photophysics of the three diphenylpolyene chromophores *trans*-stilbene, diphenylbutadiene, and diphenylhexatriene. This can be attributed, to a large extent, to the changes in the relative positions of the first two excited-state surfaces, $^1A_g^*$ and $^1B_u^*$, within the polyene series. The fluorescence intensities and lifetimes of these compounds are a function of the symmetry of the state from which emission occurs, the coupling between the two lowest energy excited singlet states, and the height of the barrier to nonradiative decay processes. However, despite their differences, there remains in each case a competition between fluorescence and nonradiative decay that is sensitive to the environment of the solute molecule.

The decrease in fluorescence with increasing temperature shown in Figure 4 for each of the diphenylpolyenes in hydrocarbon solvents reflects a thermal barrier to the available pathways of nonradiative decay, including excied-state rotation. The slopes obtained from Arrhenius plots of these data vary among these compounds and result in the calculation of activation energies for nonradiative decay that are in the range 0.2–5.3 kcal/mol.[29] The height of the barrier to rotation about a carbon–carbon double bond in the excited singlet state might be expected to depend on both the molecular structure and the viscosity of the environment in a manner analogous to that of the stilbenes.[16] However, the relative fluorescence quantum yield vs temperature curves plotted in Figure 5, in which there is an *increase* in the quantum yield as T_c is approached and then a decrease above T_c, indicate that some additional factor must influence the fluorescence behavior of diphenylpolyenes in phosphatidylcholine vesicles.

Fluorescence Intensities. The fluorescence studies shown in Figures 6–12 in which the solute:lipid ratio was varied demonstrate a strong concentration dependence of this phenomenon. There are several possible explanations for such an effect. The most obvious is that at high concentrations of solute relative to lipid in the vesicles there could be aggregation of the solute molecules that decreases as the temperature is increased. This can be discounted for two reasons. First, all absorption and fluorescence spectra appeared to be monomeric; the formation of microcrystals of these compounds is accompanied by a substantial blue shift of the absorption spectrum which was not observed for any of the vesicle preparations. Second, no hysteresis was observed; the temperature curve could be regenerated upon cooling the bilayer and remeasuring the fluorescence as a function of temperature.

A second possibility is that at the lower temperatures, below T_c, the highly ordered bilayer forces a diphenylpolyene molecule out of planarity or imposes some strain on the molecule, causing it to assume either a non-trans or an s-cis conformation. This may be supported by the small changes observed in the vibronic structure of the absorption spectrum of DPH when incorporated in DPPC vesicles at 25 and 41 °C shown in Figure 3. The lower temperature spectrum is quite similar to that of the spectrum of the trans,cis,trans isomer of DPH.[49,50]

A third explanation may be that the concentration effect results from a lower solubility of the longer diphenylpolyenes in bilayers relative to that of the surfactant stilbenes. As the vesicle is heated, solute molecules that are associated with the interface may become soluble in the bilayer interior where they would exhibit an enhanced fluorescence relative to that at the polar interface. Thus, the partitioning of the solute molecules may be sensitive to the phase of the vesicle such that below T_c guest-impurity molecules are zone refined. However, Lentz et al. have suggested that DPH does *not* preferentially partition into lipid bilayers of different phases or composition.[51,52]

Fluorescence Lifetimes. To distinguish between the latter two possibilities, it is necessary to examine the fluorescence lifetime data of these solute/vesicle systems. Although the fluorescence decays generally show two components, average lifetimes may be calculated from the integrated intensities. This allows a comparison of the fluorescence intensity curves with the average lifetimes vs temperature. For a given solute:lipid ratio, the fluorescence lifetime of DPH (Tables II and III) does not appear to be as sensitive to the temperature and therefore phase of the vesicles as is the intensity (Figures 6 and 9). This is not surprising in view of the fact that k_f is *not* independent of temperature for

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these molecules. In other words, the fluorescence lifetime of DPH in homogeneous solution has been shown to increase slightly with increasing temperature while the quantum yield decreases.[30] From phase modulation ratio measurements, Barrow and Lentz observed a decrease in τ_f from 8.7–8.8 to 8.35 ns as the lipid/DPH ratio decreased from 750 to 150.[35] However, they did not observe corresponding decreases in the fluorescence intensities of DPH/DPPC vesicle systems. Also, below T_c average lifetimes of DPH ranged from 9.6 to 12.6 ns for different vesicles in their studies.

