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Fluorescence Depolarization Studies of Phase Transitions and Fluidity in Phospholipid Bilayers. 1. Single Component Phosphatidylcholine Liposomes†

B. R. Lentz,‡ Y. Barenholz, and T. E. Thompson*

ABSTRACT: The fluorescence depolarization associated with the hydrophobic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene is used to monitor the changes in fluidity accompanying the gel–liquid crystalline phase transition in synthetic phosphatidylcholine dispersions. The parameters of the phase transition are determined for both large, multilamellar liposomes and small, single-lamellar vesicles. These parameters are compared with those obtained using other techniques. In addition, the data are interpreted in terms of two limiting molecular models, which in turn offer insight into the structural differences between multilamellar liposomes and small vesicles.

The fluidity of the lipid bilayer component of biological membranes has been shown by many studies to influence a variety of membrane functions. This parameter, which has been defined operationally by a number of different techniques, is dependent on both lipid composition and temperature. The most dramatic alteration in bilayer fluidity occurs as a result of the phase transition from the gel to liquid crystalline states exhibited by many lipid bilayer systems. Recent studies, summarized by Lee (1975), have suggested that some of the physiologically important properties of biological membranes may derive in part from lateral phase separation and from compressibility changes in the bilayer accompanying the phase transition.

In this paper and the following one, we report studies of the fluidity and of the phase transition characteristics in small, single-lamellar vesicles and multilamellar liposomes prepared from pure synthetic phospholipids and from mixtures of these compounds. A fluorescent probe molecule, 1,6-diphenyl-

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received March 23, 1976.* This investigation was supported by National Institutes of Health Research Grants GM-14628, and HL-17576. Barry Lentz was the recipient of a National Institutes of Health Post Doctoral Fellowship.

‡ Current address: Department of Biochemistry and Nutrition, University of North Carolina School of Medicine, Chapel Hill, North Carolina, 27514.

[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; POPC, 1-palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; DMPC, DPPC, DSPC, and DOPC are 1,2-dimyristoyl-, 1,2-dipalmitoyl-, 1,2-distearoyl-, and 1,2-dioleyl-3-*sn*-phosphatidylcholine, respectively; NMR, nuclear magnetic resonance; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

1,3,5-hexatriene (DPH),[1] first introduced by Shinitzky and Barenholz (1974), is used to monitor fluidity within the hydrophobic portion of the phospholipid bilayer and to detect changes in fluidity accompanying the gel–liquid crystalline phase transitions in these systems. A preliminary report establishing the feasibility of this approach has been presented elsewhere (Lentz et al., 1975).

Particular attention in this research has been focused on the thermal characteristics of small, single-lamellar, phospholipid vesicles first characterized by Huang (1969). The small radius of curvature of bilayers in this type of system is responsible for a set of physical properties which differ markedly from those of planar bilayers. Perhaps the most striking of these differences are the transbilayer compositional asymmetries seen in bilayers of vesicles having radii of curvature between 100 and 120 Å (Litman, 1973; Michaelson et al., 1973; Huang et al., 1974). Since the plasma membranes of cells and the membranes of subcellular organelles frequently are characterized by regions exhibiting radii of curvature in this range, the studies on small, single-lamellar vesicles suggest that very small radii of curvature regions of biological membranes may have special properties of physiological importance (Thompson et al., 1974).

Materials and Methods

Preparation of Synthetic Phosphatidylcholines. 1,2-Diacyl-3-*sn*-phosphatidylcholines (DMPC, DPPC, DSPC, and DOPC)[1] were synthesized by the method of Cubero Robles and Van den Berg (1969). 3-*sn*-Glycerolphosphorylcholine prepared from egg phosphatidylcholine (Chadha, 1970) and Sigma fatty acids (99+% pure) were used in the synthesis. The crude phosphatidylcholines were twice purified by silicic acid column chromatography and multivalent cations removed by ethylenediaminetetraacetic acid (EDTA) extraction as previously described (Suurkuusk et al., 1976). The pure phosphatidylcholines were dissolved in spectral grade chloroform and stored at −20 °C until required.

1-Palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine (POPC) was a gift from Dr. M. Roseman of this laboratory. The synthesis, purification, and characterization of this compound are described elsewhere (Roseman, M., Lentz, B. R., Sears, B., Gibbes, D., and Thompson, T. E., manuscript in preparation).

The purity of all the phosphatidylcholines was established by thin-layer chromatography on silica gel G plates with the solvent chloroform–methanol–H_2O (65:25:4). The chromatographs were heavily loaded with 1 and 2 μmol of phosphatide. Iodine staining of the chromatograms of all synthetic phosphatidylcholines showed one heavy spot and a very faint second spot having an R_f slightly larger than phosphatidylcholine. No other spots were observed. The ratio between the contaminant and the dipalmitoylphosphatidylcholine was less than 0.01 as calculated from the relative amount of [^{3}H]palmitic anhydride incorporation during synthesis. No other radioactive impurities were observed. Fatty acid analysis of the purified phosphatidylcholine showed that DMPC, DPPC, and DSPC contained greater than 99% of the expected fatty acid, while DOPC contained more than 98% oleic acid. In the case of 1-palmitoyl-2-oleylphosphatidylcholine, 99% of the fatty acid in position 1 was palmitic acid, while position 2 was 98% esterified with oleic acid. All phosphatidylcholines were stable for at least 6 months when stored as described.

