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Partition of a Fluorescent Molecule between Liquid-Crystalline and Crystalline Regions of Membranes

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Summary. We have determined the partition coefficient of the fluorescent molecule perylene between liquid crystalline and crystalline regions of vesicle membranes formed from binary mixtures of several lipids. We measured the fluorescence intensity of perylene in these vesicles as a function of temperature and used the intensity profiles, together with a theory developed in a previous paper, to determine the partition coefficient defined as the ratio of the concentration of perylene in the liquid-crystalline (fluid) regions of the membrane to the concentration in the crystalline (solid) phase. In vesicles composed of dipalmitoyl phosphatidylcholine/distearoyl phosphatidylcholine (dppc/dspc) mixtures and of dipalmitoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine (dppc/dppe) mixtures, the partition coefficient is close to unity. Its value is 1.04 ± 0.18 for dppc/dsp mixtures and 1.10 ± 0.26 for dppc/dppe mixtures. In vesicles composed of dimyristoyl phosphatidylcholine/distearoyl phosphatidylcholine mixtures, the partition coefficient was more difficult to determine and its value ranged from 0.3 to 7.

Information concerning the location or locations of fluorescent molecules within a membrane is essential for the interpretation of changes in the fluorescence properties of these molecules in terms of changes in membrane parameters. The depth of a particular probe molecule from the lipid bilayer surface has generally been inferred from the structure of the molecule and the spectral properties of its emission in the membrane in comparison with its behavior in solvents of different polarities (Waggoner & Stryer, 1970; Radda & Vanderkooi, 1972; Sackman & Träuble, 1972). The effect of lipid insoluble solutes on the spectral properties of the probe have also been used to determine its location (Stubbs, Litman & Barenholz, 1976). For example, probes located in the hydrocarbon interior of the bilayer are little affected by solutes confined to the aqueous solution. X-ray (Lesslauer, Cain & Blasie, 1972) and NMR (Podo & Blasie, 1977) measurements on fluorescent-labeled multibilayers have also been used to determine the location of a probe with respect to the bilayer surface.

The extent to which a probe partitions between liquid crystalline (fluid) and crystalline (solid) regions of a lipid bilayer is also of interest because the partition coefficient between these phases determines the usefulness of the probe in studying, for example, the importance of lateral phase separations and changes in microviscosity in membrane functions. The partition of some fluorophores between

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the fluid and solid phases has been inferred from the magnitude of fluorescence-intensity changes occurring at a phase transition and from the transfer of probes from vesicles of one composition to those of another composition (Bashford, Morgan & Radda, 1976; Lentz, Barenholz & Thompson, 1976*b*; Sklar, Hudson & Simoni, 1977)

It has been suggested that the amphipathic fluorophore 12-(9-anthroyloxy)-stearic acid (AS) partitions preferentially into the fluid phase of a bilayer composed of dipalmitoyl phosphatidylcholine and dilauroyl phosphatidylcholine, lipids which do not cocrystallize (Bashford *et al.*, 1976). This suggestion was based on the observations that the probe AS did not detect the upper phase transition of this lipid mixture, although AS is sensitive to phase transitions in a mixture of dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine in which the lipids do cocrystallize (Bashford *et al.*, 1976). It is interesting to note that a spin-labeled stearic acid probe also partitions preferentially into the fluid regions of a membrane (Butler, Tattrie & Smith, 1974).

The partition of the amphipathic probe paranaric acid between liquid-crystalline and crystalline regions of the bilayer has been found to depend upon whether the chains are in a cis (α) or trans (β) conformation (Sklar *et al.*, 1977). Those in the cis conformation partition equally between the two phases and those in the trans conformation partition preferentially into the crystalline regions with a partition coefficient of about 3 (Sklar *et al.*, 1977). The partition coefficients were estimated from the magnitude of the fluorescence-intensity changes occurring at the lipid transition temperatures after the probe had reached an equilibrium distribution in a mixture of pure dimyristoyl phosphatidylcholine vesicles and pure distearoyl phosphatidylcholine vesicles (Sklar *et al.*, 1977). Similar results were obtained with a mixture of pure dipalmitoyl phosphatidylcholine vesicles and pure dielaidoyl-phosphatidylcholine vesicles (Sklar *et al.*, 1977).

Partition studies have also been done on two lipophilic fluorescent probes diphenylhexatriene (DPH) and perylene which are thought to be located within the hydrocarbon region of the bilayer and which have been used extensively in studies of the microviscosity of membranes (Shinitzky *et al.*, 1971; Cogan *et al.*, 1973; Shinitzky & Barenholz, 1974; Shinitzky & Inbar, 1974; Fuchs *et al.*, 1975; Barenholz, Moore & Wagner, 1976; Lentz, Barenholz & Thompson, 1976*a*; Lentz *et al.*, 1976*b*; Stubbs *et al.*, 1976; Feinstein, Fernandez & Sha'afi, 1975). Diphenylhexatriene has been found to partition equally between pure vesicles of dimyristoyl phosphatidylcholine in the fluid phase and pure vesicles of dipalmitoyl phosphatidylcholine in the solid phase (Lentz *et al.*, 1976*b*). On the other hand, the partition of perylene (but not DPH) in vesicles of phosphatidic acid is complex and cannot be explained in terms of a simple partition between solid and fluid phases (Jacobson & Papahadjopoulos, 1975).

The object of this paper is to determine from intensity *vs.* temperature graphs and a phase diagram the partition coefficient of perylene between the liquid-crystalline and crystalline regions of specific two-component lipid bilayers, by the theory developed in the previous paper (Yguerabide & Foster, 1979). We became interested in the partition coefficient of perylene because of studies in which we used this probe to evaluate the role of microviscosity in membrane functions (Kehry, Yguerabide & Singer, 1976; Masters, Yguerabide & Fanestil, 1978; Fos-

ter & Vandenberg, *unpublished*). It has negligible solubility in aqueous solution, a property which is convenient, although not essential, for the application of the theory developed in the previous paper. Binary bilayers with three different compositions were used for this study. These binary systems, dipalmitoyl phosphatidylcholine/distearoyl phosphatidylcholine (dppc/dspc), dimyristoyl phosphatidylcholine/distearoyl phosphatidyl choline (dmpc/dspc), and dipalmitoyl phosphatidylethanolamine/dipalmitoyl phosphatidylcholine (dppe/dppc), were chosen to be representative of the most common types of phase diagrams that have been reported for lipid bilayers. Mixtures of dipalmitoyl phosphatidylcholine and distearoyl phosphatidylcholine cocrystallize; mixtures of dimyristoyl phosphatidylcholine and distearoyl phosphatidylcholine and mixtures of dipalmitoyl phosphatidylethanolamine and dipalmitoyl phosphatidylcholine exhibit a limited solubility in the crystalline state (partial cocrystallization). A preliminary account of this work has appeared (Foster & Yguerabide, 1977).

