Thermodynamics of phosphatidylcholine-cholesterol mixed model membranes in the liquid crystalline state studied by the orientational order parameter

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ABSTRACT It is shown that good estimates of the activity of cholesterol in phosphatidylcholine-cholesterol mixed model membranes are obtained by examining the orientational order parameter S of cholestane spin probe (CSL) that is obtained from electron spin resonance by spectral simulation. By introducing thermodynamic stability conditions of liquid mixtures, the variation of activity (or S) as a function of cholesterol mole fraction is utilized to predict the concentration at which the phase separation occurs. These results for DMPC and cholesterol binary mixtures agree very well with those of Tempo-partitioning experiments. The comparison of activity coefficients and the phase boundary in DMPC/cholesterol mixtures with those of POPC/cholesterol mixtures suggests that acyl chain unsaturation leads to poorer mixing of cholesterol in phosphatidylcholine model membranes at higher temperatures (i.e., >35°C). In ternary solutions of DMPC, POPC, and cholesterol, it is found that cholesterol shows less deviation from ideality than in either of the two binary mixtures, and this implies that the phase separation occurs at higher cholesterol concentration than in either of the two binary mixtures. The present analysis suggests that there may not be a critical point in DMPC/cholesterol mixtures, even though phase separation does occur.

INTRODUCTION

Thermodynamic properties, especially the phase behavior, of phosphatidylcholine model membranes containing cholesterol have been studied extensively. By direct experimental methods such as calorimetry (1), x-ray scattering (2), and freeze-fracture electron microscopy (3), or by indirect methods such as Tempo-partitioning (4, 5), spin labeling (6, 7), and lateral diffusion measurement (8), the phase behavior below the main chain melting transition (T_m) has been reasonably well investigated. Those experiments have revealed several remarkable features. First, cholesterol broadens the chain melting transition and gradually abolishes the associated enthalpy change (1). Second, a cholesterol-rich liquid crystalline phase and pure phospholipid gel phase alternate just below T_m (3). Third, more than 20 mol% cholesterol induces a phase transition from the gel phase to the fluid phase (8). There also have been theoretical approaches to explain the effect of cholesterol on the main chain melting transition (9, 10).

By contrast to such richness of information below T_m, the thermodynamic properties of the liquid crystalline state have yet to be established for the mixtures. There is general agreement from several experiments in the location of the phase boundary at high cholesterol concentration in DMPC/cholesterol mixtures (5–7) (cf. Fig. 4). The basic disagreement arises from identifying the number of phase at the left-hand side of the phase boundary as either a single phase (2, 11) or two coexisting fluid phases (6, 12). Most recent experimental results, however, agree with a single fluid phase (2, 11).

The orientational order parameter may be defined (13, 14):

$$S = \langle D^2_{00} \rangle = \int D^2_{00} \exp\left[-V(\Omega)/k_B T\right] d\Omega \Big/ \int \exp\left[-V(\Omega)/k_B T\right] d\Omega, \quad (1)$$

where $D^2_{00} = \frac{1}{2}(3\cos^2\theta - 1)$, and $V(\theta)$ is an orienting potential. The orientational order parameter S is rather easily obtainable by NMR or ESR. It is an extremely important thermodynamic quantity that has been essential to the understanding of the thermodynamic properties of thermotropic liquid crystals (13, 15) and model membranes (16–18). We principally utilize the rigid CSL spin probe, because it has no intramolecular modes of motion affecting the spin label, and it is known to report on the overall ordering in the solution (16). (For a label on a flexible chain position one must correct the observed order parameter for the additional effects of internal motional averaging [19].)

The orienting potential is more precisely the "potential of mean torque" (20). That is, it may be described by the statistical average of the contributions of orienting forces (or torques) from the surrounding molecules. Thus, it reflects the molecular packing and the distribution of molecules around a given molecule in an average sense.