The liposome and vesicle dispersions prepared from these phospholipids were also examined at the end of each individual study for signs of hydrolysis. To do this, lipids were extracted by the method of Bligh and Dyer (1959) and chromatographed on thin-layer silicic acid plates.

Preparation of Multilamellar Liposomes. Multilamellar liposomes were prepared for fluorescence studies by the method of Bangham et al. (1967) using an aqueous phase containing 50 mM KCl (extra pure Heico) and 15% (w/v) sucrose (ultra pure Schwarz/Mann). Sucrose prevented settling of the liposomes in the cuvette during the course of an experiment. The water used to prepare solutions was first deionized, then distilled from alkaline $KMnO_4$, and finally glass distilled. To a 4.5-ml portion of this suspension (0.5 mM in lipid), 1 μl of 2 mM DPH in tetrahydrofuran was added with rapid agitation. The remaining portion (2.25 ml) was retained for a light scattering blank. Samples were then gently swirled for at least 2 h above the transition temperature to allow equilibration of the DPH with the liposome bilayers. Data presented in the Results section show that the time required for DPH penetration at these temperatures was less than 90 min.

Preparation of Small, Single-Lamellar Vesicles. Small, single-lamellar vesicles, homogeneous in size, were prepared in 50 mM KCl at a temperature above the lipid phase transition using a Heat Systems W-350 Sonifier following a modification of Huang's procedure (Huang, 1969) which eliminates the molecular sieve fractionation step (Suurkuusk et al., 1976). Extensive autocorrelation light scattering studies carried out on small vesicle dispersions of a variety of phospholipids prepared by this procedure indicate that these dispersions are equivalent to Huang dispersions in all respects (Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, F., in preparation). These studies showed that the radii of DMPC and DOPC vesicles did not exceed 120 and 150 Å, respectively. To 4.5 ml of the supernatant (usually 0.4 to 0.8 mM in lipid), 1 μl of 2 mM DPH in tetrahydrofuran was added with rapid vortexing. Tetrahydrofuran (0.5 μl) without DPH was added to the remainder of each small vesicle preparation (2.25 ml), and this sample was used as a light scattering blank. Tests showed that the addition of this small amount of tetrahydrofuran had no measurable effect on the 90° scattering of representative small vesicle samples. Samples both with and without DPH were incubated above their phase transition temperature for at least 90 min in order to allow equilibration of the DPH with the vesicle wall. At all times, except during the centrifugation procedure, the samples were maintained above the temperature of their phase transition. The concentrations of lipid in dispersions were determined as inorganic phosphate by the Bartlett procedure (1959).

Fluorescence Measurements. Fluorescence depolarization of DPH as a function of temperature was measured by a method similar to that described by Shinitzky and Barenholz (1974) using a modified Perkin-Elmer MPF-3 spectrofluorometer (Suurkuusk et al., 1976). All measurements were made in the ratio mode. DPH was excited at 360 nm and its fluorescence was detected at 430 nm using instrument filter 39 as a cut-off for wavelengths below 390 nm. In all experiments, the mole ratio of phosphatidylcholine to DPH was in the range 500–2000. Control studies showed that a ratio as low as 50 had no effect of the fluorescence depolarization results. An average cooling rate of 20 °C per h was used. That this rate was sufficiently slow to allow continuous temperature equilibration within the cuvette was established by equilibrating the sample for 15 min at selected temperatures and comparing data so obtained with data obtained at these temperatures during a temperature scan. In order to be certain that small, single-lamellar vesicles were in fact studied, dispersions were stored above the phase transition temperature before use and

a cooling scan was used in all studies. This precaution was necessary since Suurkuusk and co-workers (1976) have shown that single-lamellar DPPC vesicles maintained below, but not above, their phase transition temperature exhibit time-dependent changes in their thermotropic behavior.

The general theory of the fluorescence probe depolarization technique as applied to membrane systems has been outlined in detail by Shinitzky et al. (1971). The use of DPH as a fluorescent probe specific for the hydrophobic region of lipid bilayers has been described by Shinitzky and Barenholz (1974). The technique is based on determination of the rotational motion of the probe molecule. This motion is characterized by the anisotropy parameter, r, as defined by:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} = \frac{(I_{\parallel}/I_{\perp}) - 1}{I_{\parallel}/I_{\perp} + 2} \quad (1)$$

where $I_{\parallel}$ is the fluorescence intensity parallel to and $I_{\perp}$ is the intensity perpendicular to the plane of polarization of the excitation beam. For the geometry of our sample chamber, the total fluorescence, F, is given by $I_{\parallel} + 2I_{\perp}$.

In all experiments the fluorescence anisotropy and total fluorescence intensity of DPH were calculated after correction for light scattering. The correction was made as described by Shinitzky et al. (1971). There was no depolarization due to light scattering since dilution of liposomes labeled with DPH had no effect on the fluorescence anisotropy.

As pointed out by Shinitzky and Barenholz (1974) the emission and excitation spectra of DPH in phosphatidylcholine dispersions indicate that the observed fluorescence originates from DPH within the hydrophobic region of the phospholipid bilayer. In addition, since we found the shapes of these spectra to be unaffected by the gel–liquid crystalline phase transition, the DPH probe must remain in the hydrophobic region of the bilayer at all temperatures studied.