Materials and Methods

Lipids

Dipalmitoyl phosphatidylcholine (dppc), dimyristoyl phosphatidylcholine (dmpc), and distearoyl phosphatidylcholine (dspc) were purchased from Sigma or Calbiochem. Chloroform solutions of the lipid, about 20 mg/ml, were stored in the freezer until use. The purity of the lipids was judged from thin layer chromatography and gas liquid chromatography. For thin layer chromatography the plates were loaded with about 1 μmol of each lipid and developed with chloroform/methanol/water volume ratios (65 : 25 : 4). Only one spot was observed for each lipid after the plates were visualized with rhodamine, iodine, and sulphuric acid and the head groups were estimated to be greater than 99% pure. No lysolecithin nor lysophosphatidylethanolamine was detected, and contamination with lyso-products was estimated to be less than 1%, by comparison with runs made with lysolecithin. From gas liquid chromatography, the lipid chains were found to be greater than 99% homogeneous, the heterogeneity in each case amounting to no more than a few tenths of a percent. In dspc and in dmpc the contamination with other chain lengths was estimated to be less than 0.1%; in dppc and dppe there was a 0.5% to 0.7% contamination with an 18-carbon chain.

Fluorescent Probes

Perylene was purchased from Aldrich (99%) and stored in the freezer in an acetone solution (about 40 μg/ml) until use. N-phenyl-1-naphthylamine (NPN) was a gift from H. Träuble.

Formation of Vesicles

Nonsonicated dispersions of the lipid in buffer were prepared by the method of Bangham (Bangham, Standish & Watkins, 1965), a method which has been shown to produce

multilammellar vesicles. We elected not to sonicate the vesicles because of uncertainties which have been reported concerning the structure, stability and possible asymmetry of different lipids in sonicated bilayers (Litman, 1973; Michaelson, Horwitz & Klein, 1973; Berden, Barker & Radda, 1975; Lentz *et al.*, 1976*a,b*; Suurkuusk *et al.*, 1976; for a review, *see* Yguerabide, 1978). During preparation of the dispersions the temperature was maintained above the highest transition temperature of the lipids. For perylene-labeled vesicles, aliquots of the lipid and the probe dissolved in organic solvent were added to a round-bottomed flask and mixed. The mixed solvent was then evaporated under a stream of nitrogen; 3 to 8 ml heated buffer, together with several glass beads, were added to the flask; and the sample was agitated manually for 30 to 60 sec. For NPN-labeled vesicles, a similar procedure was followed, except that NPN was added to the buffer. For both fluorescent probes, the lipid/probe ratio was about 1000:1. The dispersion contained about 0.3 mg lipid/ml and had an OD of about 0.5 at 500 nm, as measured in the Cary-14 spectrometer. Some test runs were made with other lipid/probe ratios and with other lipid concentrations for perylene in pure dppc. In the range 360 : 1 to 3600 : 1, the transition temperature observed was relatively insensitive to the lipid/probe ratio and to the lipid concentration. All runs were made with 0.1 M sodium phosphate buffer at pH 7.5, although dispersions with dppe in this buffer had a tendency to floculate and settle out. A test run with dppe in 0.01 M phosphate indicated that a buffer of lower ionic strength might have been a better choice for dppe.

Fluorescence Measurements

Fluorescence measurements were made in an instrument constructed in the laboratory by one of the authors (JY). The sample solution (stirred with a magnetic button in the cuvette) was excited with a Bausch and Lomb xenon lamp or a HBO 200 watt AC mercury lamp through a monochromater and an interference filter (Corion 405-nm Hg filter, bandwidth 10 nm for perylene; Corion 334 Hg filter for NPN). The emission was observed at right angles through a cut-off filter (Corning 3-73 filter for perylene; Corning 3-75 filter for NPN). For perylene the signal from scattered light under these conditions was about 3 to 4% of the fluorescence signal and was due primarily to Raman scattering, as judged from the spectrum. Some runs were made without the emission filter in order to observe the change in scattering over the phase transition. The lamp intensity was monitored during the run with the use of a quartz plate beam splitter and rhodamine B counter. Current from the photomultiplier (EMI 6256S in the rhodamine B counter and either EMI 6256S or RCA 1P28 for the fluorescence emission) was measured with a picoameter (Keithley 414S), the output of which was fed into a ratio meter (PAR 230). The output of the ratio meter, which is proportional to the ratio of the fluorescence and the lamp intensities, drove the y-input of a Hewlett-Packard 7004B x-y recorder. Abrupt changes in geometry of the arc of the zenon lamp, for which the ratio mode described above does not compensate, were minimized by placing a magnetic stirrer adjacent to the lamp. The sensitivity of the instrument was constant within a few tenths of a percent for several hours. Current oscillations due to the AC component of the mercury lamp were damped with an RC filter before the picoameter. The intensity measurements were made with polaroid filters located in the excitation and emission beams, exciting with vertically polarized light and monitoring emission at 55° from the vertical. (The response of the emission photomultiplier to vertically and horizontally polarized light differed by about 5%.) With this arrangement of polaroid filters, the signal from the sample is proportional to the intensity and is not affected by changes in polarization.

The temperature of the sample was monitored with a temperature probe placed directly in the cuvette, and the output of this thermometer (Hydrolab Model ET100) was fed into the x-input of the x-y recorder. The temperature varied by about 0.3 °C as a function of position in the cuvette. The temperature of the sample was controlled by means of a thermostated cuvette holder through which water was circulated from a Lauda K-2/R temperature bath. Since the sample was initially above the transition temperature, the I *vs.* T profiles were recorded by cooling the sample at a rate of about 0.1 to 0.8 °C per min. However, both heating and cooling runs were often made to check hysteresis and reproducibility. The data presented here are from the cooling experiments.

Results and Analysis

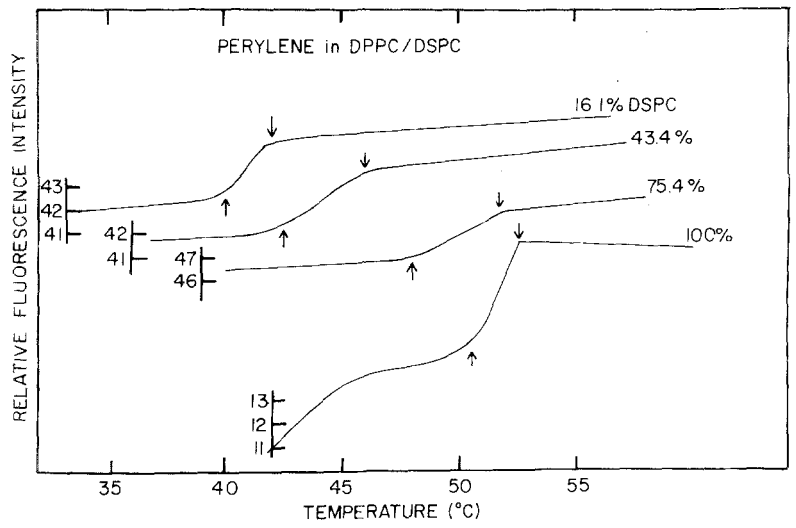
Intensity vs. Temperature Profiles

The temperature dependence of the fluorescence intensity measured for perylene in lipid mixtures of dppc/dspc, dppc/dppe and dmpc/dscp are shown in Fig. 1*a-c*. The graphs are shown as recorded by cooling except that the zero base line has been displaced to facilitate the presentation of the graphs. At temperatures above and below the phase transition region, the fluorescence intensity is not strongly dependent on temperature, the rate of change being less than 0.25% per degree. The phase-transition region in most of the graphs is marked by abrupt changes or breaks in slope at two temperatures. These temperatures are shown by arrows in the plots and are interpreted as the temperatures for the onset and termination of a phase transition. The fluorescence intensity decreases in going from the fluid to the solid phase for the pure lipid vesicles by about 5–10% for dppc and dmpc, 15–25% for dspc, and greater than 40% for dppe. The magnitude of the fluorescence-intensity change during a transition for a mixed lipid vesicle is between the magnitudes displayed by the pure lipid vesicles. A "prephase" transition is seen at still lower temperatures for mixtures of phosphatidylcholine and for the mixture with 20% mole fraction phosphatidylethanolamine. The transitions in pure lipids are sharp, occurring within a temperature range of less than 1° in PC vesicles and 2-3° in PE vesicles. The transition temperatures observed for the pure lipids are in agreement with those found by calorimetry (Hinz & Sturtevant, 1972), by ESR (Shimshick & McConnell, 1973), and by fluorescence measurements with other fluorescent probes (Vanderkooi & Chance, 1972; Lentz *et al.*, 1976*a*). With mixtures of lipids, the transitions take place over a more extended range of temperatures between the transition temperatures of the pure components. Fig. 2*a-c* shows I *vs.* T profiles obtained with NPN as fluorescent probe.