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Furthermore, it is reasonable to expect that the orienting potential of the ith component in a multicomponent system would reflect the average local molecular composition of surrounding molecules. Consequently, we may consider the possibility of a connection between the order parameter of the ith component and its activity in solution (21).

The primary objective of the present work is to demonstrate the success we have had in relating the activity of a component in multicomponent model membranes to the orientational order parameter. These results indicate a general approach to obtain the activity coefficients of the components of model membranes. For a binary solution, such as a DMPC/cholesterol mixture, we can calculate the activity coefficient of the second component from that of the first by integrating the Gibbs-Duhem equation (21, 22), and generalization exists for ternary, etc., solutions. Thus, our approach would allow a complete determination of the thermodynamic behavior of the mixture.

We have studied binary mixtures of DMPC and cholesterol, and of POPC and cholesterol. For the binary model membranes we utilize the thermodynamic stability conditions of liquid mixtures to obtain the concentration at which the mixture will have a phase separation at a given temperature. By comparing the results of two binary mixtures, the effect of acyl chain unsaturation on nonideality and on phase separation is examined. The possibility of a critical point is also examined. We have performed the same analysis on ternary mixtures of DMPC, POPC, and cholesterol with the hope of understanding the effects of mixing in model membranes. (Note that DMPC and POPC are known to be completely miscible [23].)

MATERIALS AND METHODS

1,2-Dimyristoyl-*sn*-glycero-phosphatidylcholine (DMPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphatidylcholine (POPC) were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) and they were used without any further purification. Cholesterol was obtained from Sigma Chemical Co. (St. Louis, MO) and recrystallized in ethanol. The 3-doxyl derivative of cholestan-3-one (CSL) was purchased from Syva Co. (Palo Alto, CA) and also recrystallized in ethanol.

The well-aligned homeotropic multilayer plate samples are prepared by modified hydration-evaporation technique described elsewhere (24, 25). For DMPC, POPC, and cholesterol ternary mixtures the ESR spectra were taken in conjunction with the dynamic imaging experiment, in which the sample was initially prepared to have an inhomogeneous spin probe (CSL) distribution (25). On the other hand, for DMPC/cholesterol mixtures, the samples with homogeneous spin probe distribution were used just to measure ordinary ESR spectra. The CSL concentration was ~0.5 mol%. (Given the small concentration of CSL, we have not found any dependence of order parameter of CSL on its homogeneous vs. inhomogeneous distribution.) The order parameter of CSL in POPC/cholesterol mixtures was taken from our previous work (21).

The orientational ordering potential of CSL was obtained by nonlinear least-square ESR spectral simulation, which was performed on the Cornell supercomputer. The same magnetic tensor parameters were used for all spectra: A = (5.6, 5.3, 34) in gauss and g = (2.0081, 2.0061, 2.0024) (18). We simulated the spectra taken at that sample orientation for which the external magnetic field and the director of the molecule coincide. The details of the computational procedure and the calculation of order parameters from orienting potentials are described elsewhere (26–28).

RESULTS AND DISCUSSION

Because the orientational order parameter $S_i(\{x\}, T)$ is an intensive thermodynamic property of the ith component of the solution, the variation of $S_i(\{x\}, T)$ as a function of x_i should be related to its activity in solution. (Here $\{x\}$ refers to the collective mole fractions of the various components.) We expect $S_i(\{x\}, T)$ will be linear with x_i if the solution is ideal; otherwise it would deviate from linear behavior (analogous to the partial pressure in an ordinary isotropic nonideal solution). We have previously pointed out that the orientational order parameter could offer a way to measure the activity coefficient in anisotropic solutions (21). That is, when $S_i(\{x\}, T)$ varies nonlinearly with x_i, the replacement of x_i by the activity of the ith component should "relinearize" the functional dependence, just as it does other intensive properties of the components of a solution (e.g., the partial pressure). Thus, we shall write

$$S_i(x_i, T) - S_i(0, T) = ba_i(\{x\}, T) = bx_i\gamma_i(\{x\}, T), \quad (2)$$

where a_i is the activity of the ith component and γ_i is its associated activity coefficient. That is, in Eq. 2 we are neglecting any, presumably small, higher order terms in a_i.

