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Theory of Lipid Bilayer Phase Transitions as Detected by Fluorescent Probes

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Summary. We present a quantitative theory that relates the fluorescence intensity *vs.* temperature (*I vs. T*) profile of a fluorescent-labeled two-component lipid bilayer to the phase diagram of the bilayer and the partition coefficient K of the fluorophore between fluid and solid phases of the bilayer. We show how the theory can be used to evaluate K from experimental *I vs. T* profiles and the appropriate phase diagrams as well as to understand the different shapes of *I vs. T* profiles obtained with particular fluorophores and phase diagrams. Using calculated *I vs. T* graphs, we discuss the meaning of parameters, such as midpoint of the phase transition and onset and termination of a transition, which are often used to characterize phase transitions on the basis of fluorescence intensity *vs.* temperature profiles.

Lipophilic and amphiphilic fluorescent probes have played a useful role in the study of certain properties of biological membranes such as lipid phase transitions, microviscosity, and structural organization of lipid bilayers (For reviews, *see* Radda, 1975; Vanderkooi & McLaughlin, 1976; Yguerabide & Foster, 1978). Lipid phase transitions have been detected and phase diagrams constructed by monitoring temperature-dependent changes in fluorescence intensity or polarized emission of these probes, while microviscosity and structural organization have been studied by polarized fluorescence measurements of probes embedded in specific transverse regions of the membrane. The primary interest in many of these studies has been to establish relations between changes in the functional state of a membrane and changes in its conformation and dynamics. The interpretation of changes observed in emission parameters in terms of changes in properties of a membrane, however, are often complicated by lack of information concerning the distribution of the fluorescent probe between possible gel (solid) and liquid crystalline (fluid) phases of a membrane.

Several methods have been used to study the phase distribution of fluorescent as well as ESR probes in synthetic bilayers of known lipid composition. McConnell and colleagues (Shimshick & McConnell, 1973; Wu & McConnell, 1975) developed a mathematical model that relates the order parameter of an ESR probe to the phase diagram of a two-component bilayer and used the model to estimate partition coefficients for two water soluble ESR probes from measurements of order parameters *vs.* temperature. The partition coefficient of the fluorophore di-

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phenylhexatriene (DPH) has been estimated from changes in fluorescence polarization which occur when DPH partitions between labeled vesicles of one lipid in the fluid (or in the solid) phase to unlabeled vesicles of another lipid in the solid (or in the fluid) phase (Lentz, Barenholz & Thompson, 1976). A partition coefficient of 1 was estimated for the partition coefficient of DPH between the fluid phase of dimyristoyl phosphatidylcholine and solid phase of dipalmitoyl phosphatidylcholine (Lentz *et al.*, 1976). The partition coefficients of cis- and trans-paranaric acid have been estimated from changes in the ratio of the fluorescence intensity above and below the lipid phase transitions, which occur when paranaric acid transfers from vesicles composed of one lipid to those of another (Sklar, Hudson & Simoni, 1977).

In this paper we develop a quantitative theory with which to calculate the partition coefficient K of a fluorophore between fluid and solid phases of a bilayer from experimental intensity *vs.* temperature data and a phase diagram. In addition, the theory gives insight into relations between the shape of an intensity *vs.* temperature graph, the phase diagram, and the partition coefficient. The theory is developed initially for water insoluble fluorophores, such as perylene, which are confined to the bilayer and do not partition into the water phase. Later sections discuss the applicability of the theory to more general cases, including water soluble probes.

In the present paper we use the theory to study relations between I *vs.* T plots and phase diagrams and discuss on the basis of theoretical plots of I *vs.* T the meaning of parameters usually used to characterize these plots, such as midpoint of the transition and onset and termination of the transition. In the following paper (Foster & Yguerabide, 1978) we use the theory to evaluate from experimental I *vs.* T plots, the partition coefficient for perylene in several two component liquid bilayers. The theoretical expressions presented in this paper have been previously presented in outline form (Foster & Yguerabide, 1977).

Theory

1. *Water Insoluble Probes*

a) *Bilayers with Lipids that Cocrystallize.* We consider a lipid bilayer composed of two lipid components A and B that cocrystallize. The overall composition of the bilayer is described by the overall mole fractions X_A and X_B of A and B, respectively. More explicitly, if N_A and N_B are the total number of moles of A and B in the system, then X_A and X_B are defined as $X_A = N_A/N$ and $X_B = N_B/N$ where $N = N_A + N_B$. At any temperature within the phase transition region of the bilayer, the bilayer consists of a liquid crystalline (fluid) and a gel (solid) phase. The composition of the fluid phase is described by the mole fractions X_{AF} and X_{BF} of A and B in this phase. (These mole fractions are expressed in terms of the total number of moles of lipid N_F in the fluid phase, i.e., $X_{AF} = N_{AF}/N_F$ and $X_{BF} = N_{BF}/N_F$ where N_{AF} and N_{BF} are the number of moles of A and B, respectively, in the fluid phase; *see* Eq. (1) below.) Similarly the mole fractions X_{AS} and X_{BS} describe