The fluorescence anisotropy is not related in a simple way to the absolute temperature. However, the anisotropy can be related to the apparent microviscosity, η, of the probe environment (Shinitzky et al., 1971). For a homogeneous phase, the natural logarithm of the viscosity should be a linear function of $1/T$ (Eyring, 1936; Eyring and Jhon, 1969). The anisotropy may be related to the apparent microviscosity experienced by the probe by means of a modified version of the Perrin equation

$$r_0/r = 1 + [kT\tau]/[\eta v(r)] \quad (2)$$

Here r_0 is the upper limit value for r in a medium of infinite viscosity; τ, the lifetime of the excited state; η, the apparent microviscosity; and $v(r)$ the effective rotational molar volume of the probe, which is itself a function of r. k is the Boltzmann constant and T is the absolute temperature. In the work reported here, we have used the functional dependence of v on r for DPH determined by Shinitzky and Barenholz (1974) and a measured value of r_0 (0.362) in agreement with these authors. Values of the fluorescence lifetimes for DPH in liposome dispersions at various temperatures were estimated from a limiting low temperature value of 11.4 ns following the procedure of Shinitzky and Barenholz (1974). This procedure gave a value of $\tau = 10.1 \pm 0.5$ ns at 21 °C for DMPC small vesicles and a value of $\tau = 10.6 \pm 0.5$ ns at 20 °C for DPPC multilamellar liposomes. These values are in good agreement with a value of 9.8 ns obtained for small, single-lamellar DMPC vesicles at 21 °C by the phase modulation technique (Spencer, 1970) in the laboratory of G. Weber. They are also in reasonable agreement with $\tau = 9.6$ ns obtained at 20 °C for large, multilamellar DPPC liposomes by single photon counting (Yguerabide, 1972) in the laboratory of L. Brand. Both independent measurements gave a single lifetime for the excited state of the fluorophore.

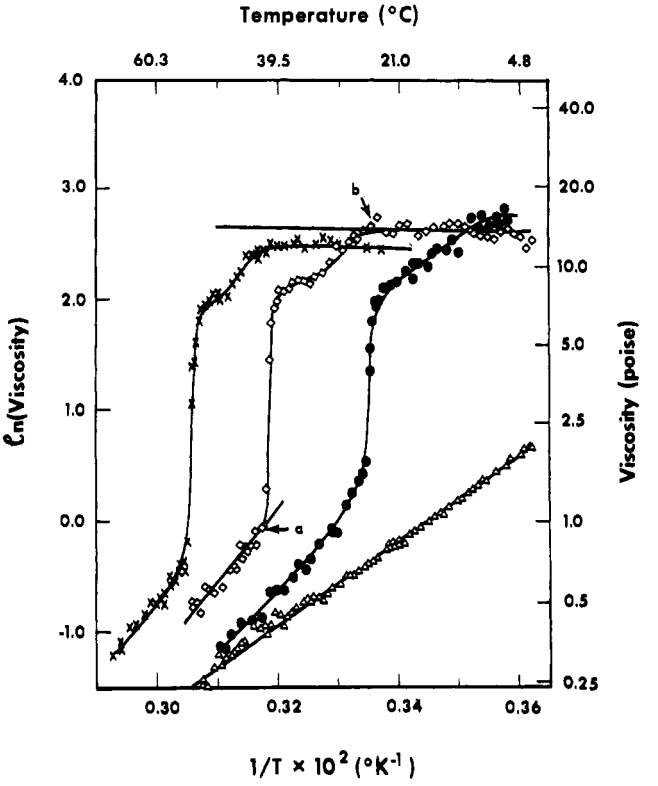

FIGURE 1: Plot of natural logarithm of the apparent microviscosity vs. (temperature)$^{-1}$ for dispersions of large, multilamellar vesicles prepared from (△) DOPC; (●) DMPC; (◇) DPPC; (X) DSPC. Arrows a and b designate limits of the transition range (see text).

The large number of data points for each experiment allowed the use of statistical methods for characterization of the plots. All calculations, plotting, and statistical analysis were done using a CDC 6400 computer in conjunction with a Cal Comp plotter.

Results

Figures 1 and 2 illustrate the effect of temperature on the microviscosities derived from fluorescence anisotropies for dispersions prepared from the four pure phosphatidylcholines. The results for multilamellar liposome preparations are presented in Figure 1, while those for small single-walled vesicles are presented in Figure 2. These data are presented as Arrhenius plots of the microviscosity. In the temperature range studied, only liposomes and vesicles prepared from DOPC show the linear Arrhenius plot expected from the theory of eyring and Jhon (1969). Liposomes and vesicles prepared from fully saturated phosphatidylcholines give Arrhenius plots which show two abrupt changes in slope. For multilamellar liposomes the temperature midway between these abrupt changes corresponds well with the temperature of the calorimetrically observed high-temperature phase transitions. Table I contains a summary of the physical parameters of these transitions in multilamellar liposomes obtained by fluorescence depolarization, scanning calorimetry, dilatometry, and spin-label partitioning. Data for the low-temperature transition are also included in Table I. Comparison of the data in Table I shows that good agreement exists among the parameters characterizing the high-temperature transitions determined by the various methods. This is not the case, however, for the