Thermodynamics provides the following stability conditions for binary liquid mixtures. (In the Appendix we review the derivation of thermodynamic stability conditions.) As long as the binary mixture is in stable equilibrium, the first derivative of the chemical potential of a component with respect to its mole fraction will be positive:

$$\left(\frac{\partial \mu_2}{\partial x_2}\right)_{T,P} > 0. \quad (3)$$

Whereas the mixture will be unstable (phase separation) if

$$\left(\frac{\partial \mu_2}{\partial x_2}\right)_{T,P} < 0. \quad (4)$$

Thus, at the phase boundary the derivative vanishes:

$$\left(\frac{\partial \mu_2}{\partial x_2}\right)_{T,P} = 0. \quad (5)$$

Because the chemical potential of the ith component is

$$\mu_i = \mu_i^0(T) + RT \ln a_i, \tag{6}$$

we readily find from Eqs. 2 and 3 that

$$\frac{\partial \ln |\Delta S_2(x_2, T)|}{\partial x_2} > 0, \tag{7}$$

where $\Delta S_2(x_2, T) = S_2(x_2, T) - S_2(0, T)$, if the binary mixture is stable. Whereas from Eq. 5 the phase separation is predicted when

$$\frac{\partial \ln |\Delta S_2(x_2, T)|}{\partial x_2} = 0, \tag{8}$$

It is obvious from Eq. 8 that the orientational order parameter will reach an extremum at the phase boundary.

We have found it useful to fit our experimental results of order parameter of CSL spin probe in phosphatidylcholine/cholesterol mixtures with a functional form:

$$S_{CSL}(x, T) - S_{SCL}(0, T) = b \frac{x \exp [d(T)(1 - x)^2]}{1 + c(T)x^q}, \tag{9}$$

where x denotes the mole fraction of cholesterol in the mixtures. Although Eq. 9 is utilized in an empirical sense to interpolate between data points, we rationalize its features below. The best fitting values of parameters b, d, and c at the different temperatures for DMPC/cholesterol, POPC/cholesterol binary model membranes, and DMPC/POPC/cholesterol ternary model membranes are given in Table 1. The parameter q was chosen to best fit at all three temperatures for each model membrane of different phosphatidylcholines. For DMPC/cholesterol and for DMPC/POPC/cholesterol the best value of q is 2, whereas $q = 5/4$ gives good results for POPC/cholesterol mixtures. The variations of order parameter as a function

TABLE 1 Fits of $S_{CSL}(x, T)$ to Eq. 9

Temperature	q	b	c	d
DMPC and cholesterol mixtures				
°C				
35	2	1.58	10.6	0.167
45	2	1.42	7.7	0.142
55	2	1.23	4.9	0.123
POPC and cholesterol mixtures				
35	5/4	1.39	4.32	1.02
45	5/4	1.36	4.14	1.05
55	5/4	1.52	4.42	0.92
DMPC/POPC/cholesterol mixtures				
35	2	1.01	4.55	0.33
45	2	1.06	3.91	0.22
55	2	1.00	3.23	0.33