the composition of the solid phase. The values of the mole fractions X_{AF} and X_{AS} at any temperature T are given by the fluidus and solidus graphs of the phase diagram of the two component system as shown in Fig. 1. The values of X_{BF} and X_{BS} can be determined from X_{AF} and X_{AS} with the expressions

$$X_{AF} + X_{BF} = 1 \tag{1}$$

$$X_{AS} + X_{BS} = 1. \tag{2}$$

The total number of moles of lipid (A and B) in the fluid phase N_F and in the solid phase N_S are related to the mole fractions X_A, X_{AS} and X_{AF} in the phase diagram by the expression (Castellan, 1971)

$$N_F/N_S = (X_A - X_{AS})/(X_{AF} - X_A). \tag{3}$$

(This equation is obtained from the conservation relations

$$N_A = X_{AF}N_F + X_{AS}N_S$$
$$= X_A(N_A + N_B)$$

which allow us to write, using the equality $N_A + N_B = N_S + N_F$,

$$X_{AF}N_F + X_{AS}N_S = X_A(N_S + N_F).$$

Rearranging this expression gives

$$(X_{AF} - X_A)N_F = N_S(X_A - X_{AS})$$

from which Eq. (3) follows.) The observed fluorescence intensity of a water insoluble probe located in the bilayer is the sum of contributions from probe molecules in each of the bilayer phases. For a system with a maximum of two phases (a single fluid and a single solid phase), the fluorescence intensity at any temperature is given by the expression (assuming that the probe occupies a single homogeneous site in each phase)

$$I = a[Q_S P_S + Q_F P_F] \tag{4}$$

where a is an instrument constant, Q_S is the product of the fluorescence efficiency and the extinction coefficient of the probe in the solid phase, P_S is the number of moles of probe in the solid phase, and Q_F and P_F are the corresponding quantities in the fluid phase. In general, Q_S, Q_F, P_S and P_F are temperature and composition dependent. The total amount of probe P_O in the bilayer is given by

$$P_O = P_S + P_F. \tag{5}$$

We define the partition coefficient K for the probe between solid and fluid phases by the expression

$$K = C_F/C_S \tag{6}$$

where C_F and C_S are the concentrations of the probe in the fluid and solid phases, respectively. If V_F and V_S are the volumes of the fluid and solid phases, we can write from Eqs. (5) and (6)

