Temperature Dependence of 1,6-Diphenyl-1,3,5-hexatriene Fluorescence in Phospholipid Artificial Membranes†

Mary P. Andrich and Jane M. Vanderkooi*

ABSTRACT: The fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene in phospholipid vesicles is a function of the physical state of the lipid. Below the phase transition, the polarization approaches the theoretical maximum for total immobilization while above the phase transition the fluorescence becomes nearly completely depolarized. The discontinuity in the temperature dependence of polarization occurs within a temperature range under 5 °C in the case of pure phospholipids, but for mixed phospholipids occurs over a temperature range greater than 20 °C. From these data, phase diagrams describing the gel-sol equilibrium can be constructed; the phase diagrams correspond well with those described in the literature which were constructed using spin-label probes or from x-ray diffraction patterns. The marked change in polarization at the phase transition may be related to the packing of the probe molecule into the lipid bilayer: fluorescence measurements on oriented bilayers indicate that below the phase transition the long axis of the probe is oriented perpendicular to the plane of the membrane while above the transition the probe is oriented randomly relative to the plane of the membrane.

The importance of the physical state of the lipid bilayer in regulation of membrane function is apparent from two points of view:

1. Compelling evidence is available that membrane components are able to diffuse laterally (Frye and Edidin, 1970) or rotate about an axis perpendicular to the membrane surface (Cone, 1972; Brown, 1972).

2. The temperature dependence of the activity of membrane enzymes and the transport of metabolites and ions can be interpreted in terms of the mobility of the membrane lipids (Overath et al., 1970; Wilson and Fox, 1971; Mavis and Vagelos, 1972; Papahadjopoulous et al., 1973).

Techniques which have been employed to study membrane phase transitions include spin-label (Scandella et al., 1972; Caron et al., 1974; Hubbell and McConnell, 1968) and fluorescent probe analysis (Vanderkooi and Martonosi, 1971; Vanderkooi and Chance, 1972; Shinitzky et al., 1971; Cogan et al., 1973), as well as more direct techniques such as nuclear magnetic resonance analysis (Sheetz and Chan, 1972; Horwitz et al., 1973), x-ray analysis (Luzzati et al., 1968; Luzzati, 1968), and differential scanning calorimetry (Ladbrooke and Chapman, 1967).

Shimshick and McConnell (1973) first interpreted the response of a membrane "probe" molecule in terms of lateral phase separations which occur in phospholipid dispersions. The spin-label 2,2,6,6-tetramethylpiperidine-1-oxyl was observed by them to partition differentially in lipid below and above the phase transition, enabling construction of phase diagrams. Although the use of fluorescent probes to sense changes in the "membrane fluidity" is well established (Shinitzky and Inbar, 1974; Vanderkooi et al., 1974), a similar complete physical description of the response of a fluorescent dye in terms of the gel-sol phase diagram of the lipid is not available. In this paper, we describe the fluorescent behavior of the dye 1,6-diphenyl-1,3,5-hexatriene (DPH[1]) as a function of temperature in phospholipid dispersions. DPH is a small lipophilic molecule which has previously been shown to partition into membranes of cells (Shinitzky and Inbar, 1974). We show here that the polarization of DPH fluorescence in membranes is a function of membrane fluidity and, from the temperature dependence of polarization, construct phase diagrams of the lipids. In addition, we give data concerning the location and orientation of the probe molecule in the lipid bilayer.

Experimental Section

Materials

L-α-Dimyristoyllecithin and L-α-dipalmitoyllecithin were supplied by Sigma Chemical Co. (St. Louis, Mo.); L-α-distearoyllecithin came from Applied Science Laboratories (State College, Penna.). Purity of the lipids was analyzed by thin-layer chromatography on silica gel G plates using chloroform, methanol, and water as solvent in the volume ratio 65:25:4. All three lipids were determined pure by the presence of only one spot for each after staining with iodine. DPH was obtained from Dr. R. Cooper, University of Pennsylvania, and from Aldrich Chemical Co. (Milwaukee, Wis.). D_2O for the NMR experiment was procured from Wilmad Glass Co. (Buena, N.J.). Distilled and deionized water was used throughout the experiments. All other reagents were of the highest purity commercially available.