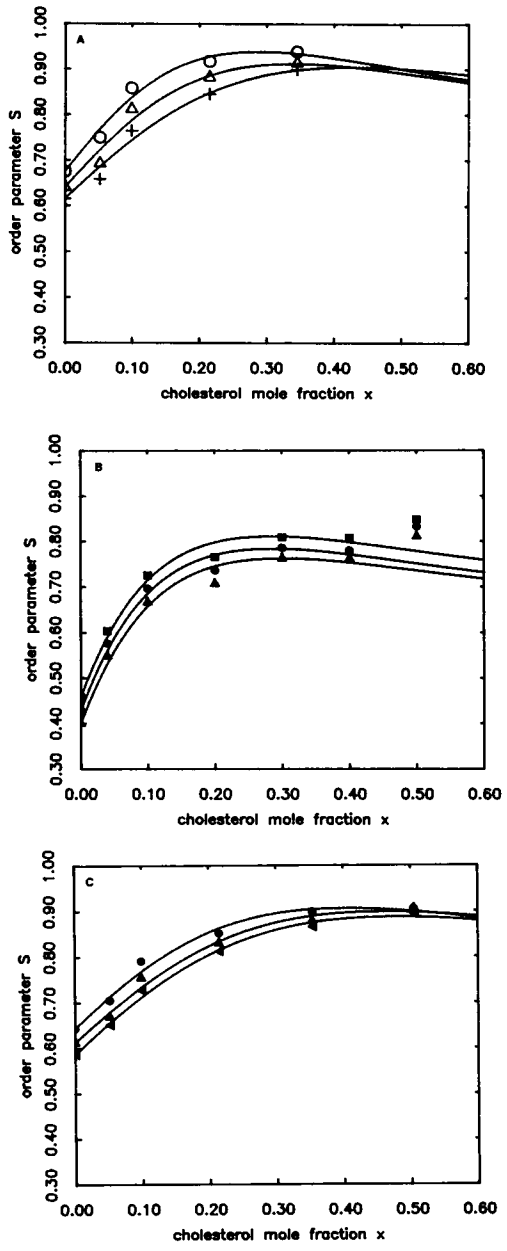

FIGURE 1 (A) Plots of order parameter S of CSL vs. cholesterol mole fraction x in DMPC and cholesterol mixtures. The best fit with Eq. 9 is shown by a curve for each temperature: 35 (○), 45 (△), and 55°C (+). (B) Same type of plots for POPC and cholesterol mixtures: 35 (■), 45 (●), and 55°C (▲). The order parameter at $x = 0.5$ has not been included in fitting. Deviation from Eq. 9 at this high concentration probably indicates that the activity of cholesterol may not follow the hypothetical behavior of Eq. 9 at x larger than x_t. (C) Same type of plots as A for DMPC, POPC, and cholesterol mixtures: 35 (●), 45 (▲), and 55°C (◀).

of x and the result of fitting for each model membrane are illustrated in Fig. 1, *a–c*.

Because CSL spin probe is known to report on cholesterol due to its structural similarity, we may consider S_{CSL} and S_{chol} comparable (21, 29). Consequently, from Eqs. 2 and 9 we obtain the activity of cholesterol as a function of x at constant temperature:

$$a_{chol}(x, T) = x\gamma_{chol}(x, T) = \frac{x \exp [d(T)(1 - x)^2]}{e^{d(T)}[1 + c(T)x^q]}. \quad (10)$$

This is similar to the behavior of the activity of a component in a regular solution (30) except for the modification $1/(1 + cx^q)$. This modification was chosen to express the tendency toward saturation observed for the order parameter of CSL spin probe at high cholesterol concentration. The coefficient e^d in the denominator is just added to follow the Henry's law convention for the solute ($\gamma_{chol} \rightarrow 1$ as $x \rightarrow 0$). The variation of the activity coefficient γ_{chol} with x in three different model membranes as obtained from Eq. 10 is compared in Fig. 2 for 45°C.