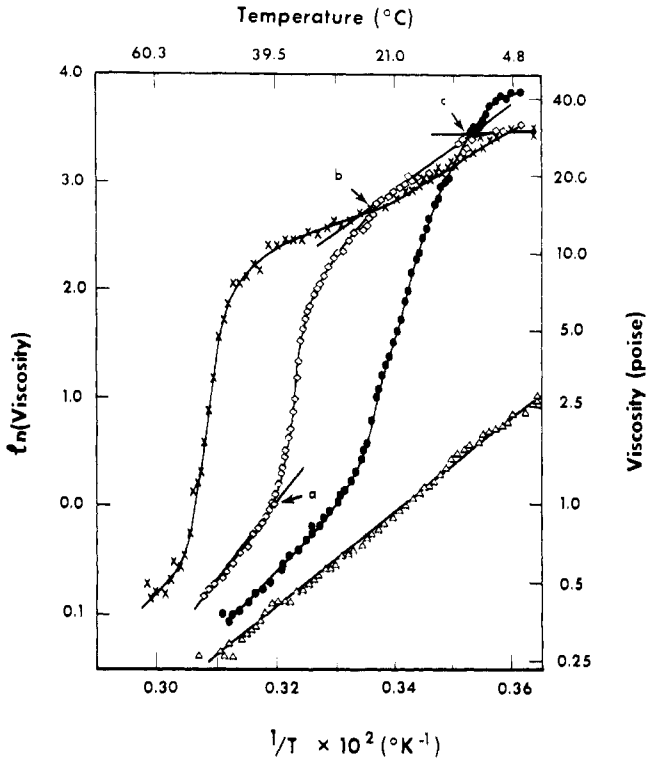

FIGURE 2: Plot of natural logarithm of the apparent microviscosity vs. (temperature)$^{-1}$ for dispersions of small, single-lamellar vesicles prepared from (△) DOPC; (●) DMPC; (◇) DPPC; (X) DSPC. Arrows a and b designate limits of the transitions range, while arrows b and c delimit a possible low temperature structural change (see text).

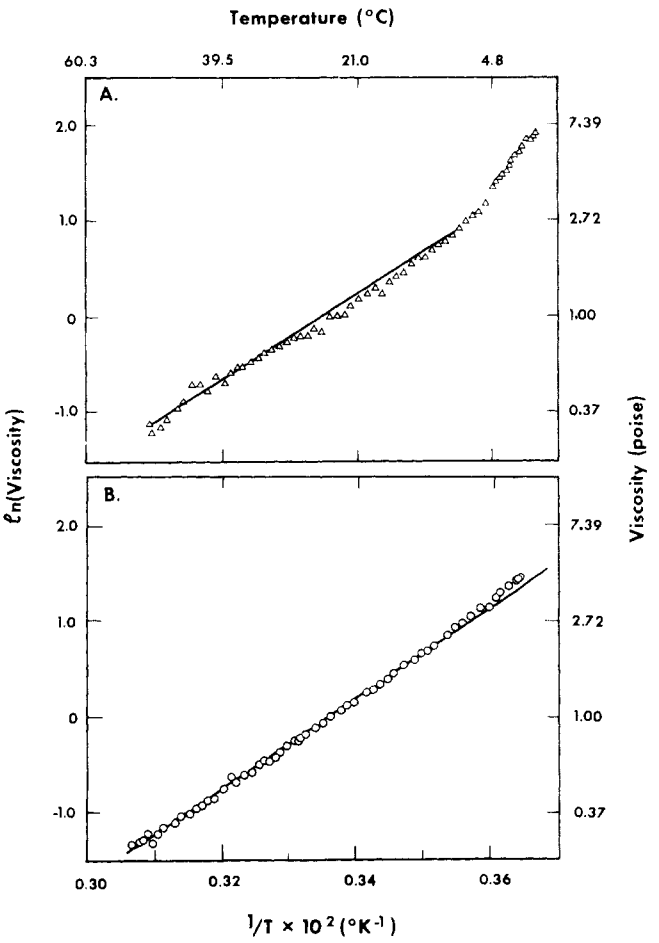

FIGURE 3: Plots of natural logarithm of the apparent microviscosity vs. (temperature)$^{-1}$ for dispersions prepared from POPC. (A) Large, multilamellar liposomes. (B) Small, single-lamellar vesicles.

low-temperature transition (see Discussion). In spite of this discrepancy, it is interesting to note that the temperature difference between the low- and high-temperature T_m values determined fluorimetrically decreases with increasing acyl chain length as first noted by Chapman and co-workers (1967).

The phase transition characteristics for small, single-lamellar vesicles derived from Figure 2 are summarized and compared with literature values in Table II. It is apparent that the small vesicles exhibit a single rather broad transition in contrast to the behavior of large, multilamellar liposomes. Furthermore, the T_m value for this transition lies between the low- and high-temperature T_m values obtained for the same phosphatidylcholine in multilamellar liposomes. Similar results have been reported by Sheetz and Chan (1972) and Suurkuusk et al. (1976) for DPPC.