$$K = (P_F/P_S)(V_S/V_F) \tag{7}$$

$$= [P_F/(P_O - P_F)](V_S/V_F) \tag{8}$$

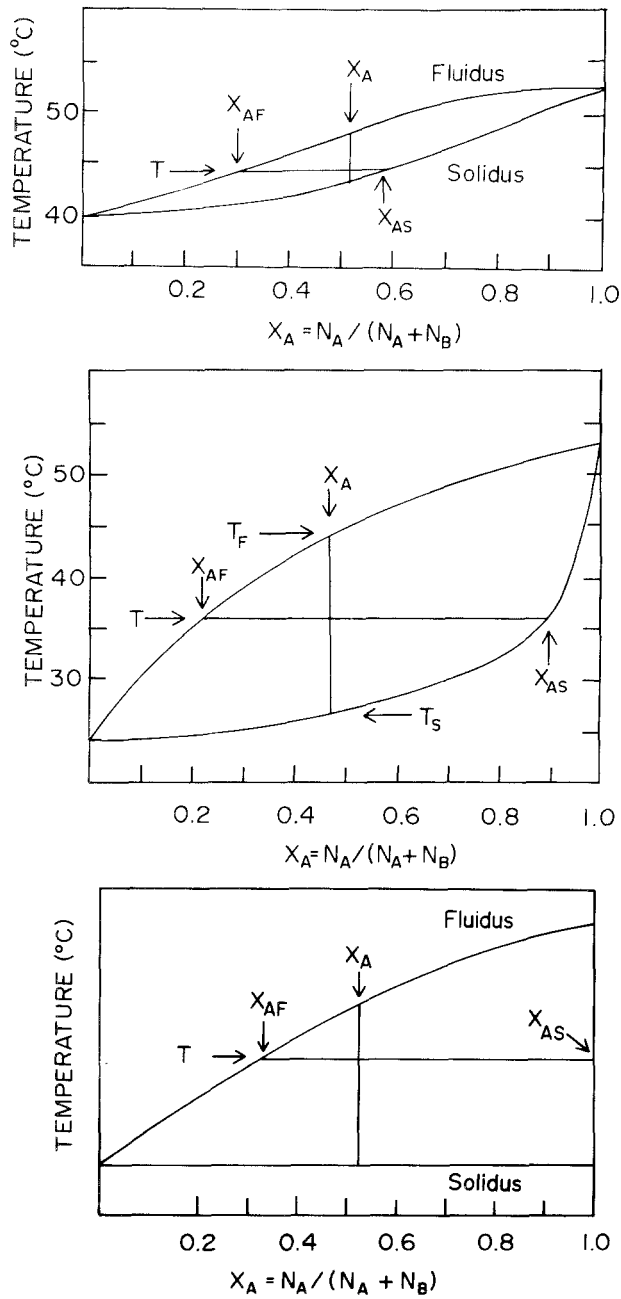


Fig. 1. Phase diagrams for (*a*) a binary mixture of lipids which cocrystallize, (*b*) a binary mixture of lipids which partially cocrystallize and (*c*) a binary mixture of lipids which do not cocrystallize. X_A is the mole fraction of component A in the mixture. The mole fractions of A in the fluid and in the solid phase at any temperature T for a bilayer with composition X_A are indicated by X_{AF} and X_{AS}, respectively. The beginning and end points of the transition for the mixture X_A are indicated by the temperatures T_F and T_S. Note that in Fig. 1*c*, the value of X_{AS} is 1 for all values of T and X_A which fall in the region between the solidus and fluidus graphs. The reason is that, for mixtures which do not cocrystallize, the system in this region consists of a pure solid phase (hence $X_{AS} = 1$) and a solution of composition X_{AF}

Solving Eq. (8) for P_F, substituting the resulting expression into Eq. (4), and rearranging terms, we get

$$\bar{I} = y/(1 + y) \tag{9}$$

where

$$\bar{I} = (I - I_S)/(I_F - I_S) \tag{10}$$

$$I_S = a\, Q_S P_O \tag{11}$$

$$I_F = a\, Q_F P_O \tag{12}$$

and

$$y = (V_F/V_S)K. \tag{13}$$

The intensity I_S is the fluorescence intensity that would be measured at the prevailing temperature if all of the probe molecules in the bilayer were in the solid phase. Similarly I_F is the fluorescence intensity if all of the probe molecules were in the fluid phase. We will refer to $\bar{I}$ as the normalized intensity. The meaning and usefulness of this intensity in the analysis of *I vs. T* plots is discussed below.

Equations (9) to (13) provide quantitative relations with which to predict (as described below) *I vs. T* graphs, for any fluorescent labeled two-component bilayer, given the appropriate phase diagram and assumed dependence of K, Q_F and Q_S on temperature and composition. Conversely, the expressions can be used to evaluate K from experimental *I vs. T* graphs as described in the following paper.

Inspection of Eqs. (9) to (13) reveals that the variation of fluorescence intensity with temperature can be conveniently separated into two parts. One part accounts for (i) the dependence on temperature of the amount of bilayer in the fluid (V_F) and solid (V_S) phases (which reflects the phase diagram) and (ii) the possible dependence of the partition coefficient on temperature and lipid composition. The other part accounts for variations of Q_S and Q_F (which reflect fluorescence efficiencies and extinction coefficients) with temperature and composition. Equation (9) indicates that $\bar{I}$ is that part of the fluorescence intensity which reflects the the variations of N_F, N_S and K with temperature. On the other hand, I_S and I_F reflect the variation of the fluorescence I with temperature due to changes in Q_S and Q_F with temperature and composition. More specifically, we can write

$$I = \bar{I}(I_F - I_S) + I_S \tag{14}$$

which shows explicitly the dependence of I on $\bar{I}$, I_F and I_S.

$\bar{I}$ *vs. T* for a bilayer with a given composition X_A can be predicted with Eqs. (9) to (13) and a phase diagram as follows. For a two-component system where the lipids cocrystallize we can write from Eq. (13)

$$y = (N_F/N_S)\,(D_S/D_F)K \tag{15}$$

where D_F and D_S are the densities of the bilayer in the fluid and solid phases, respectively. As mentioned above, the values of X_{AS} and X_{AF} at any temperature T and composition X_A are given by the fluidus and solidus graphs of the phase diagram. These values of X_{AS} and X_{AF} can be used to calculate N_F/N_S at any temper-

ature with Eq. (3), and the values of this ratio together with an assumed value for K and the appropriate value for D_S/D_F, used to calculate y with Eq. 15). $\bar{I}$ can then be calculated with Eq. (9). The dependence of $\bar{I}$ on temperature can thus be predicted from the phase diagram.

In this paper we will confine ourselves primarily to the calculation and discussion of $\bar{I}$ vs. T plots for the phase diagrams of Fig. 1. These normalized intensity plots not only give insights into relations between intensity in the region of a phase transition, and, for example, the fraction of bilayer in the fluid phase but are convenient in the analysis of experimental data. That is, experimental data can be conveniently analyzed by converting the experimental *I vs. T* into $\bar{I}$ *vs. T* plots by using Eq. (10). This conversion, however, requires knowledge of the dependence of I_S and I_F on temperature and composition. Thus in preparation for the analyses of experimental data in the following paper, we now discuss methods for evaluating the dependence of I_S and I_F on temperature and composition from experimental *I vs. T* plots.