Methods

Preparation of Samples. For the fluorescence experiments, DPH in tetrahydrofuran was added to lipid samples suspended in 0.1 M ammonium acetate. The highest final concentration of tetrahydrofuran was 0.1%. Unless otherwise noted, samples were then sonicated using a Branson sonifier for 1 min at maximum power output. The sample

† From the Department of Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, Pennsylvania 19174. *Received September 8, 1975.* This work has been supported by grants from the National Institutes of Health (GM 12202 and 21699). J.M.V. is the reciprient of an NIH Career Development Award GM 00053. Use was made of the Middle Atlantic Regional NMR Facility (supported by National Institutes of Health Grant RR-542) and of University of Pennsylvania Computer Center.

[1] Abbreviations are: NMR, nuclear magnetic resonance; DPH, 1,6-diphenyl-1,3,5-hexatriene; DML, L-α-dimyristoyllecithin; DPL, L-α-dipalmitoyllecithin; DSL, L-α-distearoyllecithin.

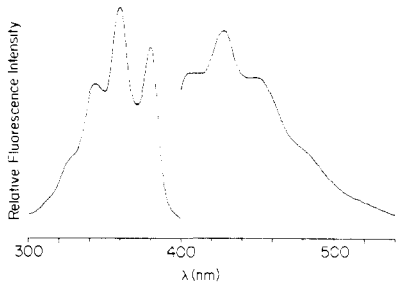

FIGURE 1: Uncorrected excitation and emission spectra. The sample contained DPH (0.2 μM) in 0.1 mg/ml (0.13 mM) of DSL and 0.1 M ammonium acetate, at 23 °C. Excitation and emission wavelengths were at 360 and 430 nm, respectively. Bandpass was 5 nm.

size for sonication was 1–3 ml, and the normal size probe was used. The sample was allowed to warm during sonication (this, along with the small sample size, facilitates sonication). In the case of the samples for the NMR experiment, the tetrahydrofuran was evaporated prior to suspension in D_2O, and sodium acetate was added for internal reference.

Fluorescence Measurements. Steady-state fluorescence polarization was measured at 90 °C relative to the exciting beam using an Hitachi MP-2A fluorescence spectrometer. Polacoat 4B polarizers were used to polarize both exciting and emitting beams. The fluorescence polarization, P, was calculated according to:

$$P = (I_{VV} - I_{VH}G)/(I_{VV} + I_{VH}G) \quad (1)$$

where I_{VV} and I_{VH} are observed intensities measured with polarizers parallel and perpendicular to the vertically polarized exciting beam, respectively. G is a factor used to correct for the inability of the instrument to transmit differently polarized light equally.

Temperature was regulated by circulating water which heated or cooled the sample cell holder. The temperature of the sample was measured prior to each measurement by means of a digital thermometer with a thermistor probe. Temperatures were accurate to within 1 °C.

Oriented multilayers were prepared on glass microscope coverslips by allowing the mixture of DPH and DPL to air-dry overnight. The relative humidity was 87% and temperature was about 24 °C. During the experiment, the coverslip was placed in the cell holder in three different positions: one perpendicular (I) and two parallel (II, III) to the incident beam (see Figure 6). Four polarization measurements were taken in each position: combinations of exciting and emitting light either vertically or horizontally polarized—for a total of 12 measured intensities. Due to symmetries of the lipid bilayer, only 4 of these 12 quantities are independent (denoted by $\alpha,\beta,\delta,\gamma$). These were calculated, after Yguerabide and Stryer (1971), for the case where there is no rotation of chromophores and both the absorption and emission transition moments lie along the same axis:

$$\alpha = \frac{6\pi}{4}\int_0^{\pi/2} p(\phi)\sin^5\phi\, d\phi \quad (2)$$

$$\beta = \frac{\pi}{2}\int_0^{\pi/2} p(\phi)\sin^5\phi\, d\phi$$

$$\gamma = 2\pi\int_0^{\pi/2} p(\phi)\cos^2\phi\sin^3\phi\, d\phi$$

$$\delta = 4\pi\int_0^{\pi/2} p(\phi)\cos^4\phi\sin\phi\, d\phi$$

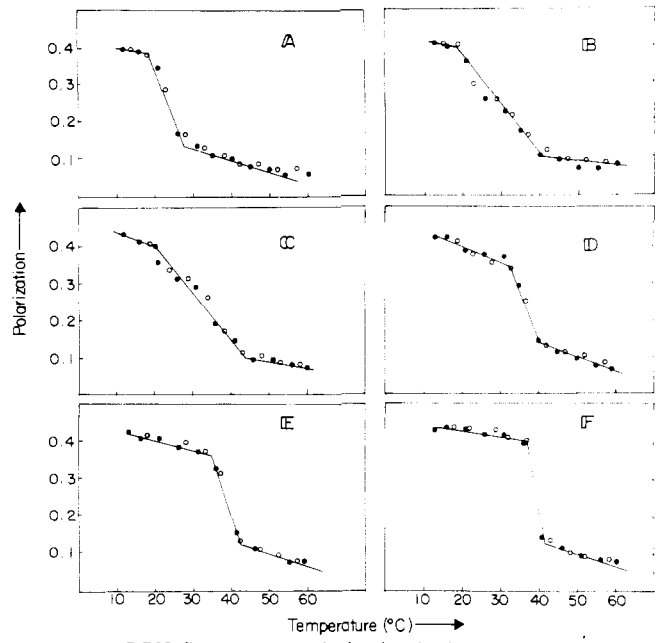

FIGURE 2: DPH fluorescence polarization in DML and DPL mixed dispersions. Varying molar concentrations of DML and DPL were mixed to give a total lipid concentration of 0.14 mM in 0.1 M ammonium acetate, pH 6.7. DPH was added to a final concentration of 0.2 μM, and the samples were then sonicated. Temperature profiles were measured by scanning from the highest temperature to the lowest and back to the highest: (●) descending temperature; (○) ascending temperature. Excitation and emission were at 360 and 430 nm, respectively. (A) 100% DML; (B) 80% DML, 20% DPL; (C) 60% DML, 40% DPL; (D) 40% DML, 60% DPL; (E) 20% DML, 80% DPL; (F) 100% DPL in molar ratios.

where $p(\phi)$ is the distribution of transition moments in the bilayer.

The fluorescence lifetime, τ, of DPH was measured with an Ortec photon-counting fluorescence lifetime instrument as previously described (Vanderkooi et al., 1974). Excitation and emission wavelengths were isolated using appropriate interference filters. The data were transferred from the multichannel analyzer to a PDP-10 computer via cassette magnetic tape. The data were deconvoluted for the lamp function and analyzed for exponential decay using a nonlinear, least-squares analysis.

Nuclear Magnetic Resonance. NMR spectra were taken on a Varian NR-220 NMR spectrometer equipped with a Fourier transform accessory. The sample temperature measured with a thermistor thermometer was controlled to ±0.5 °C.

Results

Characterization of Fluorescence Parameters. The fluorescence excitation and emission spectra of DPH in distearoyllecithin vesicles are presented in Figure 1. The wavelengths of maxima for excitation and emission are not detectably altered by the gel–sol phase transition of the lipid and the peaks correspond to the maxima reported by Shinitzky and Inbar (1974) in hydrophobic solvents. On the other hand, the other fluorescence parameters of the DPH probe are affected by the physical state of the lipid and can be used to detect gel–sol phase transitions. The fluorescent lifetime of DPH in dimyristoyllecithin at 23 °C is 10.0 ± 0.4 ns; at 15 °C it was determined to be 10.4 ns and at 30 °C it is 8.9 ns. The decrease in fluorescence lifetime with increasing temperature is proportional to a decrease in the fluorescence intensity.