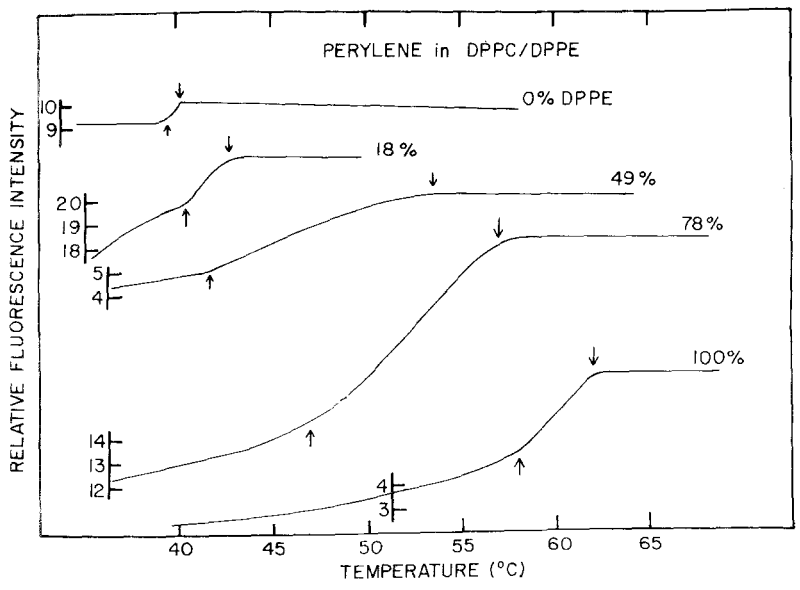
Determination of Phase-Diagram

The method that we use to determine partition coefficients requires phase diagrams. Phase diagrams for the binary systems used in this study have been reported in the literature by several investigators (Phillips, Ladbrooke & Chapman,

1970; Shimshick *et al.*, 1973; Leè, 1975; Sklar *et al.*, 1977). The phase diagrams however, are sensitive to a variety of conditions which may differ between laboratories, such as small amounts of impurities, method of preparation of vesicles, temperature gradients, and rate of temperature change. To maintain self consistency, we have determined the required phase diagrams from the fluorescence intensity *vs.* temperature graphs shown in Fig. 1*a–c*. To do this, we have assumed that for each lipid composition, the breaks shown by the arrows in the *I vs. T* plots correspond to the initiation and termination of the phase transitions and



A



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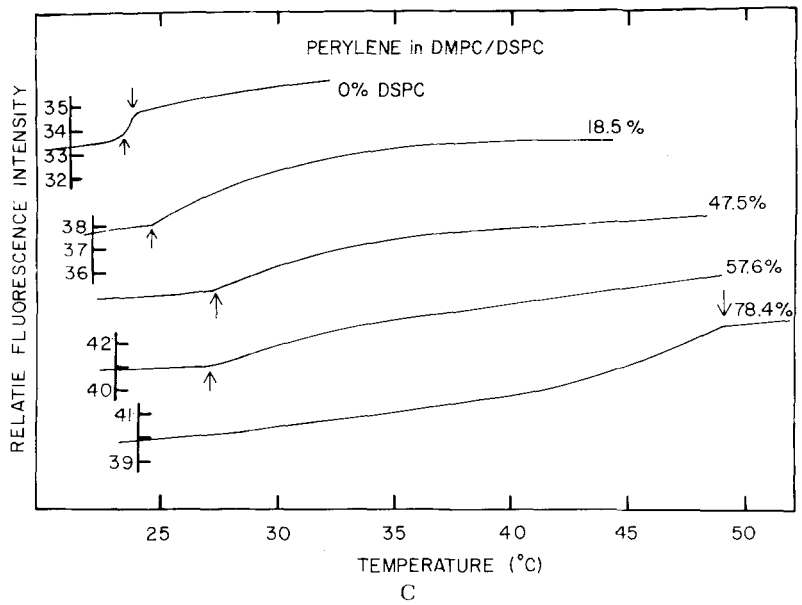


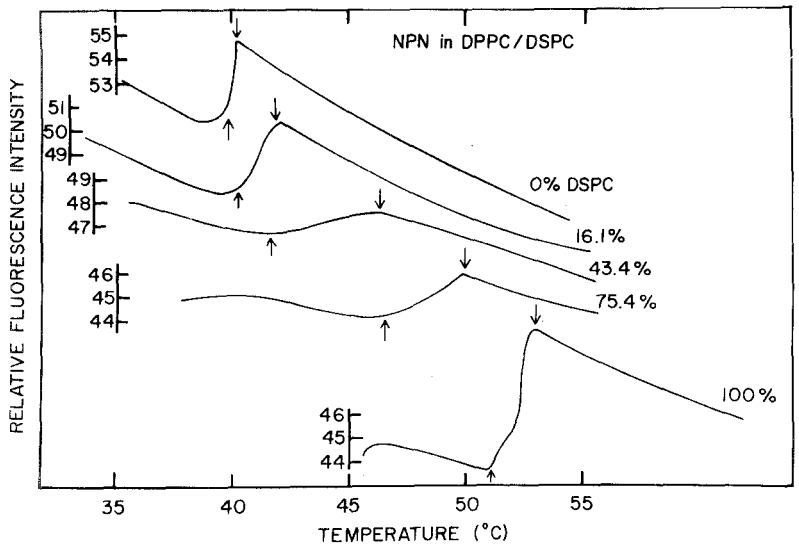
Fig. 1. Fluorescence intensity *vs.* temperature profiles for perylene-labeled vesicles composed of two different lipids. Lipid mixtures are (*a*) dppc/dspc, (*b*) dppc/dppe and (*c*) dmpc/dspc. The percent next to each graph corresponds to percent mole fraction of (*a*) dspc, (*b*) dppe and (*c*) dspc in the lipid mixtures. The relative intensity scale for each mole fraction is to the left of the corresponding graph. The scales of the different graphs cannot be compared since no care was taken to prepare vesicle suspensions with comparable intensities. The zero intensity baseline for each graph has been displaced so that for each lipid mixture the phase transition graph for the lowest mole fraction appears at the top of the plot while the highest mole fraction is at the bottom of the plot. The arrows point to the temperatures which we estimate for the apparent onset and termination of a transition by the procedure described in a previous paper

thus give the points on the liquidus and solidus graphs of the phase as described in the previous papers. (The validity of this assumption is discussed further below.) However, there were some lipid mixtures, especially in the dmpc/dspc system, in which the change in intensity during the phase transition was gradual and the break points could not be established with a reasonable degree of certainty. The part of the solidus or fluidus graph corresponding to these mixtures therefore could not be accurately determined, as also discussed further below. The points on the fluidus and solidus graphs which could be determined from the perylene data are shown as crosses in Fig. 3*a--c*.