The excess partial molar free energy (i.e., the excess chemical potential) of the ith component in a multicomponent solution is related to the activity coefficient by the equation (31):

$$\Delta\mu_i^{ex} = RT \ln \gamma_i. \quad (11)$$

It is noticed from Fig. 2 that γ_{chol} in all phosphatidylcholine/cholesterol mixtures we have studied is <1 for all x. This is also true at the other temperatures. This implies that the excess partial free energy is always negative. The negative partial free energy of cholesterol means that cholesterol has a tendency to associate to form cholesterol-rich domains. Fig. 2 shows that γ_{chol} in DMPC/cholesterol mixtures is higher than that in POPC/cholesterol mixtures for all compositions, but this difference is found to decrease with decreasing T. It suggests that cholesterol molecules have a tendency to associate with each other more in POPC model membranes than in DMPC model membranes. In other words, acyl chain unsaturation tends to lead to cholesterol expulsion from the vicinity of phospholipid molecules. Given the relative T dependence of γ_{chol}, it would suggest that the partial molar entropy of mixing of cholesterol is less for POPC/cholesterol mixtures, and possibly the enthalpy of mixing is greater. (Note that $\Delta\mu_{imix} = \Delta h_{imix} - T\Delta s_{imix}$ and $(\partial\Delta\mu_i/\partial T)_p = -\Delta s_i$.) On the other hand, the γ_{chol} in DMPC/POPC/cholesterol ternary mixture is larger than in either of the binary mixtures (except for γ_{chol} at 55°C, where it is almost identical to that in DMPC/cholesterol mixtures). This suggests that the mixing of the two types of phospholipid leads to less self-association of cholesterol molecules.

The association of cholesterol in the mixtures will lead to phase separation at sufficiently high cholesterol concentration. Indeed, an extra broad peak appears at high x in POPC/cholesterol mixtures as can be seen in Fig. 3. (A careful examination of Fig. 3 shows an apparent dependence of the location of this peak on x as well as a narrowing of the full spectral width of the main component. This latter spectral feature is mainly due to the increase in S at $x = 0.5$ shown in Fig. 1 *b*. In the simple case of two phases [with two components] at equilibrium, the addition of one component should not affect the nature or properties of each phase, but only their relative amounts. The difference we see could be due to failure to reach complete equilibrium; we generally waited 1 h after each temperature change before collecting data. We found this sufficient for nearly all our cases [i.e., $x \leq 0.4$]

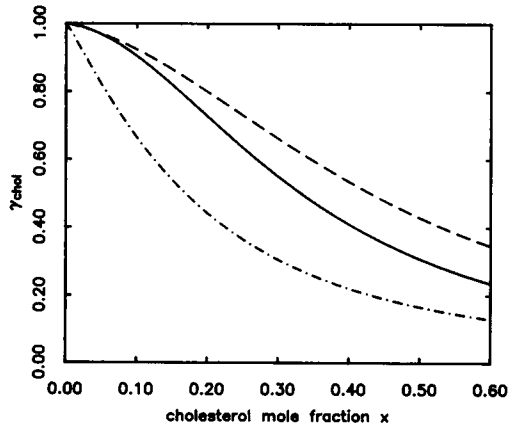

FIGURE 2 Plot of activity coefficient of cholesterol vs. x at 45°C: DMPC/cholesterol (—), POPC/cholesterol (-·-·-) and DMPC/POPC/cholesterol mixtures (---).

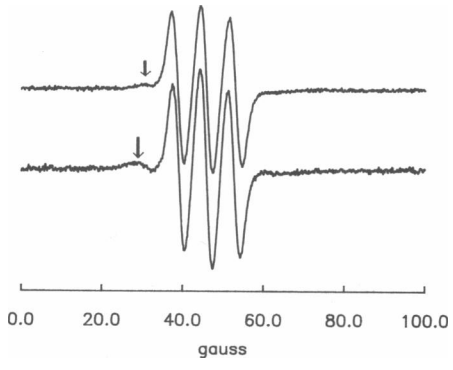

FIGURE 3 The ESR spectra of CSL in POPC/cholesterol mixtures at 45°C for $x = 0.4$ (*top*) and for $x = 0.5$ (*bottom*). The angle (Ψ) between the ordering axis and the applied magnetic field (H_0) was 0°. The extra peak indicated by the arrow may be due to CSL in a rather highly disordered second phase.

in which we only observe a single phase, but in the presence of two phases this might not be long enough. In fact, under the polarizing microscope we did observe, for $x = 0.5$ and POPC/cholesterol mixtures, needle-shaped microcrystals whose appearance changes only slowly as the temperature changes. Further work is needed to clarify this matter, but we briefly mention an alternative possibility. This would be that the microdomains of a phospholipid-rich phase [yielding the three-line ESR spectrum] and a cholesterol-rich phase, are small enough in extent that they modify each others' properties either directly or by exchange of molecules. This alternative seems less likely in view of our observation under the polarizing microscope of crystals of macroscopic size.)