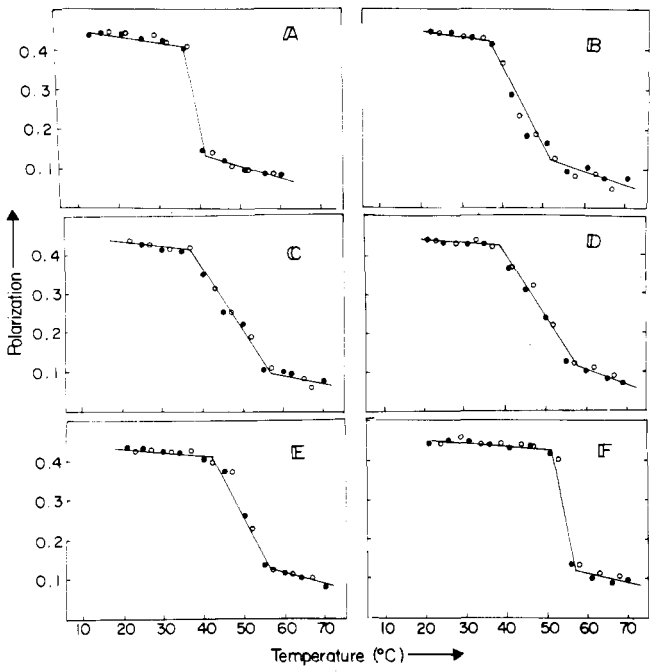

FIGURE 3: DPH fluorescence polarization in DPL and DSL mixed dispersions. Conditions were the same as described in Figure 2. (A) 100% DPL; (B) 80% DPL, 20% DSL; (C) 60% DPL, 40% DSL; (D) 40% DPL, 60% DSL; (E) 20% DPL, 80% DSL; (F) 100% DSL in molar ratios.

Table I: Polarized Fluorescence Intensities.[a]

Excitation and Emission Component	I	II	III
I_{VV}	α	α	δ
I_{VH}	γ	β	γ
I_{HV}	β	γ	γ
I_{HH}	γ	γ	β

[a] From Yguerabide and Stryer (1971).

The fluorescence parameter which is, however, most dramatically altered by the lipid phase transition is the fluorescence polarization. Sharp discontinuities in the temperature dependence of DPH polarization are observed at 23, 40, and 54 °C for the DML, DPL, and DSL suspensions, respectively (Figures 2-4). These temperatures correspond to the temperature at which these lipids undergo phase transitions as detected by differential scanning calorimetry (Chapman et al., 1967; Hinz and Sturtevant, 1972). The breaks in the curves for the polarization profiles are sharp, and the transition occurs within 5 °C of the literature values. This can be interpreted to mean that the probe does not greatly alter the packing of the hydrocarbon chains below the phase transition and, therefore, does not cause a large depression in the melting point. Not shown in the figures are experiments in which DPH concentration was increased to 2 μM; essentially the same results were obtained. The sharp discontinuity of DPH fluorescence polarization at the phase transition of the lipid disappears when the lipids of different fatty acid chain length are mixed. The temperature range at which the transition occurs is 4 °C for the pure lipid, but is broadened to around 26 °C for the lipid vesicles composed of 60:40 molar ratio of DML and DPL or DPL and DSL.

More complicated behavior is obtained for the temperature dependence of DPH polarization in mixed phospholipid vesicles in which the fatty acid side chains differ by more than two carbons. In Figure 4 the temperature profiles of DPH polarization in dimyristoyllecithin-distearoyllecithin mixed vesicles are presented. For the sample in which the lipids are mixed, but not sonicated together, two distinct phase transitions can be discerned (Figure 6A). The samples which are sonicated together (Figures 4B, C, E, and F) also give rise to discontinuities but the transitions are less sharp.