The phase transition properties determined by fluorescence depolarization summarized in Tables I and II were obtained as follows. With large, multilamellar liposomes, the regions of abrupt change in the microviscosity are well defined. The transition range was determined as illustrated in Figure 1 for DPPC. Points a and b delimit the phase transition. The limit temperatures were difficult to determine in the region between the low- and high-temperature transitions, especially when these transitions were closely spaced. In the case of small vesicles, the transition was well defined on the high-temperature side as is shown in Figure 2. However, on the low-temperature side of the transition, it was not possible to draw a single straight line through all data. In each case, at least two and sometimes three lines were needed to fit the data below the transition. Points b and c of Figure 2 delimit one such line. Whether or not the curvature in this region reflects subtle reorganizations in the lipid bilayers of small vesicles below the transition is as yet unclear. It may be noted, however, that calorimetric studies of DPPC small vesicles (Suurkuusk et al., 1976) show baseline anomalies below the phase transition. In order for the fluorescence depolarization data to be consistent with the calorimetric data, it is necessary to take the low-temperature limit of the phase transition to be determined by the first straight line that could be fit to the data below the transition (i.e., point b, Figure 2).

Most naturally occurring phosphatidylcholines have an unsaturated fatty acid esterified in position 2 of thee glyceryl-phosphorylcholine and a saturated fatty acid (usually palmitic) esterified in position 1. For this reason the thermotropic characteristics of synthetic 1-palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine (POPC) were examined. Arrhenius plots of the microviscosity of POPC multilamellar liposomes and small vesicles are presented in Figure 3. The data in Figure 3A suggest that POPC in multilamellar liposomes may undergo a phase transition beginning about 8 °C. Recorded in Figure 3B is the Arrhenius plot of the microviscosity for small vesicles composed of POPC; this plot is essentially linear to about 4 °C. The absence of any suggestion of a transition in the small vesicles composed of POPC may be due to the broadening and shifting of the transition as observed for other small vesicle systems. Recently, a differential scanning calorimetry study has reported a phase transition for POPC centered at 3 °C (op den Kamp et al., 1975), while an earlier study had given a value of −5 °C (De Kruyff et al., 1973).

Incorporation of DPH into Liposomes. The incorporation

TABLE I: Phase Transition Parameters of Large Multilamellar Vesicles.

			High-Temp Transition			Low-Temp Transition			
Reference	Method	Lipid	T_{m_1}	Range[a]	ΔT^a	T_{m_2}	Range[a]	ΔT^a	$T_{m_1}-T_{m_2}$[j]
This work	DPH fluorescence depolarization	DMPC	24.4 ± 0.5[b]	22.5–29.6	7.1		9.3–14.1[c]	4.8	12.7
		DPPC	41.1 ± 0.5[b]	37.3–42.4	5.1		25.2–33.9[c]	8.7	11.5
		DSPC	54.0 ± 0.5[b]	50.7–55.1	4.4		42.5–47.9[c]	5.4	8.8
Hinz and Sturtevant (1972)	Differential scanning calorimetry	DMPC	23.70 ± 0.09			13.5 ± 0.2			10.2
		DPPC	41.75 ± 0.06	40.3–44.0[e]	3.7	34.0 ± 0.2	30.3–36.5	6.2	7.7
		DSPC	54.24 ± 0.03[d]	52.8–56.0[e]	3.2	49.1 ± 0.2	47.5–51.2	3.7	5.1
Suurkuusk et al. (1976)	Differential scanning calorimetry	DPPC	41.2 ± 0.1	39.4–42.2[f]	2.8	34.4 ± 0.1	33.2–36.7[f]	3.5	6.8
Nagle (1975)	Dilatometry	DPPC	41.5[g]	41.3–42.4[g]	1.1		32.9–36.1[c]	3.2	
Sheetz and Chan (1972)	Dilatometry	DPPC	41.5[h]	39–44[h]	5				
Shimshick and McConnell (1973)	Partitioning of Tempo spin-label probe	DMPC	23.2	21–24[i]	3	10.1	7–11[i]	4	13.1
		DPPC	40.5	38–42[i]	4	29.5	25–31[i]	6	11.0
		DSPC	54.0	52–56[i]	4	46.1	42–49[i]	7	7.9

[a] Temperature range of the transition. [b] Probable standard error assigned on the basis of the ttemperature spread between data points. I_m assigned as the midpoint of the range of maximum change in microviscosity. [c] No basis for assigning T_m; only range can be defined. [d] Corrected value. Table I of Hinz and Sturtevant has 58.24 °C, but Figure 3 of that paper clearly indicates a transition temperature of about 54 °C. [e] Taken from Figure 1 or Figure 3 of Hinz and Sturtevant (1972). [f] Taken from Figure 1 of Suurkuusk et al. (1976). [g] Taken from Figure 1 of Nagle (1973) (25% w/w dispersion). T_m taken as temperature of ½ change. [h] Taken from Figure 8 of Sheetz and Chan (1972). T_m taken as temperature of ½ change. [i] Taken from Figure 2 of Shimshick and McConnell (1973). [j] T_{m_2} is defined operationally as the midpoint of the range of the transition in those cases where only a range can be accurately defined.

TABLE II: Phase Transition Parameters of Small Single-Lamellar Vesicles.