Once we have empirically obtained a good functional form of $\Delta S_{chol}(x, T)$ or $a_{chol}(x, T)$ as in Eq. 9 or 10, it is easy to locate the concentration of cholesterol x_l at which the limit of stability of the mixture is reached, and the phase separation starts to occur (cf. Eq. 8). By solving Eq. 8 we found x_l for a given mixed model membrane at three temperatures. The results are plotted in the temperature-composition phase diagram (Fig. 4).

For DMPC/cholesterol mixtures, they are compared with reported results from various other experiments. Our prediction of the phase boundary turns out to agree very well with the results of Shimshick and McConnell (5) obtained by Tempo-partitioning. In the POPC/cholesterol mixture the phase separation occurs at lower cholesterol mole fraction compared with DMPC/cholesterol mixture, at temperatures above 35°C. (However, below 35°C, it appears from extrapolations in Fig. 4 that this is reversed. This may be appropriate considering that POPC undergoes its main chain melting transition ~20°C below that of DMPC. Note that the entropic effects are less important at lower T.) That is, the saturated DMPC mixes more with cholesterol than does the unsaturated POPC above 35°C. In DMPC/POPC/cholesterol ternary mixtures, however, the phase separation is predicted to occur at higher cholesterol concentration than in either of the binary model membranes. This probably implies that the entropy of mixing in ternary mixtures contributes to sustain more cholesterol than in binary mixtures.

Because the activities of components in mixtures are related to each other by the Gibbs-Duhem equation (cf. Eq. 20), we can calculate the activity of the second component, once we know the activity of one component vs. the composition in a binary mixture. By integrating the Gibbs-Duhem equation with Eq. 10 for a_{chol} in DMPC/cholesterol mixtures we find

$$a_{DMPC}(x_{DMPC}, T) = \gamma_{DMPC}(x, T)(1 - x) = (1 - x)^{(1-c)/(1+c)} \cdot (1 + cx^2)^{-1/(1+c)} \exp\left[dx^2 - \frac{2c^{1/2}}{1 + c} \tan^{-1}(c^{1/2}x)\right], \quad (12)$$

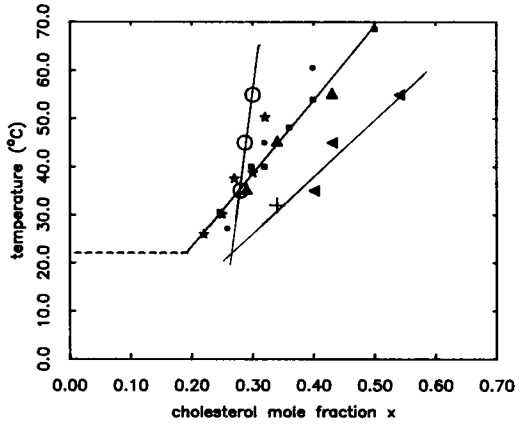

FIGURE 4 The temperature-composition phase diagram for phosphatidylcholine-cholesterol mixtures. Our results are: DMPC/cholesterol (▲); POPC/cholesterol (○); DMPC/POPC/cholesterol (◀). For DMPC/cholesterol mixtures these results are compared with reported ones: (■) (5); (☆) (6); (●) (7). For the ternary mixtures the point at $x = 0.34$ and $t = 32$°C (+) is obtained from lateral diffusion measurements by the ESR imaging method (Shin, Y.-K., J. K. Moscicki, and J. H. Freed, submitted for publication). The dotted line represents the main chain melting transition for DMPC.