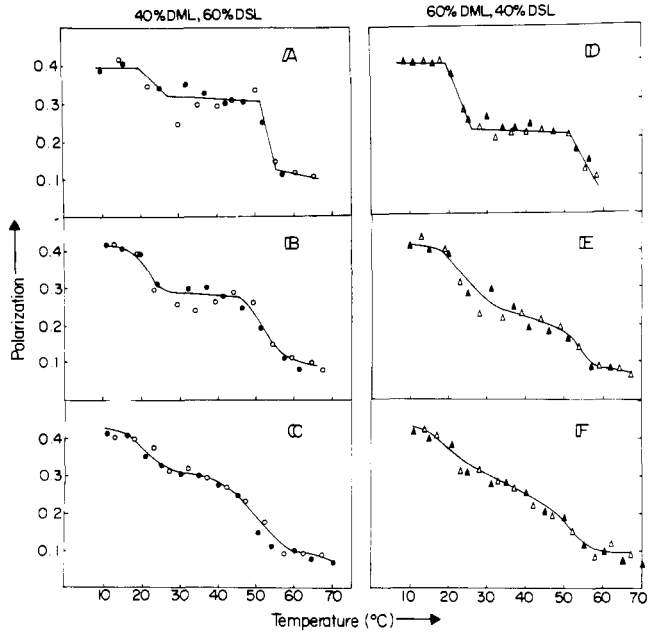

FIGURE 4: DPH fluorescence polarization in DML and DSL mixed lipid dispersions. 6A-C: 40% DML and 60% DSL (molar ratio); 6D-F: 60% DML and 40% DSL (molar ratio). All samples contained 0.2 μM DPH, 0.14 mM total lipid concentration and 0.1 ammonium acetate, pH 6.7. Samples represented by Figures 6A and 6D were not sonicated after the two lipid dispersions (previously sonicated) were mixed. 7B-F were sonicated after mixing lipids and DPH. Temperature profiles for 7B and 7E were performed by starting at 23 °C, ascending to highest temperature, descending to lowest, and ascending to 23 °C. 7C and 7F were obtained by descending from the highest temperature to the lowest, and then ascending to the highest. Closed symbols denote descending temperature while open symbols are ascending temperature. Emission and excitation wavelengths were 430 and 360 nm, respectively.

The DPH fluorescence temperature profiles for the mixed lipid systems resemble those obtained by Shimshick and McConnell (1973) using the spin-label probe 2,2,6,6-tetramethylpiperidine-1-oxyl. These authors interpret the results in terms of phase separations; that is, at intermediate temperatures there is a separation in two dimensions of the solid phase. Using the same interpretation, phase diagrams can be constructed from the data presented in Figures 2 and 3. The temperature marked T_1 refers to the temperature below which all the lipid is in the paracrystalline state; T_2 refers to the temperature above which all the lipid is in the melted, disordered state (Figure 5). This interpretation is supported by x-ray analysis (Luzzati et al., 1968; Tardieu et al., 1973) and by the observation that proteins and lipids and hydrocarbon probes can diffuse laterally in the membrane (Frye and Edidin, 1970; Cone, 1972; Brown, 1972; Vanderkooi and Callis, 1974; Hubbell and McConnell, 1968). The phase diagrams which one obtains with DPH are similar to the ones which have been obtained using a spin-label probe (Shimshick and McConnell, 1973),