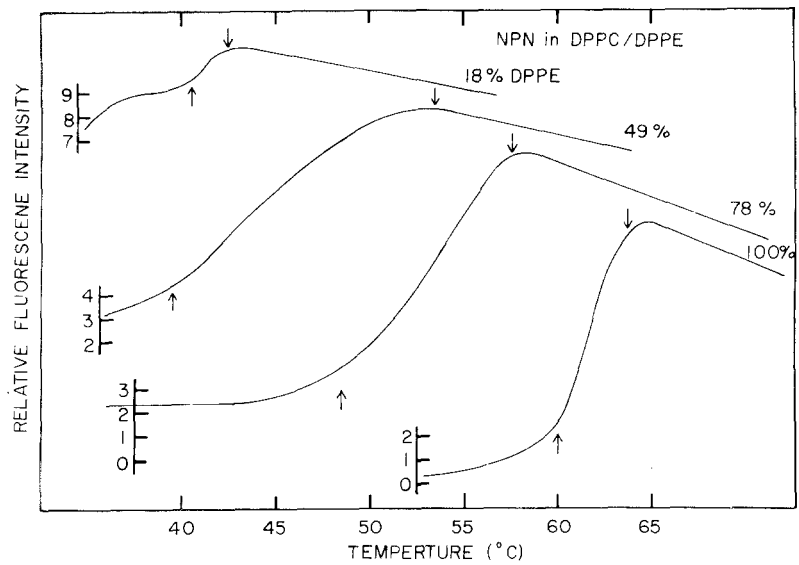
Since calculations presented in the previous paper indicate that the apparent break points on an *I. vs. T* plot for a given fluorophore do not always correspond to points on the liquidus and solidus graphs, we redetermined the phase diagrams from the *I vs. T* graphs of NPN shown in Fig. 2*a-c*. This probe has been used to monitor phase transitions in several systems, and for one system the temperature range of the phase transition as determined by NPN was found to be in agreement with the temperature range determined by X-ray diffraction (Overath *et al.*,

1975). The points on the liquidus and solidus graphs determined from the NPN data are shown as squares in Fig. 3*a-c*.

Within experimental uncertainty good agreement is generally found between the break points observed for a given lipid mixture with perylene and with NPN. The smooth curves drawn through the perylene and NPN points yield the phase diagrams that we use in the following sections to determine the partition coefficient for perylene between the solidus and fluidus phases. The phase diagram for



A



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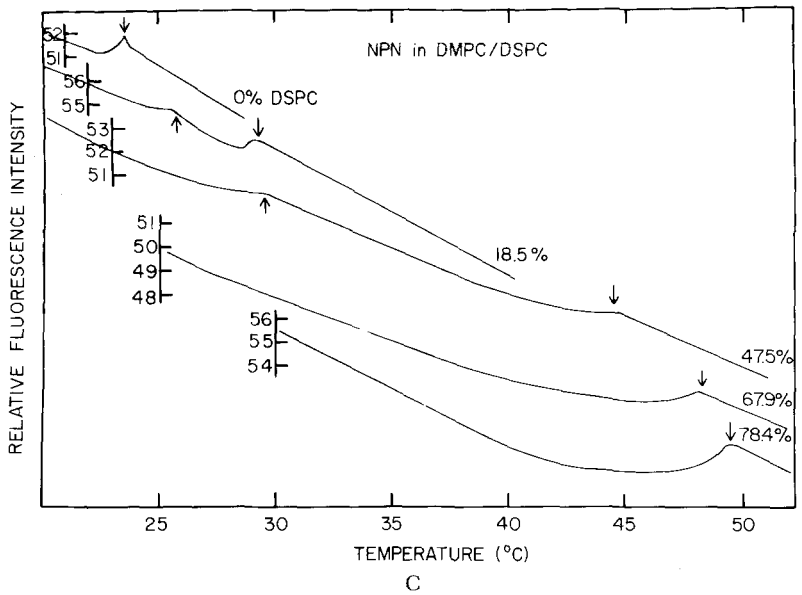

Fig. 2. Fluorescence intensity *vs.* temperature profiles for NPN-labeled vesicles of two different lipids. Lipid mixtures are (*a*) dppc/dspc, (*b*) dppc/dppe and (*c*) dmpc/dspc. Percents next to each graph correspond to mole fractions of (*a*) dspc, (*b*) dppe and (*c*) dspc. For further details, *see* legend of Fig. 1

dppc/dspc agrees well qualitatively with those determined by ESR (Shimshick *et al.*, 1973) and calorimetric measurements (Phillips *et al.*, 1970). The phase diagram that we determine for dppe/dppc, however, differs noticeably from that determined by ESR (Shimshick *et al.*, 1973) and also from those determined by fluorescence measurements of paranaric acid (Sklar *et al.*, 1977) and of chlorophyll *a* (Lee, 1975). We do not have an explanation for these differences. For consistency in our analysis we use the phase diagrams determined from our data.

The solidus graph of the phase diagram for dmpc/dspc is not well determined from our data for mole fractions of dspc greater than about 0.7, and the fluidus graph is not well determined for mole fractions less than about 0.4. Similar difficulties have been encountered by other investigators who have studied this phase diagram by ESR (Shimshick *et al.*, 1973) and by calorimetric measurement (Phillips *et al.*, 1970). Figure 3*c* shows two solidus and two fluidus graphs which can be drawn through the experimental points for the system dmpc/dspc.

We also attempted to determine the phase diagrams by measuring light scattering as a function of temperature. Right angle light scattering increases by 10–50% in going from the fluidus to the solidus phase and has been used previously to determine phase transitions (Overath & Träuble, 1973). In our spectrofluorimeter the light scattering measurements can be done simultaneously with the fluorescence intensity measurements, and it would have been advantageous to have this independent means of determining the phase diagram of our sample. However, although the dppc/dppe scattering I *vs.* T graphs were monophasic, our preliminary experiments with dmpc/dspc and dppc/dspc mixtures indicated that these