			Transition		
Reference	Method	Lipid	T_m	Range[a]	ΔT^a
This work	DPH fluorescence depolarization	DMPC	20.9 ± 0.5[b]	14.3–27.4	13.1
		DPPC	36.4 ± 0.5[b]	29.7–40.6	10.9
		DSPC	51.3 ± 0.5[b]	46.5–53.8	7.3
Suurkuusk et al. (1976)	Differential scanning calorimetry	DPPC	36.9 ± 0.1		
Sheetz and Chan (1972)	Dilatometry	DPPC	39[c]	33–44[c]	11

[a] Temperature range of the transition. [b] Probable standard error assigned on the basis of the temperature spread between data points. T_m assigned as the midpoint of the range of maximum change in microviscosity. [c] Taken from Figure 8 of Sheetz and Chan (1972). T_m taken as temperature at ½ total change in the volume across the transition region.

of DPC into small vesicles can be easily followed since DPH has nearly zero fluorescence intensity in an aqueous environment. The effect of the gel–liquid crystalline phase transition on the incorporation of DPH into DMPC and DPPC small vesicles is shown in Figures 4A and 4B, respectively. In these figures the fraction of DPH incorporated into the lipid bilayer is plotted as a function of time. The fraction incorporated is given by the total fluorescence intensity, F, at any time divided by the total fluorescence intensity at a very long time, F_∞.

Analysis of these data clearly shows that DPH incorporation does not follow first-order kinetics. At least two separate processes can be detected. Of greater interest, however, is the marked change in uptake of the DPH below the temperature range of the gel–liquid crystalline phase transition. Comparison of Figures 4A and 4B shows that above the phase transition, incorporation is nearly identical in both lipids and quite rapid (DMPC at 33 and 45 °C; DPPC at 45 °C). Within the range of the transition (see Table II), incorporation is still rapid (DMPC at 21 °C and DPPC at 33 °C). Comparison of the results for DMPC and DPPC at 33 °C indicates that the rate of dye uptake is similar if the vesicles are at a temperature within the range of the phase transition or just above the tra-isition. However, if the temperature is below the lower limit of the phase transition range for one lipid, a marked difference in the rate of DPH uptake occurs (compare DPPC with DMPC at 21 °C). Uptake is nearly identical and very slow for both lipids below their phase transitions.

The uptake of DPH by multilamellar liposomes is a more complex process than uptake by small vesicles. The fluorescence intensity increases in a stepwise fashion after addition of a small amount of DPH in tetrahydrofuran. After about 90 min above the phase transition the intensity levels off and

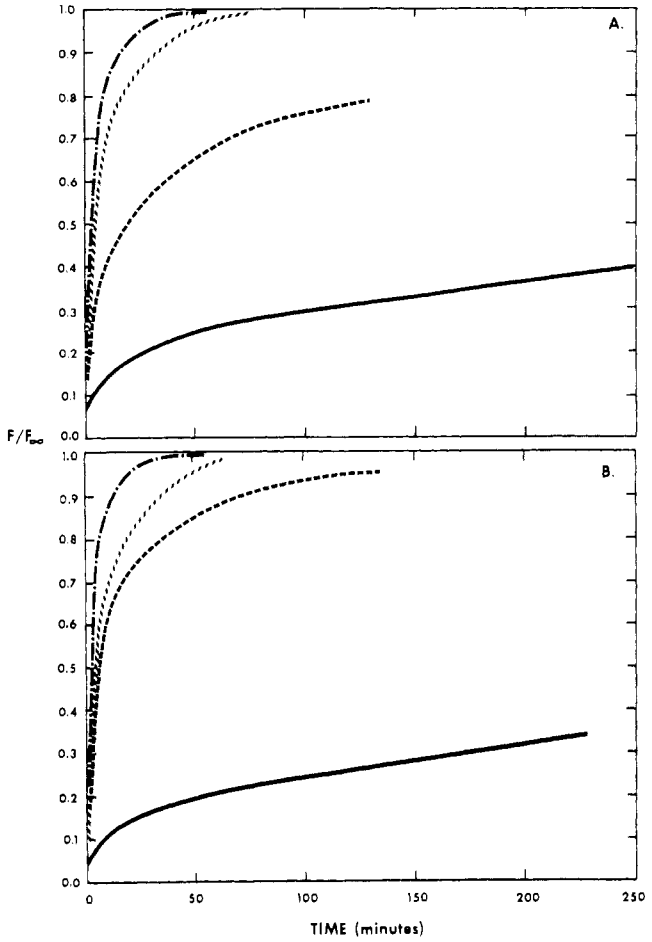

FIGURE 4: DPH incorporation into small, single-lamellar vesicles of DMPC (A) and DPPC (B) as a function of time at four temperatures (—) 3-9 °C; (— — —) 21.1 °C; (- - -) 33.0 °C; (- · · · -) 45.5 °C.

thereafter remains constant. The stepwise increase is probably due to the multi-layered structure of large Bangham-type vesicles.

Discussion

The use of eq 2 is based on three assumptions which should be considered in more detail (Weber, 1953): (1) the DPH excited state relaxes with a single lifetime; (2) the rotating fluorophore behaves as a classical body undergoing Brownian rotation; and (3) the resistance to rotation of the fluorophore can be described by hydrodynamics as applied to macroscopic bodies. Assumption 1 is reasonably well satisfied in the case of DPH dispersed in pure lipid systems since a single lifetime was found for DMPC and DPPC (see Materials and Methods). In bilayers composed of mixed lipid in the temperature range of the gel-liquid crystalline phase transition, however, there probably coexist regions of different fluidity. The detailed behavior of the observed anisotropy in such cases is considered in the following paper (Lentz et al., 1976). Although assumptions 2 and 3 cannot be strictly valid for molecular motions, their use may be justified as follows. The detailed molecular interactions that hinder the random rotational motions of a probe molecule in an oriented lipid bilayer must be different from the integrated interactions expressed as a macroscopic viscosity. Only in a relative sense can the hindered motions in these two systems be compared. Thus, an increase in the rotational freedom of DPH in a lipid bilayer is associated with decrease in macroscopic viscosity that accompanies an increase in rotational freedom of DPH in a hydrocarbon oil. It is in this relative sense that the apparent viscosities obtained by means of eq 2 are used.