The simplest case is where I_S and I_F depend on temperature but not on composition of the fluid and solid phases (which also changes with temperature as indicated by the values of N_S and N_F). As discussed in the following paper, this approximation applies to bilayers composed of lipids with the same polar heads. In this case the values of I_S and I_F can be evaluated at any temperature in the region of a phase transition by extrapolation of the *I vs. T* graphs from below and above the phase transition region as shown in Fig. 2. Thus above the phase transition $P_S = 0$ and the intensity at any temperature is given by Eq. (12), whereas below the phase transition $P_F = 0$ and the intensity at any temperature is given by Eq. (11). Extrapolations of these intensities into the region of the phase transition can therefore be used to estimate the dependence of I_F and I_S on temperature in this

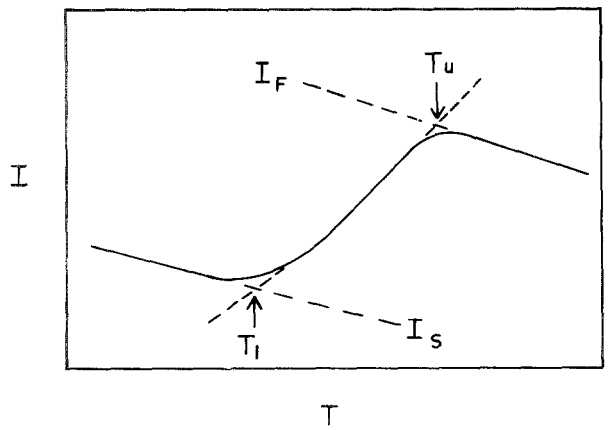


Fig. 2. Determination of I_S and I_F in the region of a phase transition by linear extrapolation of *I vs. T*, assuming no dependence of I_S and I_F on lipid composition. The temperatures T_u and T_l identify the apparent temperatures for the onset and termination of the transitions, respectively, as identified by the crossing points of the extrapolated lines shown in the figure

region. Several methods of extrapolation can be used based on the following observations. Studies of the effect of temperature on the fluorescence efficiencies of fluorophores indicate that for many fluorophores the main effect of temperature is on the specific rate for internal conversion k_i and that the temperature dependence of this rate can be expressed as (Pringsheim, 1949)

$$k_i = k_i^0 \exp(-E/RT) \tag{16}$$

where E is the activation energy, R the gas constant and T the absolute temperature. According to this expression, a plot of log k_i *vs.* $1/T$ is linear. Because of this linear dependence it is common practice to make linear extrapolations of intensity *vs.* temperature data using plots of log $1/I$ *vs.* $1/T$. This method of extrapolation, however, is not strictly correct as seen from the following expressions. The intensity of a fluorophore in a homogeneous environment is given by the equation

$$I = I_0 k_e/(k_e + k_i) = I_0/[1 + (k_i^0/k_e)\exp(-E/RT)] \tag{17}$$

where k_e is the specific rate for light emission. This expression indicates that a plot of log $1/I$ *vs.* $1/T$ is in general complex and is strictly linear only when $(k_i^0/k_e)\exp(-E/RT) \gg 1$ (assuming that k_e is not temperature dependent). In this limit, Eq. (17) reduces to

$$I = I_0(k_e/k_i^0)\exp(E/RT). \tag{18}$$

On the other hand, when E/RT is much smaller than 1, we can expand $\exp(-E/RT)$ in terms of a power series and, retaining only first order terms, write

$$(1/I) = (1/I_0)[1 + (k_i^0/k_e)(1 - E/RT)]. \tag{19}$$

Thus for this condition, a plot of $1/I$ *vs.* $1/T$ should be linear and the plot can be used for linear extrapolations. These simple calculations indicate that the best method of extrapolation depends on the relative values of k_e, k_i and E. In the literature, plots of log $1/I$ *vs.* $1/T$, I *vs.* $1/T$ and simply I *vs.* T have been used for linear extrapolation although the justification for the method used is usually not given.

In general, the correct method of extrapolation for a probe in a homogeneous environment with P_O independent of temperature requires curve fitting of Eq. (17) to the experimental data. In practice, I_F and I_S are not strong functions of temperature for fluorophores such as perylene and often appear to be linear in I *vs.* T plots. In our studies with perylene we have therefore assumed that I_F and I_S can be linearly extrapolated from above and below the phase transition, respectively, in plots of I *vs.* T as shown in Fig. 2.

For bilayers composed of lipids with different polar heads, I_F and I_S in the region of the phase transition may depend not only on temperature but also on composition of the fluid and solid phases which also changes with temperature. A method for evaluating the dependence of I_F and I_S on temperature and composition in the region of the phase transition is as follows. We assume that the intensity I_S varies linearly with the mole fractions X_{AS} and X_{BS} (the mole fractions of lipid A and lipid B, respectively, in the solid phase). In place of Eq. (11) we

may then write

$$I_S = a(X_{AS}Q_{AS} + X_{BS}Q_{BS})P_O \tag{20}$$

$$= X_{AS}I_{AS} + X_{BS}I_{BS} \tag{21}$$

$$= X_{AS}I_{AS} + (1 - X_{AS})I_{BS} \tag{22}$$

where I_{AS} and I_{BS} are the intensities of the probe in the solid phase of pure lipids A and B, respectively. In this expression, I_{AS} and I_{BS} by definition depend only on temperature and account completely for the dependence of I_S on T. On the other hand, the mole fractions X_{AS} and X_{BS} account for the dependence on composition. The dependences of I_{AS} and I_{BS} on temperature are evaluated from the experimental I *vs.* T graphs for the probe in, respectively, a pure bilayer of A and a pure bilayer of B. A similar expression may be written for I_F, with analogous definitions for X_{AF}, X_{BF}, I_{AF} and I_{BF}, i.e.