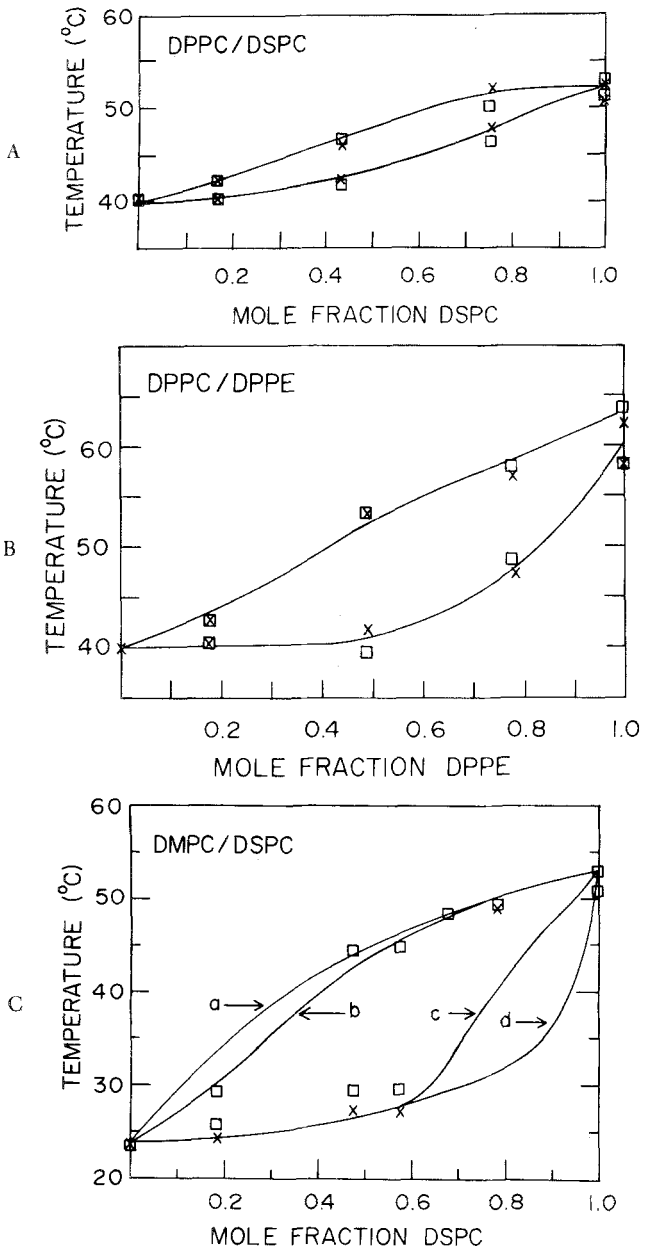

Fig. 3. Phase diagrams for the binary mixtures (*a*) dppc/dspc, (*b*) dppc/dppe and (*c*) dmpc/dspc. The points on the liquidus and solidus graphs were obtained from the graphs of Figs. 1 and 2 as discussed in the text. The crosses represent data points obtained from the perylene graphs of Fig. 1, while the squares represent data points from the NPN graphs of Fig. 2. The phase diagram of dmpc/dspc was difficult to determine. The various solid lines drawn for the liquidus and solidus graphs of this system represent different phase diagrams which we considered in our analysis of experimental data

graphs were complex. They were biphasic and changed with successive temperature. During the first scans, the scattering graph showed some biphasic fluctuation 2 to 3 degrees above the transition temperature. This fluctuation decreased on successive runs and was not observable after about 10 runs. Light scattering data was therefore not used in the construction of the phase diagrams.

A question that arises from the discussion of light scattering is whether the abrupt increase in perylene fluorescence during the solid to fluid phase transition is due to changes in light transmission produced by changes in light scattering, instead of an increase in fluorescence efficiency as we have assumed. To test this possibility, we recorded *I vs. T* for fluorescein dissolved in a dppc vesicle suspension that had an optical density (due to light scattering) of 0.6 at 500 nm. Since fluorescein presumably does not enter bilayers, it is expected that any abrupt changes in its fluorescence intensity reflect changes due to light scattering. The change in fluorescence intensity, which we detected experimentally in going from the solid to fluid phase, was only about 0.2%, indicating that the increase in fluorescence intensity recorded for perylene is essentially due to an increase in fluorescence intensity. This conclusion is reinforced by the observation that the magnitude of the percent change in perylene fluorescence is independent of the optical density of the scattering solution within experimental error. It should be noted that optical densities due to light scattering have no absolute meaning but depend on distance between detecting photomultiplier and solid angle of detection, i.e., they depend on the instrument used for the measurement. Moreover, the relation between changes in light scattering detected at right angles and accompanying changes in light transmission is complex. Large percentage changes in right angle scattering can be due to small percentage changes in light transmission. Therefore, the effect of light scattering on fluorescence intensity cannot easily be inferred from scattering measurements but must be measured experimentally as described above.

Determination of Partition Coefficients of Perylene

To determine the partition coefficient K for perylene between bilayer solid and fluid phases from *I vs. T* plots, we use the theory presented in the previous paper. According to this theory, the intensity I at each temperature T is related to K and to the total amounts of lipid in the fluid and in the solid phase, measured in terms of the relative volumes V_F and V_S of the fluid and solid phases, respectively, by the following expressions. Defining $\bar{I}$ and y as

$$\bar{I} = (I - I_S)/(I_F - I_S) \tag{1}$$

$$y = (V_F/V_S)K \tag{2}$$

we can write from the theory

$$\bar{I} = y/(y + 1) \tag{3}$$

where I_S and I_F are the fluorescence intensities at the temperature T if the probe were completely in the solid and fluid phases, respectively. *K is defined as the ratio*

of the concentration of the probe in the fluid to that in the solid phase. If we let N_S and N_F equal the total number of moles of lipid in the solid and fluid phases, respectively, and D_S and D_F equal the densities for these phases, then we can write for y

$$y = (N_F/N_S)(D_S/D_F)K . \tag{4}$$

For each temperature and overall lipid composition X_A (where X_A is the overall mole fraction of component A in the binary lipid mixture), the ratio N_F/N_S may be determined from the phase diagram using the expression

$$N_F/N_S = (X_A - X_{AS})/(X_{AF} - X_A) \tag{5}$$

where X_{AS} and X_{AF} are the mole fractions of the lipid component in the solid and fluid phases. The values of X_{AS} and X_{AF} are given by the solidus and fluidus graphs of the phase diagram as described in the previous paper. We assign a value of 1.035 to D_S/D_F (Wilkinson & Nagle, 1977).

We have used two different procedures to evaluate K from the experimental I *vs.* T graphs and the expressions presented above. In both procedures we first convert the experimental I *vs.* T plot for each composition X_A to an $\bar{I}$ *vs.* T plot. In the first method we compare this plot with theoretical plots calculated with Eqs. (3) to (5) using the phase diagram and assumed values of K. The value of K which gives best agreement is taken as the value of the partition coefficient. In the second method, we calculate K with the expression

$$K = [\bar{I}/(1 - \bar{I})]\,(D_F/D_S)(N_S/N_F) \tag{6}$$

using experimental values of $\bar{I}$ and values of N_F/N_S obtained from the phase diagram.

To convert I to $\bar{I}$ we must have values of I_F and I_S at each temperature T. In general, these values depend directly not only on temperature but also on composition of the fluid and solid phases, which also changes with temperature. Analysis of our experimental data indicates that for mixtures of phosphatidylcholines I_S and I_F can be assumed to depend only on T and not on composition. For these mixtures we obtained values of I_F and I_S by linear extrapolation of I_F from above and I_S from below the phase transition as discussed in paper I. However, for perylene in mixtures of phosphatidylcholine and phosphatidylethanolamine we had to consider the dependence of I_S on composition in the solid phase. Other reports also indicate that the fluorescence intensity of perylene in membranes below the transition depends on the head group of the lipid (Jacobsen *et al.*, 1975). In our previous paper we described a procedure for calculating the dependence of I_F and I_S on temperature and composition. Although this procedure works well for calculating theoretical plots, it encounters practical problems when applied to the conversion of experimental data from I to $\bar{I}$. More specifically, the procedure requires comparison of different I *vs.* T graphs, but experimentally it is difficult to record these plots under conditions where relative intensities can be compared. We have therefore used the following alternative procedure to convert I to $\bar{I}$.