where x denotes again x_{chol}. By analogy to the partial pressure (i.e., $p_i - p_i^0 = a_i p_i^0 - p_i^0 = (a_i - 1)p_i^0$), we would expect the order parameter of DMPC will depend on x according to

$$S_{DMPC}(x, T) - S_{DMPC}(0, T) = b'[1 - a_{DMPC}(x, T)] = b'[1 - \gamma_{DMPC}(x, T)(1 - x)]. \quad (13)$$

(We previously used the empirical functional form $\Delta S_{16\text{-PC}}[x] = b''\gamma_{POPC}x$ to fit our data of the phospholipid spin probe [16-PC] in POPC-cholesterol mixtures, because the fit with Eq. 13 was only partially successful [21]. This could be due to the influence of the internal motions on the measured order parameter, which is known to be significant, because the spin is attached at a flexible end-chain segment [16, 19, 31a].) We show in Fig. 5 the variation of the order parameter of the DMPC molecule as a function of cholesterol mole fraction x as predicted by Eqs. 12 and 13. It shows the maximum at x_l, which has been guaranteed by the Gibbs-Duhem equation. It is also compared with the outer maximum splitting of ESR spectra of 5-stearic acid spin probe in dispersion samples of DMPC/cholesterol (that is expected to report on the ordering of DMPC), which was found to reach its extremum at the phase boundary in DMPC/cholesterol mixture (7). Our prediction agrees rather well with the maximum splitting data. The discrepancy at low x could result from the fact that the maximum splitting is not in itself an accurate measure of the true thermodynamic order parameter. (The latter would also require measure-

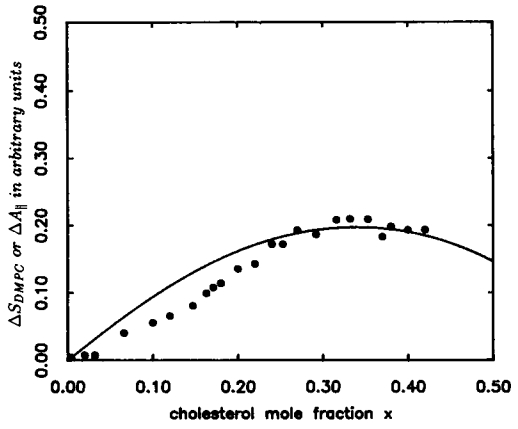

FIGURE 5 $\Delta S_{DMPC}(x)$ (—) predicted by Eq. 13 is compared with $\Delta A_{\parallel}(x)$ (●) of 5-stearic acid spin probe in reference 7. $\Delta A_{\parallel}(x)$ denotes $A_{\parallel}(x) - A_{\parallel}(0)$ and Eq. 13 defines $\Delta S_{DMPC}(x)$. They are arbitrarily scaled to overlap.

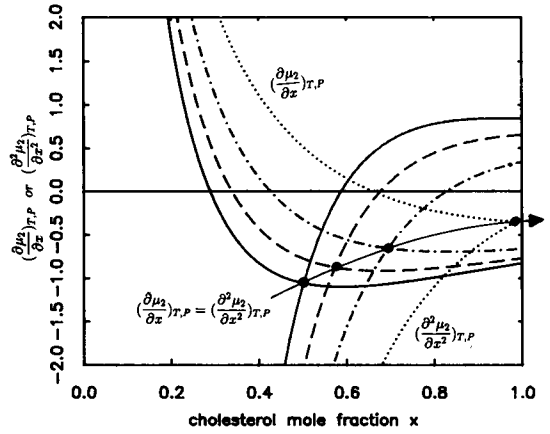

FIGURE 6 Plots of the first and second derivatives of the chemical potential vs. x at different temperatures. The curves which vary from positive to negative with increased x represent first derivatives. The temperatures are at 35 (—), 45 (---), 55 (-·-·-) and 65°C (····). The locus of points (●—●—●) for which $\partial\mu_2/\partial x = \partial^2\mu_2/\partial x^2$ shows that a critical composition x_c, at which the first and second derivatives of the chemical potential cross zero simultaneously, does not exist at atmospheric pressure.