$$I_F = X_{AF}I_{AF} + (1 - X_{AF})I_{BF}. \tag{23}$$

We have so far assumed that fluorescent probes occupy single sites in each of the solid and fluid phases of a lipid bilayer. Nanosecond fluorescence spectroscopic measurements, however, indicate that this is not usually the case. Thus, for example, perylene in a completely fluid bilayer displays at least two different lifetimes which indicates that it occupies more than one site in the fluid phase (Cogan *et al.*, 1973; *see also* Papahadjopoulos *et al.*, 1973). Although the theory presented above can easily be generalized to include multiple sites, the introduction of more parameters into the theory is not justified from a practical point of view. Instead, we assume in the application of the expressions presented here to experimental data that K, Q_S and Q_F represent average values for the different sites.

b) Bilayers with Lipids that Do Not Cocrystallize. The equations presented above are for systems which never have more than two phases at any temperature, as is the case for a system of lipids that cocrystallize. However, a two-component system in which the lipids do not cocrystallize can, in general, have three phases, namely, one fluid phase composed of A and B, one solid A phase, and one solid B phase. The phase diagram for such a system is shown in Fig. 1*c*. If the fluorescence quantum yield is the same in the solid A and solid B phases, then Eq. (9) and the methods of analyses described above still apply even when three phases are present, but Eq. (3) for calculating the relative amounts of lipid in the fluid and solid phases from the phase diagram has a discontinuity at the transition temperature T_B of the lower-melting lipid. The nature of this discontinuity can be seen as follows. In the region of the phase diagram (Fig. 1*c*) between the fluidus and solidus graphs, the system consists of a single fluid phase composed of A and B and a pure solid A phase. The ratio N_S/N_F in this region is given by Eq. (3) with $X_{AS} = 1$, i.e.

$$N_F/N_S = (X_A - 1)/(X_{AF} - X_A). \tag{24}$$

When the temperature of the system is lowered and first reaches the temperature T_B, component A has been completely converted to pure solid A phase, while component B begins to solidify from a pure fluid B phase to a pure solid B phase. The system at this point consists of three phases (pure liquid A, pure solid A, and pure solid B). As heat is removed, B continues to solidify until it is completely converted into pure solid B. The temperature remains at the value T_B during this conversion (in practice T_B has a range of 2 to 3 °C). Thus at the temperature T_B, the fraction of lipid in the solid phase is given by

$$N_S/N = X_A + N_{SB}/N \tag{25}$$

where $N = N_S + N_F$ and N_{SB}/N is the fraction of B in the solid phase. The value of N_{SB}/N cannot be determined from the phase diagram but depends on the amount of heat removed after reaching the temperature T_B. For the purpose of evaluating I *vs.* T, it is not necessary to consider the details of how heat is removed. Instead, we have merely to take into consideration that at that temperature T_B the fraction of lipid in the solid phase changes from the value

$$N_S/(N_S + N_F) = X_A \tag{26}$$

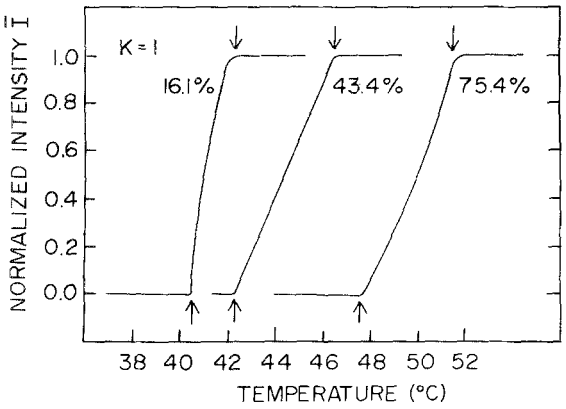
when the temperature T_B is first reached to the value

$$N_S/(N_S + N_F) = X_A + X_B \tag{27}$$

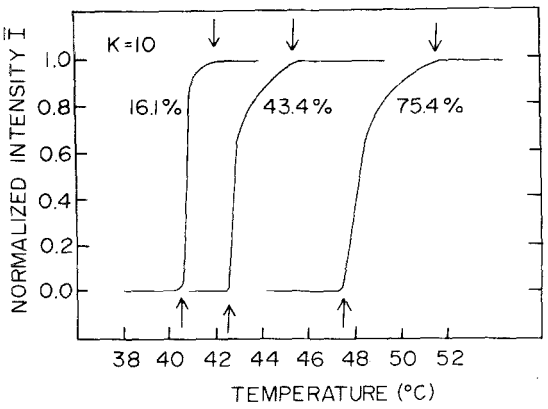
when all B is converted to the solid phase. This results in a sharp change of fluorescence intensity at the temperature T_B in a plot of I *vs.* T. The magnitude of the change in fluorescence intensity at this point is determined by the relative values of X_A and X_B. When the fluorescence quantum yield is different in the two solid phases, Eq. (9) still applies but Eq. (22) must be used to calculate I_S.