We first took into account the direct temperature dependence of I_S and I_F by linear extrapolation of the experimental graphs from below and above the transition and then made corrections for the dependence of I_S on lipid composition. No

correction was made for composition dependence of I_F, since the fluorescence intensity of perylene in membranes above the transition temperature seemed to be independent of lipid composition. The method used to determine the dependence of I_S on composition was based on the following observations. The magnitude of the change in fluorescence intensity during the phase transition in dppc/dppe vesicles depends on the mole fraction of dppe in the mixture shown in Fig 1*b*. In pure dipalmitoyl phosphatidylcholine membranes the intensity of perylene below the transition was about 93% of the intensity above the transition; in pure dipalmitoyl phosphatidylethanolamine membranes, the intensity of perylene below the transition was about 36% of the intensity above the transition. Mixtures of dppc and dppe show intermediate changes in intensity, and we find that the change in intensity has approximately a linear dependence on the mole fraction of dppe. Based on these observations, we assume that at any temperature on a phase transition graph, I_S (corrected for temperature and composition dependence) is given by the expression

$$I_S = I_{SO}[1 - 0.57(X_{AS} - X_A)] \tag{7}$$

where I_{SO} is the value of I_S corrected for temperature dependence by linear extrapolation of intensity from below the transition, X_A is the total mole fraction of dppe in the mixture and S_{AS} is the fraction of dppe in the solid phase at the temperature T as determined from the phase diagram.

Figure 4 shows experimental and theoretical plots of $\bar{I}$ *vs.* T for different mixtures of dppc/dspc. The experimental values of $\bar{I}$ are represented by circles with error bars and were obtained from the plots of Fig. 1. The error bars are based on

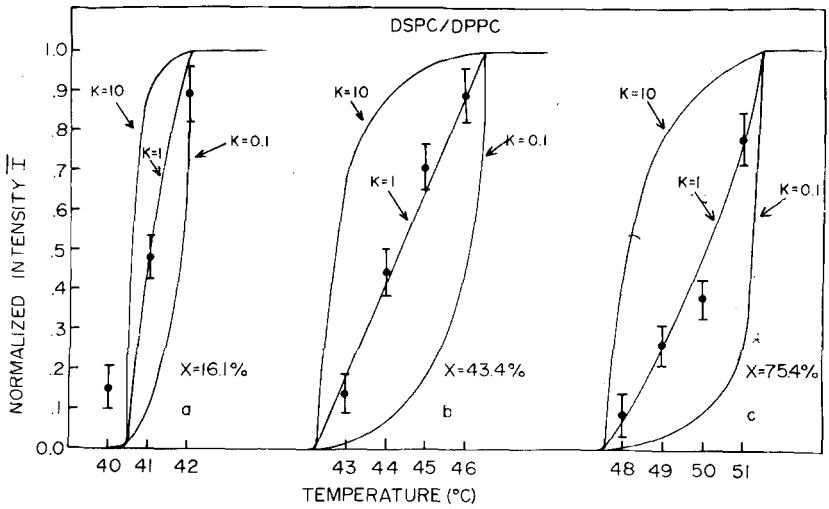

Fig. 4. Comparison of theoretical and experimental normalized intensity *vs.* temperature graphs for perylene in dspc/dppc vesicles. The theoretical graphs were calculated from the graphs in Fig 1*a* and the phase diagram in Fig. 3*a* as described in the text. Mole fractions X of dspc are (*a*) 0.161, (*b*) 0.434 and (*c*) 0.754. Error bars are estimates of the uncertainty in the normalized intensities. Solid lines are theoretical graphs assuming partition coefficients K equal to 0.1, 1, and 1.0

estimates of uncertainties in the experimental evaluations of I and include uncertainties in reading I from graphs of I *vs.* T as well as uncertainties in the reproducibility of I at a given T when the temperature is scanned several times and when the sample is prepared fresh and scanned. The combined errors give an uncertainty in $\bar{I}$ of 0.05 to 0.07 units, and in the figures we have drawn the error bars centered about the values of $\bar{I}$ determined at each temperature from the experimental curves. The theoretical plots of $\bar{I}$ *vs.* T were calculated for different values of the partition coefficient K with Eqs. (3) and (5) and the phase diagram of Fig. 3*a*. Visual comparison of experimental and theoretical plots indicates that the fit is good for a partition coefficient around $K = 1$. To determine the goodness of fit more objectively, we have calculated the mean chi square, χ^2/N, between the experimental $\bar{I}_E$ and theoretical $\bar{I}$ values for different values of K using the equation

$$\chi^2/N = (1/N) \sum_{i=1}^{N} (\bar{I}_T - \bar{I}_E)_i^2/(U_i)^2 \tag{8}$$

where N is the number of experimental points, U_i is an estimate of uncertainty in $\bar{I}_E$. The values of U_i are based on estimated uncertainties in the evaluation of I as mentioned above and range between 0.05 and 0.07. Figure 5 shows a plot of χ^2/N for different values of K for the mixtures of 0.434 and 0.754 dspc mole fractions. The 0.16 mole fraction has not been used in the analysis because the theoretical graphs are too close to each other to allow the determination of K with any meaningful precision. Figure 5 shows that the best fit (minimum value of χ^2/N) is in the range $K = 1 \pm 0.5$ for the two mixtures. The range indicated for K is for values of χ^2/N less than 6. We have also calculated values of K using Eqs. (3) to (6)

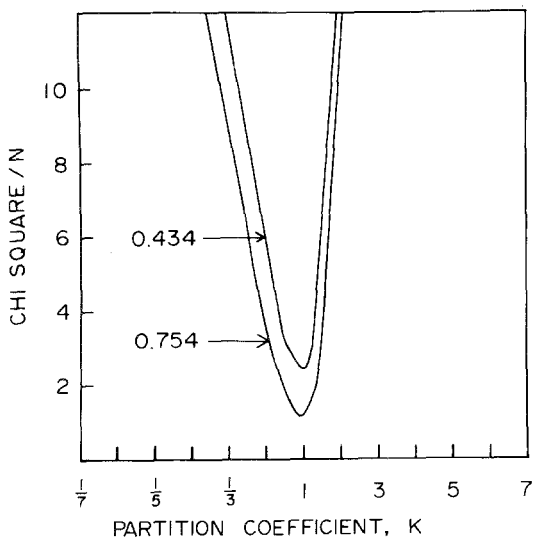

Fig. 5. Mean chi square *vs.* partition coefficient K for perylene in dppc/dspc vesicles for 0.434 and 0.754 mole fractions of dspc.

as stated above. The results, shown in Table 1, indicate that K is very close to 1 at all temperatures for the two mole fractions and that within the uncertainties in the experimental values of $\bar{I}$, K is independent of T or mole fraction composition. The weighted average $\bar{K}$ from both dppc/dspc mixtures is 1.04 ± 0.18 where $\bar{K} = (\Sigma K_i/\sigma_i^2)/(\Sigma 1/\sigma_i^2)$. In this sum K_i and σ_i represent, respectively, values for the partition coefficient and the root mean square deviation for a particular mole fraction i. Considerations based on the theory of propagation of errors indicate that if K is indeed independent of temperature and the error in the value of $\bar{I}$ at each temperature is around 0.05 to 0.07, then K can be established for these mixtures with a precision of about 20%.

Theoretical and experimental $\bar{I}$ *vs.* T graphs for mixtures of dppc/dppe are shown in Fig. 6. The best values of K, based on values of χ^2/N as described above, are 1.0 ± 0.7 and 2 ± 1.1, respectively, for mixtures with dppe/dppc mole ratios of 0.49 and 0.78. The weighted average $\bar{K}$ for values of K determined with Eqs. (3) to (6) is 1.10 ± 0.26.