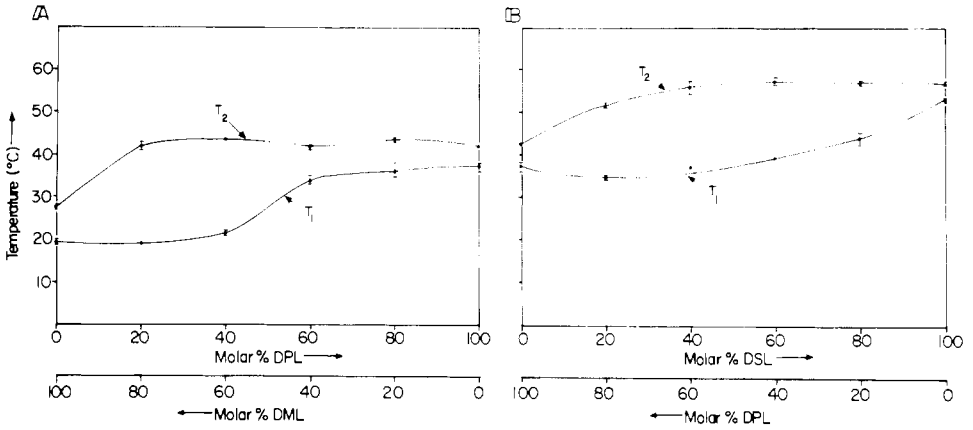

FIGURE 5: Phase diagrams for DPL–DML and DSL–DPL vesicles. Phase diagrams were constructed from the data given in Figure 2 for DPL–DML mixtures (Figure 5A) and in Figure 3 for DPL–DSL mixtures (Figure 5B). Two temperature points for each experiment mark the beginning (T_1) and end (T_2) of each phase transition and are plotted as a function of molar percent lipid. Each point represents an average of four experimental points, and two experiments.

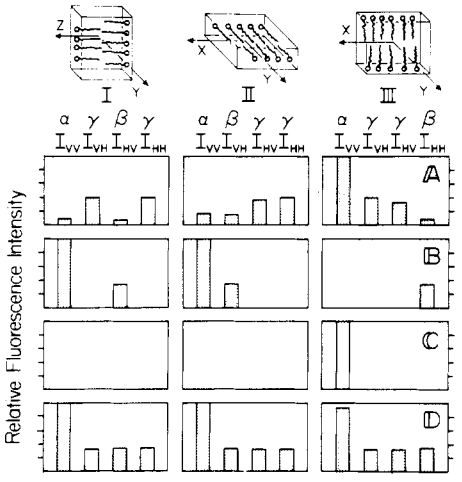

FIGURE 6: Polarized fluorescence intensities in oriented phospholipid multilayers. (A) Experimental polarized fluorescence intensities of a sample composed of dipalmitoyllecithin and DPH in 6500:1 molar ratio. Total lipid on the plate was about 1 μmol. Sample preparation is described in Experimental Section. (B) Calculated polarized fluorescence intensities assuming that the transition moment is parallel to the *xy* plane. (C) Calculated intensities assuming that the transition moment is perpendicular to the *xy* plane. D. Calculated intensities for transition moments which are oriented randomly in the sample but are not free to rotate. Calculated intensities for B and C are from Ygeurabide and Stryer (1971).

suggesting the two probes are used to detect the same physical phenomena.

It should be noted in Figures 2–4 that only one transition is observed for each lipid with the DPH probe. There is no evidence of the "pretransition" observed with differential scanning calorimetry (Ladbrooke and Chapman, 1967) which can be detected using 2,2,6,6-tetramethylpiperidine-1-oxyl (Shimshick and McConnell, 1973). The pretransition has been attributed to the tilting of the chains with respect to the plane of the lipid lamellae (Tardieu, et al., 1973; Ranck et al., 1974). The difference in response between the two probes can be interpreted in terms of the physical properties of the probes. The spin-label probe is a small molecule whose response to the phase transition is determined by a different partition coefficient above and below the phase transition. On the other hand, DPH is a lipophilic molecule, and the fluorescence dipolarization technique which we are using depends primarily upon the rotational diffusion of the probe. The data would suggest that the "pretransition" does not cause sufficient disorder in the hydrocarbon core of the membrane to detectably alter the rotational diffusion of DPH.