2. Water Soluble Probes with Very Low Fluorescence Efficiency in Water

A theory for water soluble probes such as N-phenyl-l-naphtylamine (NPN) must take into consideration the fact that P_O is dependent on temperature and composition, since such probes partition into the water phase. This requires, in general, introduction of partition coefficients for partitioning of probe between the bilayer and the bathing electrolyte. However, when the interest is in evaluating the partition coefficient of the probe between solid and fluid phases from I *vs.* T plots, the expression and methods presented above can be used to a good approximation for the evaluation of K as shown by the following arguments. According to Eqs. (11) and (12), I_S and I_F depend not only on Q but also P_O. Thus when I_S and I_F are extrapolated into the region of the phase transition as shown in Fig. 2 and described above, the extrapolated values include the variations of P_O with temperature. Values of $\bar{I}$ calculated with Eq. (10) and the extrapolated values of I_S and I_F are then approximately independent of the partitioning into the bathing electrolyte and are described by Eqs. (9) and (11) to (15).


NORMALIZED INTENSITY Ī
K = 1
16.1%
43.4%
75.4%
1.0
0.8
0.6
0.4
0.2
0.0
38 40 42 44 46 48 50 52
TEMPERATURE (°C)

a


NORMALIZED INTENSITY Ī
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43.4%
75.4%
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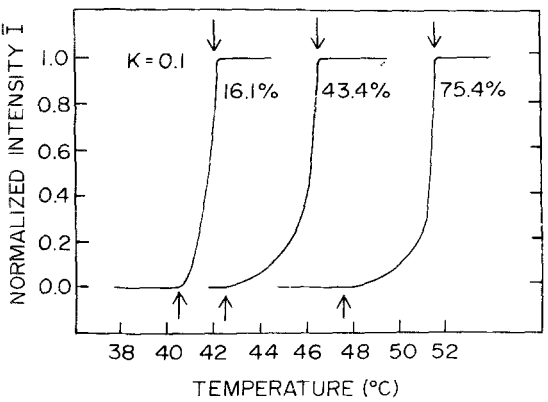
0.8
0.6
0.4
0.2
0.0
38 40 42 44 46 48 50 52
TEMPERATURE (°C)

b


NORMALIZED INTENSITY Ī
K = 0.1
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43.4%
75.4%
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0.8
0.6
0.4
0.2
0.0
38 40 42 44 46 48 50 52
TEMPERATURE (°C)

c

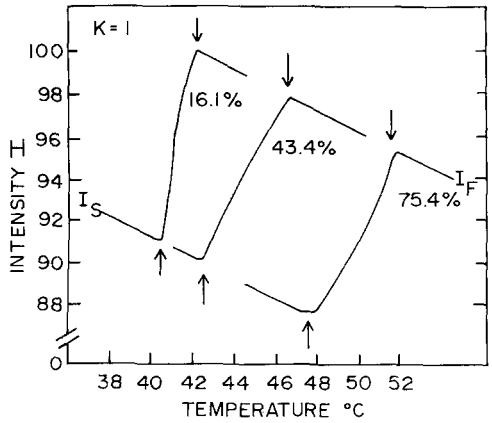

Fig. 3. Intensity profiles calculated for the phase diagram of Fig. 2*a* using Eqs. (9) to (14). The percent number next to each graph refers to mole percent (X_A) of *A* in the binary lipid mixture. (*a*) to (*c*): Normalized intensity plots calculated for different values of the partition coefficient *K*. They demonstrate the effects of *K* on the shapes of these plots. (*d*): A plot of straight intensity *I vs.* temperature *T*, demonstrating the effects of I_S and I_F (which reflect fluorescence efficiencies as described in the text) on the shapes of the intensity plots. The plots of *d* were calculated from the plots of *a* using Eq. (14). I_F and I_S were assumed to vary linearly with temperature at a rate of about −0.5% per degree. The fractional change in fluorescence intensity in going from the solid to the fluid phase was assigned a value of 10%. Arrows mark the true onset T_F and termination T_S temperatures of the transitions as determined from the fluidus and solidus graphs of the phase diagram

Calculation of Intensity Profiles

In this section we use the equations presented above to calculate intensity *vs.* temperature profiles for the two types of diagrams shown in Figs. 1*a* and *b*. In these calculations we assume that D_S/D_F has the value 1.035, recently determined by differential calorimetry for dipalmitoyl phosphatidylcholine (Wilkinson & Nagle, 1977), and that D_S/D_F and *K* do not vary with temperature. The phase diagram illustrated in Fig. 1*a* is typical of two component lipid mixtures which cocrystallize. Examples of such mixtures are dipalmitoyl phosphatidylcholine/distearoyl phosphatidylcholine (dppc/dspc) and dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine (dmpc/dppc). The diagram of Fig. 1*a* is actually that of dppc/dspc which we determined experimentally and describe in the following paper.