Fig 7 shows $\bar{I}$ *vs.* T plots for mixtures of dmpc/dspc. The phase diagram used for these plots is given by the fluidus and solidus curves *a* and *d* in Fig. 3*c*. The best values for these plots are 0.25 ± 0.2, 1 ± 0.8, 3 ± 2, and 0.5 ± 0.1, respectively, for the dspc mole fractions 0.3, 0.475, 0.576, and 0.784.

The agreement between the experimental and theoretical graphs is remarkably good, considering the simplicity of the theory, for mixtures of dspc/dppc and dppe/dppc. Values of K obtained as described above range between 1 and 2 for these mixtures. However, for mixtures of dmpc/dspc the calculated values of K show large and unsystematic variations, in the range 0.3 to 7, with changes in temperature and composition. As described in paper I, it is often difficult to determine from I *vs.* T graphs the phrase diagram for a system such as dmpc/dspc which shows partial cocrystallization. For these systems the I *vs.* T graphs have

Table 1. Partition coefficient K of perylene between fluid and solid phases of mixtures of dppc and dspc calculated with Eqs. (3) to (6) using the experimental value of $\bar{I}$ shown in Fig. 4[a]

	Mole ratio dspc	
T, C°	0.434	0.754
43	0.72	
44	1.14	
45	1.20	
46	1.11	
47		
48		1.44
49		0.97
50		0.65
51		1.14
Average	1.04 ± 0.22	1.05 ± 0.33

[a] Averages over temperature and root mean square deviations are given on the bottom line.

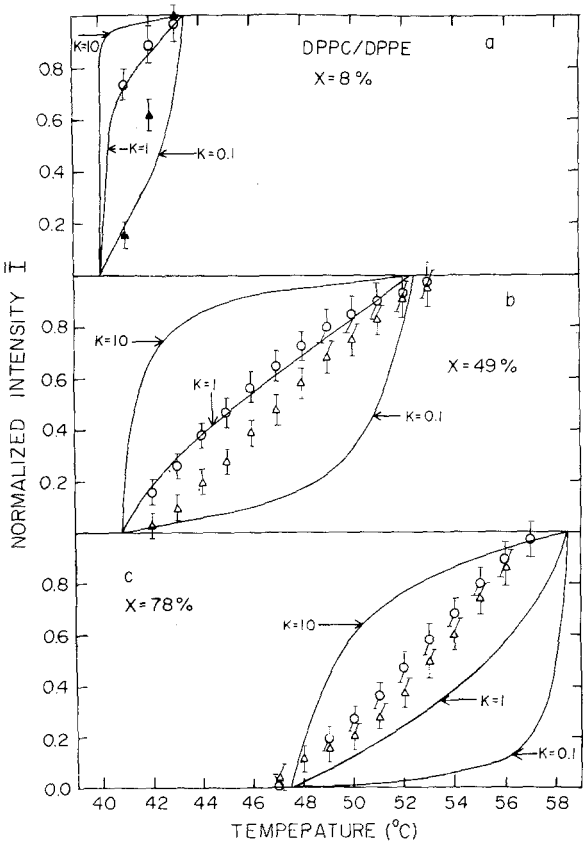

Fig. 6. Comparison of theoretical and experimental normalized intensity *vs.* temperature graphs for perylene in dppc/dppe vesicles. Theoretical graphs were calculated from the graphs of Fig. 1*b* and the phase diagram in Fig. 3*b* as described in the text. Mole fractions X of dppe are (*a*) 0.18, (*b*) 0.49, and (*c*) 0.78. Circles are values corrected for the dependence of the intensity on composition in the solid phase. Triangles are the values before correction. *See* text for further details

several inflection points within the phase transition region and, in addition, do not have sharp break points at the onset and termination of the transition. Both of these factors make it difficult to establish the appropriate temperatures for the fluidus and solidus graphs.

To determine whether the spread in the values of K that we obtained for the various dmpc/dspc mixtures is due to errors in the evaluation of the phase diagram, we analyzed the experimental data, using several different phase diagrams obtained for the fluidus and solidus graphs *a, b, c* and *d* of Fig 3*c*. However, none of the phase diagrams that we investigated led to a narrow range of values of K for the different mixtures. An alternate explanation of our results for the dmpc/dspc system is that vesicles formed from mixtures of these lipids are not homogeneous and therefore do not reflect the overall lipid composition of the mixture. That this may indeed be the case is suggested by comparing the experimental $\bar{I}$

vs. T graphs for dmpc mole fractions 0.475 and 0.576. These graphs show considerable overlap, whereas the theoretical plots indicate that the two graphs should be well separated from each other for a constant value of K. The overlap thus suggests, but does not prove, that some of the 0.475 and 0.576 vesicles may have similar compositions. A more definite conclusion requires further experimentation.

Alternate Determination of Partition Coefficient

In order to compare our results with those obtained by an alternate technique, we have determined the value of K for partitioning of perylene between the fluid

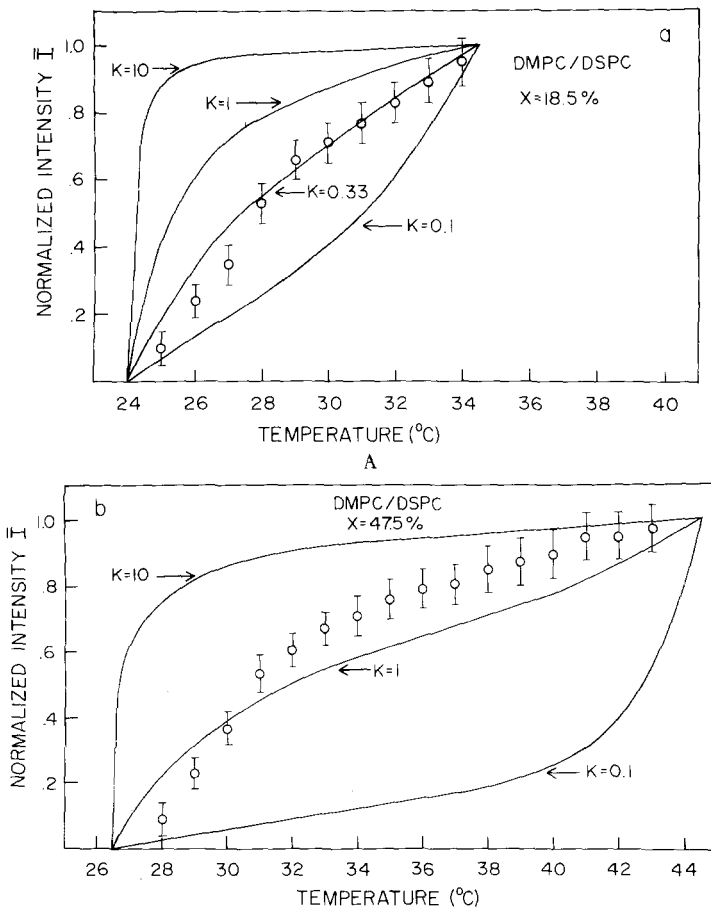

Fig. 7. Normalized intensity *vs.* temperature graphs for perylene in dmpc/dspc vesicles, calculated from the graphs in Fig. 1*c* and the phase diagram indicated by the fluidus and solidus graphs *a* and *b* in Fig 3*c*. Mole Fractions X of dspc are (*a*) 0.185, (*b*) 0.475, (*c*) 0.576, and (*d*) 0.784