Orientation of the Probe Molecule. The depolarization of DPH fluorescence at the phase transition is quite dramatic; below the phase transition, the polarization approaches the theoretical value of 0.5, while above the phase transition the DPH is nearly completely depolarized. Although temperature dependence of the fluorescence polarization of many lipophilic probes exhibits discontinuity at the phase transition, the change in polarization is often not so pronounced (Vanderkooi and Chance, 1972; Vanderkooi et al., 1974; Sackmann and Trauble, 1972; Vanderkooi and Martonosi, 1971). The degree of polarization of the fluorescent dye in the paracrystalline hydrocarbon core below the phase transition may reflect the degree of perturbation which the probe introduces; i.e., below the phase transition, a large irregularly shaped molecule which is unable to fit into the crystalline lattice will be more depolarized. Since DPH is essentially a rod-like molecule, we considered the possibility that below the phase transition the molecule is aligned along the direction of the hydrocarbon chains of the phospholipid. To test this hypothesis, oriented multilayers of phospholipid below the phase transition were prepared, and the fluorescence intensity as a function of angle and polarization was measured. The results are summarized in Figure 6. The high fluorescence intensity of δ indicates that the probe is preferentially oriented perpendicular to the plane of the phospholipid bilayer. The values of fluorescence intensity of α, β, and γ are not zero, as in the case of perfect orientation normal to the plane. Possible reasons for this include: (1) imperfect alignment of the multilayers; (2) off-axis orientation of probe molecules; (3) rotation of the probe molecules. Although the data do not rule out contributions from 1 and 2, the data do support 3. In the case of random probe orientation with no rotational motion, the ratio of α to β is 3, while in the case of rapid rotational motion about the *c* axis, the ratio of α to β will be 1 (Ygueraabide and Stryer, 1971). Our experimental results indicate that the probe is preferentially but not perfectly oriented parallel to the *c* axis, but that it can undergo rapid rotation about the *z* axis.

An attempt was made to locate the probe in the membrane by nuclear magnetic resonance techniques. The NMR spectrum of DML containing varying amounts of DPH was taken at 37 °C. Even at molar ratios of DML–

DPH of 5:1, the spectrum was unaltered. These data provide some evidence that DPH in lipid above the phase transition does not greatly alter the packing of the molecules within the bilayer.

Discussion

The data presented here illustrate that the fluorescence polarization of DPH can be used to detect lipid phase transitions and phase separations. There does not appear to be a large difference in the partitioning of the dye between the two phases. If, for example, the probe exclusively partitions into the melted lipid, we would expect the probe to detect only the onset (T_1) of the phase transition, and the transition would be displaced but not broadened. Further evidence that the probe partitions into both the lipid phases can be concluded from the data of Figure 4. In the mixed lipid samples which have been suspended together but not sonicated together, one is able to see two sharply defined transitions (Figure 4A and D). If the probe were able to partition only in the melted phase, one might predict that the probe would detect only the phase transition of the component which melts at a lower temperature.

A comment may be made about the analysis presented here. In a complete analysis of the rotational motion of a fluorescent molecule in the membrane, several things need to be taken into account, in addition to the polarization measurements. The relaxation time of rotation, ρ, is related to the fluorescent lifetime, τ, according to the Perrin equation:

$$\left(\frac{1}{\rho}-\frac{1}{3}\right)=\left(\frac{1}{\rho_0}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho}\right)$$

It is apparent that polarization is only indirectly a function of the relaxation time and that changes in the lifetime, τ, will affect the polarization observed (Weber, 1956). In addition, the meaning of rotational relaxation time needs some clarification in our situation. DPH can be approximated as a prolate ellipsoid. Since the transition moments for absorption and emission are along the long axis of the molecule, rotation about another axis normal to this axis will cause depolarization, while rotation about the long axis will not result in depolarization. A further point is the anisotropic nature of the membrane. Below the phase transition the molecule is oriented perpendicular to the plane of the membrane, while above the transition a more random distribution is obtained. Because of these uncertainties, we have expressed our data in terms of polarization rather than converting the data to rotational correlation times or "micro-viscosity". The emphasis is then placed upon the ability to detect phase transitions and to be able to construct phase diagrams.