Figure 3 shows intensity profiles calculated with the phase diagram of Fig. 1*a* for several different compositions X_A. The ordinate on the left is the normalized intensity $\bar{I}$ defined by Eq. (9) except for Fig. 3*d* where the ordinate is intensity. The normalized intensity plots represent the contributions of the phase diagram and partition coefficient *K* to the shape of the *I vs. T* plot but do not include possible variations of Q_S and Q_F with temperature and composition. The *I vs. T* plots have the same shape as $\bar{I}$ *vs. T* plots for cases where Q_F and Q_S are independent

of temperature and composition. When Q_F and Q_S vary with these parameters their effects on I can be evaluated from the normalized intensity plots with Eq. (14).

In Fig. 3*a*, the probe is assumed to partition equally between the liquid and solid phases ($K = 1$). Figure 3*d* shows a plot of I *vs.* T calculated with the data of Fig. 3*a* assuming that I_S and I_F are independent of composition but vary with temperature at the rate of -0.5% per degree. This is a stronger dependence on temperature than that observed for perylene in vesicles of dppc/dspc, but less than that observed for NPN. The amplitude of the transition (percent change in fluorescence intensity in going from the fluid to the solid phase) shown in the figure is approximately that shown by perylene in vesicles of phosphatidylcholine. Because of the many variations which can be assumed for I_F and I_S, we present our calculations in the form of normalized intensity plots. Figure 4 shows $\bar{I}$ *vs.* T plots for the phase diagram of Fig. 1*b*.

Discussion

An interesting question which arises in the analysis of experimental fluorescence intensity *vs.* temperature profiles concerns the relation between fluorescence intensity in the regions of the phase transition and the fraction of bilayer which is in the fluid phase. We will discuss this question in terms of the reduced intensity $\bar{I}$. Methods for converting experimental I *vs.* T plots to $\bar{I}$ *vs.* T are described in the following paper, and in the discussion which follows we assume that the experimental data have been converted to normalized intensity plots. From Eqs. (9) and (13), we can write

$$\bar{I} = V_F K/(V_S + V_F K). \tag{28}$$

This equation indicates that $\bar{I}$ is a direct measure of the fractional volume F_V of bilayer in the fluid phase when $K = 1$. In this case the temperature T at the midpoint of the transition ($\bar{I} = 0.5$) occurs where $V_S = V_F$. When $K \neq 1$, $\bar{I}$ is no longer a direct measure of F_V as shown by Eq. (28). Thus if the probe partitions preferentially into the liquid-crystalline phase ($K > 1$), the midpoint temperature T_M of the transition will be lower than the temperature for which $V_S = V_F$. On the other hand, if the probe partitions preferentially into the solid phase ($K < 1$), T_M is greater than the temperature at which $V_S = V_F$. These effects can be seen in the plots of Figs. 3 and 4.

A second interesting question concerns the extent to which the phase diagram of a given two-component bilayer or membrane can be constructed from experimental I *vs.* T graphs. The apparent onset and termination of a phase transition in an I *vs.* T graph is usually marked by abrupt changes or breaks in fluorescence intensity at an upper temperature T_u (onset) and lower temperature T_ℓ (termination). It is usually assumed that these temperatures can be equated, respectively, to the temperature T_F on the liquidus graph and T_S on the solidus graph corresponding to the value of X_A used to measure the I *vs.* T graph. The complete phase diagram is determined from I *vs.* T graphs measured for different values of X_A. We will discuss the validity of this assumption on the basis of $\bar{I}$ *vs.* T graphs