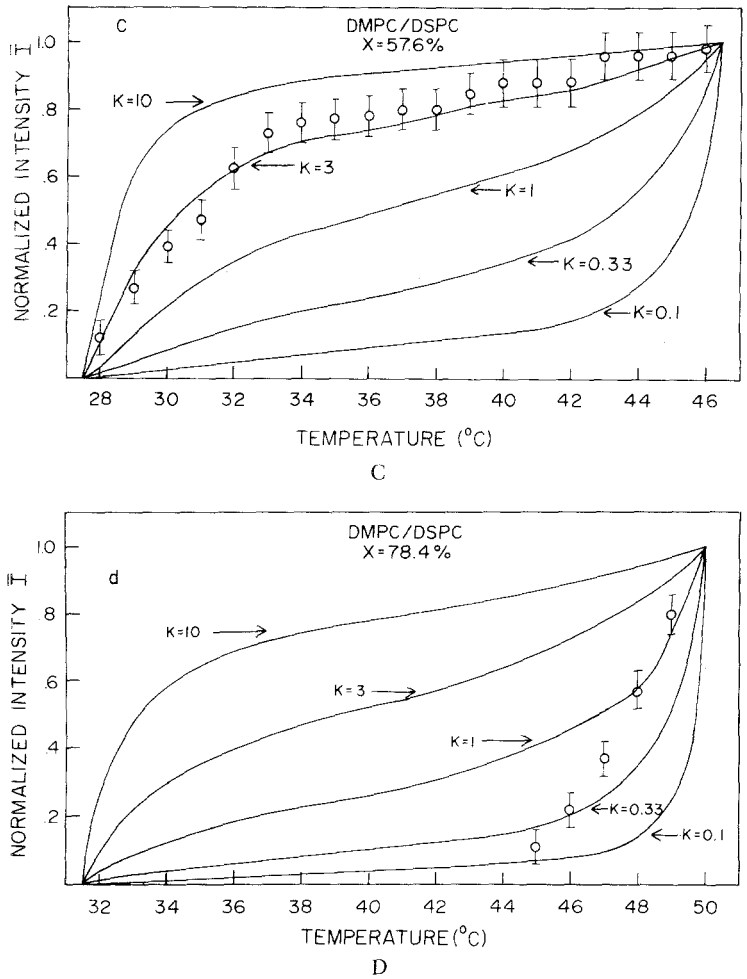

Fig. 7 Continued

phase of dppc and the solid phase of dspc using a method described by Lentz *et al.* (1976*b*). This method uses polarized fluorescence measurements to determine the partitioning of a fluorophore between the fluid phase of pure vesicles of lipid 1 and the solid phase of pure vesicles of lipid 2. These states are achieved by mixing pure single lamellar (sonicated) vesicles of lipid 1 with pure single lamellar vesicles of lipid 2 in the presence of fluorophore at a temperature T_M which is intermediate between the phase transition temperatures of the two lipids. The method assumes that (i) the probe can exchange quickly between the two types of vesicles to achieve partitioning equilibrium and (ii) that the vesicles do not fuse or exchange lipid to produce vesicles with mixed compositions.

The fluorescence anisotropies r_1 and r_2 are first determined separately for the fluorophore in vesicles of pure lipid 1 and lipid 2, respectively, at a temperature T_M. Fluorescence anisotropy is defined by the equation

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ refer to the polarized intensities when the emission polarized is, respectively, parallel and perpendicular to the direction of polarization of the exciting plane polarized light. Next the fluorescence anisotropy r_m is measured for a mixture of vesicles of pure 1 and pure 2, allowing sufficient time for the probe to equilibrate between the two types of vesicles. The measured anisotropies can then be used to evaluate the relative amounts of fluorophore in the mixture of vesicles of pure lipid 1 and lipid 2. The expressions used in this evaluation are

$$r_m = z_1 r_1 + z_2 r_2 \tag{9}$$

with

$$z_1 = f_1/[f_1 + f_2 (F_2/F_1)] \tag{10}$$

and

$$z_1 + z_2 = 1 \tag{11}$$

where f_1 and f_2 are the mole fractions of probe in vesicles of lipid 1 and lipid 2, respectively, and F_2/F_1 is the ratio of the fluorescence efficiencies of the probe in the two lipids at the temperature T_M. Using the experimental values of r_m, r_1, r_2 and F_2/F_1, we can determine the values of f_1 and f_2 with these equations. Finally the partition coefficient between the fluid phase of lipid 1 and the solid phase of lipid 2 is calculated with the equation

$$K = (f_1/x_1)/(f_2/x_2) \tag{12}$$

where x_1 and x_2 are the mole fractions of lipids 1 and 2 in the mixed vesicle system. We assume that lipid 1 has the lower phase transition temperature.

The experimental values that we determined for r_1(dppc), r_2(dspc), and $r_m(x_{\text{dppc}}/x_{\text{dspc}} = 0.75)$ with perylene at a temperature of 46°C were 0.0189, 0.0599, and 0.0376, respectively. In these experiments multilamellar vesicles were sonicated and centrifuged to produce presumably single lamellar vesicle suspensions which were then used to determine r_1 and r_2. In this mixed vesicle experiment for determining r_m, we mixed perylene labelled vesicles of dppc with unlabelled vesicles of dspc. The value of r in the mixed system assumed a constant value in less than 1 min after mixing, presumably indicating that partitioning equilibrium was quickly established. A record of I *vs.* T for the mixed system showed two sharp transitions at the phase transition's temperatures of dppc and dspc, indicating that lipid vesicles of mixed composition had not significantly formed. Values of f_1 and f_2 obtained by solution of Eqs. (9), (10) and (11) with $F_2/F_1 = 0.9$ are 0.518 and 0.482. A phosphate analysis of the vesicle suspensions by the method of Fiske and SubbarRow gave the mole ratio of lipids in the mixture $x_{\text{dppc}}/x_{\text{dspc}} = 0.75 \pm 0.11$. From Eq. (12) we obtained the partition coefficient $K = 1.4 \pm 0.3$. In a similar experiment in which perylene was initially in dspc vesicles we obtained the partition coefficient $K = 1.6 \pm 0.3$. Within experimental error the partition coefficient determined for perylene by this method does not depend on the initial distribution of the probe. This is consistent with the assumption that partitioning equilibrium has been established within the time of the experiment. The value of

K determined by this alternate method agrees well within experimental error with the value $K = 1.0 \{ 0.2$ determined by the method discussed in this paper.

Although the two methods that we have used give comparable results, there are differences in applicability which should be mentioned. The fluorescence polarization method requires, as mentioned above, (i) that the probe be sufficiently soluble in the bathing electrolyte or exchanges sufficiently fast to achieve partitioning equilibrium, (ii) that the vesicles do not fuse or exchange lipids during the time of the experiment. (iii) that the vesicles be unilamellar in order to allow the probe to equilibrate (however, multilamellar systems can be studied if the probe quickly penetrates bilayers) and (iv) that there be a difference in anisotrophy of the probe in the fluid and the solid phases. The method developed here, in turn, requires (i) that the composition of the lipid bilayer of the vesicles be uniform, (ii) that the solid and the liquid phases of the lipid bilayer of the mixed composition be in equilibrium at each temperature, (iii) that the fluorescence intensity of the probe be different in the fluid and the solid phases, and (iv) a more extensive analysis.

It can be argued that a value of $K = 1$ for sonicated vesicles is due to perturbation of the lipid arrangement in the solid phase by the high curvature of the vesicles. Our results, however, show that a value of $K \simeq 1$ is applicable to multilamellar vesicles which have curvatures comparable to living cells.

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