Summary

1. The fluorescence polarization of DPH can be used to monitor phase transitions in phospholipid vesicles.

2. From the temperature profile of depolarization of DPH in mixed phospholipid membranes, phase diagrams can be constructed.

3. The probe appears to be oriented with its long axis perpendicular to the plane of the bilayer in lecithin samples below the phase transition.

Acknowledgments

We wish to thank Mr. Harold Selick for obtaining NMR spectra and Dr. A. Horwitz for helpful criticism.

References

Brown, P. K. (1972), *Nature (London), New Biol. 236,* 35.
Caron, F., Mateu, L., Rigny, P., and Azerad, R. (1974), *J. Mol. Biol. 85,* 279.
Chapman, P., Williams, R. M., and Ladbrooke, B. D. (1967), *Chem. Phys. Lipids 1,* 445.
Cogan, U., Shinitzky, M., Weber, G., and Nishida, T. (1973), *Biochemistry 12,* 521.
Cone, R. A. (1972), *Nature (London), New Biol. 236,* 39.
Frye, L. P., and Edidin, M. (1970), *J. Cell. Sci. 7,* 313.
Hinz, H. J., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 6071.
Horwitz, A. F., Klein, M. P., Michaelson, D. M., and Cohler, S. J. (1973), *Ann. N.Y. Acad. Sci. 222,* 468.
Hubbell, W. L., and McConnell, H. M. (1968), *Proc. Natl. Acad. U.S.A. 61,* 12.
Ladbrooke, B. D., and Chapman, D. (1967), *Chem. Phys. Lipids 3,* 304.
Luzzati, V. (1968), in Biological Membranes: Physical Fact and Function, Chapman, D., Ed., New York, N.Y., Academic Press, p 71.
Luzzati, V., Gulik-Krzywicki, T., and Tardieu A. (1968), *Nature (London) 218,* 1031.
Mavis, R. D., and Vagelos, P. R. (1972), *J. Biol. Chem. 247,* 652.
Overath, P., Schairer, H. U., and Stoffel, W. (1970), *Proc. Natl. Acad. Sci. U.S.A. 67,* 606.
Papahadjopoulos, D., Jacobson, K., Nir, S., and Isac, T. (1973), *Biochim. Biophys. Acta 311,* 330–348.
Ranck, J. L., Mateu, L., Sadler, D. M., Tardieu, A., Gulik-Krzywicki, T., and Luzzati, V. (1974), *J. Mol. Biol. 85,* 249.
Sackmann, E., and Trauble, H. (1972), *J. Am. Chem. Soc, 94,* 4482.
Scandella, C. J., Devaux, P., and McConnell, H. M. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2056.
Sheetz, M. P., and Chan, S. I. (1972), *Biochemistry 11,* 4573.
Shimshick, E. J., and McConnell, H. M. (1973), *Biochemistry 12,* 2351.
Shinitzky, M., Dianoux, A. C., Gitler, C., and Weber, G. (1971), *Biochemistry 10,* 2106–2113.
Shinitzky, M., and Inbar, M. (1974), *J. Mol. Biol. 85,* 603.
Tardieu, A., Luzzati, V., and Reman, F. C. (1973), *J. Mol. Biol. 75,* 711.
Vanderkooi, J. M., and Callis, J. B. (1974), *Biochemistry 13,* 4000.
Vanderkooi, J. M., and Chance, B. (1972), *FEBS Lett. 22,* 23.
Vanderkooi, J., Fischkoff, S., Chance, B., and Cooper, R. A. (1974), *Biochemistry 13,* 1589.
Vanderkooi, J. M., and Martonosi, A. (1971), *Arch. Biochem. Biophys. 147,* 632.
Weber, G. (1956), *J. Opt. Soc. Am. 46,* 962.
Wilson, F., and Fox, C. F. (1971), *J. Mol. Biol. 55,* 49.
Yguerabide, J., and Stryer, L. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1217.