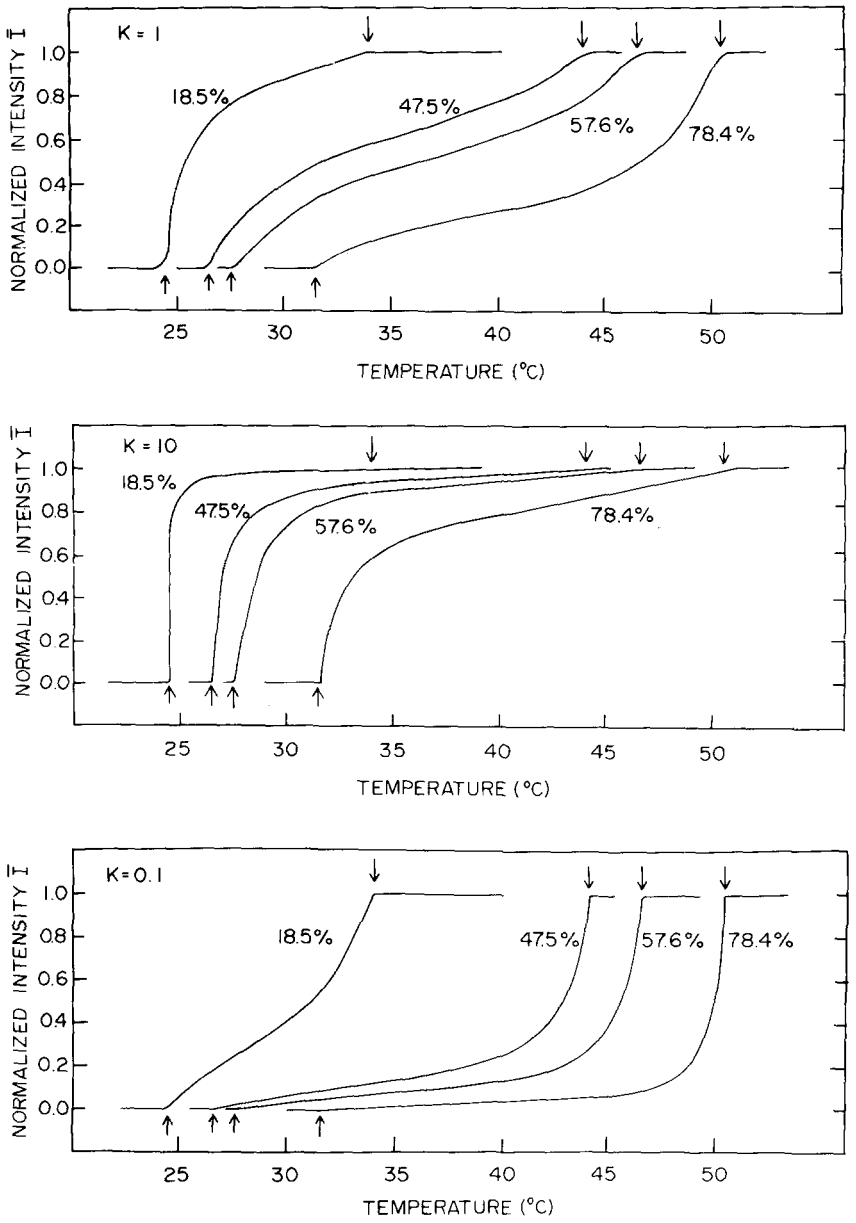

Fig. 4. Normalized intensity $\bar{I}$ *vs.* temperature T plots calculated for the phase diagram of Fig. 1*b* using Eqs. (9) to (15). The percent number next to each graph refers to mole percent (X_A) of A in the binary mixture. (*a*) to (*c*): the effects of the partition coefficient K on the normalized intensity profiles

calculated with the theory presented above. We assume in the discussion that the values of T_u and T_ℓ are determined from I *vs.* T graphs as follows. The value of T_u is selected as the temperature of the point of intersection of straight lines

drawn through the intensity points immediately above and below this region as shown in Fig. 2. The value of T_ℓ is similarly established except that the straight lines are drawn through points at the low temperature region of the transition.

Inspection of the graphs of Fig. 3 indicates that for lipids which cocrystallize, the temperatures T_ℓ and T_u for a given composition X_A agree with the temperatures T_S and T_F given by the phase diagram when $K = 1$. However, when K is much greater than 1, the $\bar{I}$ *vs.* T plot becomes rounded in the upper temperature range of the transition, and some ambiguity may arise in the evaluation of T_u for K much greater than 1. Similarly, when K is much less than 1, the $\bar{I}$ *vs.* T graph is rounded at the lower end of the transition which leads to some ambiguities in establishing a value for T_ℓ.

The situation is more complex for systems that do not cocrysallize or only partially cocrystallize. Figure 4 shows that for such systems the I *vs.* T graph for a given X_A may show several abrupt changes or inflections. This more complex behavior is due to the fact that systems which do not cocrystallize or only partially cocrystallize actually have two different solid phases as discussed above, while systems which do cocrystallize have only one solid phase. In the limit of zero cocrystallization, there should be two distinct transitions, one broad transition corresponding to the solidification of the component with the higher melting point [*see* Eq. (24)] and a narrower one corresponding to solidification of the component with the lower melting point [*see* Eq. (25)].

The intensity profiles of Fig. 4 indicate that for $K = 1$, the values for T_u and T_ℓ can be identified and agree well with values for T_F and T_S, respectively. For values of K which are much larger than 1 (*see* Fig. 4*b*), the $\bar{I}$ *vs.* T graphs are very flat in the upper region of the transition and T_u does not agree with T_F. When K is much less than 1 (*see* Fig. 4*c*), the graphs are quite flat in the lower temperature range of the transition and the value for T_ℓ does not agree with T_S.

The results above indicate that care must be exercised in the study of such membrane properties as microviscosity when probes that partition preferentially into the fluid or solid phase are used. Such probes may not be able to sense changes in the average microviscosity or other membrane parameters resulting from a change in the fraction of bilayer in the solid phase. Thus a comparison of the plots of Fig. 4*a* and *b* shows for example that for $K = 10$ and $X_A = 0.185$ a change in temperature from about 34 to 25 °C, which changes the fraction of lipid in the fluid phase from 1 to 0.5, decreases $\bar{I}$ by only 10%.

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