This paper clearly demonstrates the utility of DPH in monitoring the gel-liquid crystalline phase transitions of phospholipids in bilayer array. The thermotropic behavior of five phosphatidylcholines are reported, and, in each case, the DPH fluorescence depolarization data are in good agreement with the results obtained by other techniques. The extreme sensitivity of the fluorescence depolarization technique combined with its simplicity suggests its applicability to biological membrane studies.

Agreement between fluorescence depolarization results and calorimetrically determined data was not particularly good for the low-temperature transition displayed by multilamellar liposomes. Fluorescence depolarization gave T_m values lower by about 5 °C than the values determined by calorimetry. It is interesting, however, that the temperature of the transition determined by the electron spin resonance probe, Tempo, is in essential agreement with the depolarization of fluorescence results in Table I (Shimshick and McConnell, 1973). Since both our study and that of Shimshick and McConnell employed cooling scans, while both calorimetric studies (Hinz and Sturtevant, 1972; Suurkuusk et al., 1976) employed heating scans, it is possible that the discrepancies between these two sets of results are due to hysteresis. The discrepancy cannot be due to the presence of sucrose in the dispersion studied by fluorescence depolarization (Suurkuusk et al., 1976).

It is interesting to compare in more detail the thermotropic behavior of the various phosphatidylcholines examined. These differ either in the length of the acyl chains or in the presence of cis double bonds. Figures 1 and 2 show that the Arrhenius plots of microviscosity for the three saturated lecithins have nearly the same slope above the gel-liquid crystalline phase transition. This is true for both the small vesicles and multilamellar liposomes. The behavior of these three phosphatidylcholines in the region of their phase transitions is, however, far from identical. As shown in Tables I and II, the temperature range of the phase transition in both single-lamellar vesicles and multilamellar liposomes decreases as the length of the acyl chains increases. This leads to the reasonable speculation that with increasing chain length increased opportunities for steric interactions between the chains lead to increased cooperativity in the phase transition.

Also of interest is the difference in slopes of the microviscosity Arrhenius plots for saturated and unsaturated phosphatidylcholines above their phase transition temperatures. The plots for the multilamellar liposomes comprised of saturated phosphatidylcholines all have slopes of $6300 \pm 400\ K^{-1}$, while that for DOPC has a slope of $3700 \pm 32\ K^{-1}$. The POPC multilamellar vesicles yield an Arrhenius plot with a slope of $4150 \pm 84\ K^{-1}$, a value intermediate between but not the average of the values for saturated and unsaturated phosphatidylcholines. The Arrhenius plots for the small, single-lamellar vesicles show similar results: saturated lecithins, $6200 \pm 640\ K^{-1}$; DOPL, $4336 \pm 50\ K^{-1}$; POPC, $4860 \pm 34\ K^{-1}$. Thus, it appears that the introduction of unsaturated fatty acids into the phosphatidylcholine molecules results in a decrease in the temperature dependence of the effective microviscosity above the transition temperatures. The effects of unsaturated phosphatidylcholines in bilayers composed of mixtures of phospholipids are discussed in the accompanying paper (Lentz et al., 1976).

Bashford and co-workers (1976) have very recently reported fluorescence depolarization measurements on DOPC liposomes

using several different fluorophores. These authors describe small deviations from linear Arrhenius behavior below 30 °C in this system and others containing DOPC which they point out may be due to "cluster" formation as first suggested by Lee et al. (1974). The apparent linearity of our data for DOPC in both small vesicles and multilamellar liposomes (Figures 1 and 2) suggests that, if such "clusters" do exist, they are so small or so short-lived that the DPH molecule does not partition into them as a separate phase within the bilayer. However, it is also quite possible that the observed deviations from linearity are due to the presence of impurities. Experience in our laboratory has shown that contamination by oxidation products may markedly modify bilayer parameters.

Our results clearly show that large differences exist between the effective microviscosities within bilayers of small, single-lamellar vesicles and multilamellar liposomes. Above the gel–liquid crystalline phase transition, both the microviscosities and microviscosity activation energies are nearly identical for both types of dispersions. In the temperature range of the phase transition, however, the microviscosity activation energy for multilamellar liposomes is much larger than for small vesicles. At still lower temperatures, the multilamellar structures undergo a low-temperature transition which is not observed for the small vesicles. Tables I and II show, however, that the temperature range of the phase transition in small vesicles corresponds very closely to the temperature range between the high- and low-temperature transitions in the multilamellar liposomes. This suggests that the two discrete transitions taking place in multilamellar liposomes may merge into one apparently continuous transition in the small vesicles. The low-temperature transition has been suggested to be due to rearrangements in the polar head group portion of the phosphatidylcholine molecule (Ladbrooke and Chapman, 1969) and more recently (Rand et al., 1975) to conformational changes in the phosphatidylcholine acyl chain moiety. Our results favor the latter interpretation since we detect this transition with a probe that is located in the hydrophobic portion of the phospholipid bilayer (Shinitzky and Barenholz, 1974). Below the low-temperature transition of the multilamellar structures, further cooling has almost no effect on the microviscosity within the bilayer; and the activation energy is close to zero. The small vesicles, on the other hand, show a continuous increase in microviscosity with cooling below the phase transition, such that at about 5 °C the microviscosity within these vesicles is nearly twice that within the multilamellar species (Figures 1 and 2).