Although the average lifetimes calculated from our measurements did not change as markedly as the fluorescence intensities with the phase of the vesicle, some important observations can be made by looking at the change in relative contribution of each fluorescence component. For example, as the concentration of solute relative to lipid is decreased for DPH/DPPC (increasing lipid:solute ratio from 55 to 220 to 510), the contribution of the short-lifetime component decreases from 38 to 27 to 12% at 41 °C. Also, the magnitude of both the short- and long-lifetime components increases as the T_c is approached. This supports a picture of the vesicle in which a certain fraction of the solute molecules remains localized at the interface at temperatures below the phase transition. The proportion of such molecules depends both on the temperature of the bilayer and on the relative concentration. The lower the lipid:solute ratio, the greater that fraction, as though a saturation level exists for the solubility of a given solute molecule in the interior of phosphatidylcholine vesicles. A higher percentage of DPH molecules appears to be localized at the interface in DSPC vesicles than in DPPC vesicles (30 vs 17%). The longer chain phospholipid (DSPC) may form more tightly ordered structures that more effectively exclude guest molecules from the bilayer interior in the low-temperature "gel" phase. The somewhat random changes in both the magnitude and percentage of the short-lifetime component with temperature probably reflects a sampling of average solute location rather than a partitioning between two distinct sites.[53]

Perturbation. Remarkably, the surfactant diene and triene probes appear to be *less* well solubilized within a given bilayer than are their nonsurfactant counterparts. 4H4A shows greater fractions of the short-lifetime fluorescence component than does DPH in either DPPC or DSPC. The creation of a chromophore-containing fatty acid (or surfactant probe) should have allowed for an increase in the compatibility of the probe molecule with the lipid bilayer. That 4H4A shows a larger contribution to the shorter-lived fluorescence may indicate that the longer diphenylpolyenes are more perturbative to the bilayer environment than has previously been assumed, such that the addition of alkyl chains to them *reduces* their ability to align with the lipid chains rather than enhances it. It must be noted, however, that a proportion of the short-lifetime component of the 4H4A fluorescence may be attributable to an enhancement of delayed emission from $^1B_u^*$. The red-shifted absorption ($^1A_g \rightarrow {}^1B_u^*$) but unshifted emission ($^1A_g^* \rightarrow {}^1A_g$) of 4H4A, as compared to DPH, indicates that in 4H4A the S_2 surface is stabilized relative to S_1. This should increase the probability of thermal repopulation of S_2 from S_1 in 4H4A.

Structures in which guest molecules partially micellize within a bilayer, forming local defect sites, have been proposed by Tirrell.[54] The study of the concentration dependence of the fluorescence of DPHpPC in DPPC vesicles may support such a view. The fluorescence curves plotted in Figure 8 are similar to those obtained for DPH and 4H4A when incorporated into DPPC vesicles. However, it does not seem likely that the DPHpPC probe, a large phosphatidylcholine molecule, could be differentially solubilized at the bilayer interior and interface depending upon the phase of the vesicle. In fact, DPHpPC has been shown to preferentially partition into the fluid phase of DPPC vesicles (k_f = 3.3).[46] Rather, the solute may induce a change in the morphology of the bilayer. Parente and Lentz have suggested that DPHpPC disrupts its local environment when solubilized in lipid bilayers below T_c and may even create perturbed solute-rich local domains.[46] Micellization or similar loose aggregation of solute molecules within the bilayer might be expected to produce small changes in the absorption spectrum of DPH and be reversibly dependent on the vesicle temperature, in agreement with our results.

Anisotropy. Relatively large anisotropy values (r = 0.2–0.3) were determined from the fluorescence depolarization studies depicted in Figures 13 and 14 for each solute/lipid system at temperatures below T_c. This indicates that all of the diphenylpolyene probes experience a high degree of order in the low-temperature phase of the vesicles. Free rotation is most hindered (r = 0.28–0.30 below T_c) for the DPH probes. The difference between the anisotropy values obtained for 4H4A and DPH above T_c may be attributable to an "anchoring" of the surfactant probe into the bilayer by its acyl chains so that it continues to experience some preferential rotation about its long axis in the fluid phase. The nature of the DPH anisotropy curves correlates closely with those for the DPB probes as well as with that of the *trans*-stilbene probe 6S4A. Through the phase transition the anisotropy undergoes a sharp decrease and then levels out at a relatively low value ($r \leq 0.1$) above T_c. This rules out any artifact due to the anisotropy at low temperatures as a cause of the low fluorescence observed in that phase. This is in accord with the observation of Shinitsky that such an effect should lead to an "apparent" quenching of the fluorescence observed of up to 20%, which is not sufficient to explain our results.[55] No concentration dependence was observed for the anisotropy data. This is most likely due to the fact that the steady-state fluorescence intensity is dominated by the more strongly fluorescing solute molecules which are located within the bilayer interior. The contribution to the total fluorescence of solute located at the interface, where it might be expected to experience freer rotation, is much lower. Time-resolved fluorescence depolarization experiments and magic angle lifetime measurements are necessary to corroborate our lifetime data.

Summary

The fluorescence intensities and lifetimes of diphenylpolyene molecules are sensitive to their environment. They can, therefore, be useful probes of such microheterogeneous media as lipid bilayer membranes, provided they are used in relatively low concentrations so as not to induce unusual fluorescence behavior. The development of surfactant derivatives of these chromophores may also help provide for their specific placement within an assembly. However, our comparison between the fluorescence behavior of diphenylpolyenes and their fatty acid derivatives demonstrates that seemingly compatible solute molecules may not necessarily be considered nonperturbative to lipid environments. Ultimately, a more complete understanding of the subtleties of host–guest interactions should allow for the development of systems in which reactivity can be carefully controlled.

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