In terms of the arrangement of molecules within the bilayer, these results suggest that both liposome systems have similar molecular packing densities at temperatures above the phase transition. However, at temperatures well below the phase transition temperature, the DPH is able to undergo more rapid rotations in multilamellar liposomes than in small vesicles. The different behavior of the DPH can be rationalized on the basis of either of two simple models describing the molecular packing in multilamellar liposomes and single-lamellar vesicles.

In one model the assumption is made that, just below the low-temperature transition, the lipid molecules in multilamellar vesicles are arranged in a regular array which does not change as the temperature is lowered. This assumption is consistent with the fact that the anisotropy (or microviscosity) shows no increase with decreasing temperature below the low-temperature transition. The constant value of 0.29–0.31 for the anisotropy indicates that the probe still retains considerable rotational freedom at these low temperatures in the multilamellar species. Thus, although the structure of the lipid

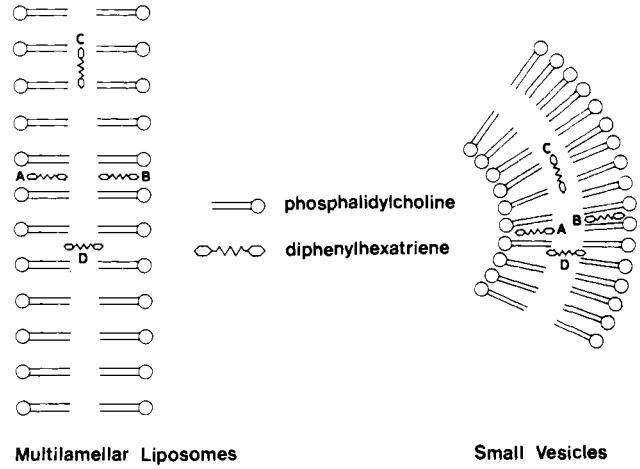

FIGURE 5: Schematic representation of fluorescence probe locations within the hydrophobic core of bilayers in multilamellar liposomes and small, single-lamellar vesicles.

array is regular and relatively fixed, it affords an occupation volume to the probe molecule which is sufficient to permit considerable rotational freedom.

In small, single-lamellar vesicles, on the other hand, at temperatures just below the phase transition, the molecular arrangement in the hydrocarbon core of the bilayer is a less ordered glass-like structure. This is the direct result of molecular packing constraints imposed by the small radius of curvature of the bilayer in these vesicles. Upon further cooling below the transition temperature, such a glass-like structure would be expected to undergo additional molecular reorganizations, each of which further restricts the motion of the probe. The result is a continuous increase in the anisotropy (microviscosity) as observed.

An alternative model which rationalizes the different rotational behavior of the probe molecule in multilamellar liposomes and small vesicles is illustrated in Figure 5. In this figure, the bilayers of both multilamellar liposomes and small vesicles are represented schematically. Several of many possible positions of the DPH probe in the bilayer are designated A, B, C, D. Each position may be considered to be a possible free energy state for the probe. Thus, there exists a temperature-dependent distribution of DPH molecules between all such possible positions. For example, entropically favored, enthalpically disfavored states will be more populated at higher temperatures and will be characterized by somewhat larger anisotropies.

In terms of this simplified model, the probe molecule has a choice of three states in the multilamellar liposomes at temperatures just below the low-temperature phase transition: A (=B), C, and D. The invariance of the fluorescence anisotropy below the phase transition suggests that either these states each impose about the same degree of rotational inhibition on the probe molecule (i.e., all result in similar anisotropies), or one state predominates at all temperatures below the gel–liquid crystalline phase transition. In the case of the small, single-walled vesicles, the probe molecules have a choice of four distinctly different states, A, B, C, and D. Thus, at temperatures just below the phase transition, an equilibrium should exist between states A, B, C, and D, such that the average anisotropy observed is similar to the average anisotropy observed in the large liposomes. As the temperature is further lowered, however, state B (the enthalpically favored state) becomes increasingly more favored relative to state A (the entropically

favored state) and the anisotropy increases, until at very low temperature, very few molecules exist in state A and the average anisotropy becomes considerably larger than in multilamellar liposomes. This model, then, accounts for the anisotropy (or microviscosity) differences observed between large, multilamellar liposomes and small vesicles at low temperature by the added variation in the microscopic environments of small vesicles relative to large ones.

Both models, representing logical extremes, are consistent with the suggestion based on proton NMR data that the molecular disorder in small vesicle bilayers is greater than in multilamellar liposomes (Sheetz and Chan, 1972). The essential difference between the two models is that the first postulates temperature-induced structural changes in the bilayer structure of small vesicles as they are progressively cooled below the phase-transition temperature, while the second requires no structural change in the bilayer, but only that the relative population of the various DPH binding sites change with temperature. Evidence for possible structural changes in the bilayers of small vesicles below the phase transition is found in the baseline anomalies reported in the excess heat capacity scans reported by Suurkuusk et al. (1976). No such anomalies were observed for multilamellar vesicles.

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