ment of the minimum splitting value $A'_{\perp}$ as well as the assumption that the rotational motion is fast enough to average out the time-dependent part of the spin Hamiltonian; otherwise, it would deviate from the true order parameter due to motional effects [32, 33].) Alternatively, it could be due to our implicit neglect of the effects of the third component, water (21), whose effects on the lipid bilayer could be modified as cholesterol is added. (In this, and our other work [21; Shin, Y.-K., J. K. Moscicki, and J. H. Freed, submitted for publication], there is no evidence for lipid phase separation for $x \leq 0.2$.)

The calculation of a_{DMPC} is an example of the use of the Gibbs-Duhem equation in a two component system. It should be noted that the Gibbs-Duhem equation is applicable to any multicomponent system. In fact, for a multicomponent solution, knowledge of the activity of one component at all compositions $\{x\}$ allows one to obtain the activities of all the components (34).

Let us now consider the possibility of a critical point. At the critical point not only is Eq. 5 fulfilled, but also the conditions

$$\left(\frac{\partial^2 \mu_2}{\partial x_2^2}\right)_{T,P} = 0, \quad \left(\frac{\partial^3 \mu_2}{\partial x_2^3}\right)_{T,P} > 0 \qquad (14)$$

would have to be satisfied simultaneously (35). For a DMPC/cholesterol mixture we fit the temperature dependence of parameters $d(T)$ and $c(T)$ by a linear approximation, and we find the best fits are $d(t) = 0.24 - 2.1 \times 10^{-3}t$ and $c(t) = 20.2 - 0.28t$, where t denotes the temperature in Celsius. The behavior of the first and second derivatives of $\ln a_{chol}$ with respect to x_{chol} given by Eq. 10 are plotted in Fig. 6 for a range of temperatures. It is found that there is no x_c in $0 < x < 1$, such that Eqs. 5 and 14 are simultaneously satisfied. Consequently, we may conclude that a critical point does not exist in DMPC/cholesterol binary model membranes (at atmospheric pressure). (In fact, an extrapolation of Fig. 6 would lead to $x_c \sim 1.6$). One should note that the existence of a critical point is not necessary for phase separation to occur (35). The latter only requires Eq. 5 to be valid for an x in $0 < x < 1$ and not Eqs. 14, and this is indeed what we have found in this work. However, we cannot at present be certain of the reliability of our simple extrapolations of $d(T)$ and $c(T)$. In fact Ipsen et al. (36) have considered a simple microscopic Potts model for DPPC/cholesterol mixtures which leads them to predict a critical point for $x < 1$. Further studies, but at higher temperatures, of the sort reported in the present work should further clarify this matter.

CONCLUSIONS

(*a*) The orientational order parameter of CSL provides a good estimate for the activity of cholesterol in model membranes.

(*b*) The order parameter, or the activity, reaches its extremum at the phase boundary.

(*c*) The phase boundary for the DMPC-cholesterol mixtures obtained by the present approach agrees well with that of Tempo-partitioning experiments.

(*d*) Acyl chain unsaturation leads to poorer mixing of cholesterol in the phosphatidylcholine model membranes

at higher temperatures (i.e., >35°C) in the liquid crystalline phase.

(*e*) In a mixed solvent consisting of two types of phosphatidylcholines (DMPC and POPC), dissolved cholesterol deviates less from ideality.

(*f*) The prediction of $S_{DMPC}(x)$ utilizing the Gibbs-Duhem equation agrees rather well with the maximum splitting data of the 5-stearic acid spin probe. This indicates that the thermodynamics of a multicomponent model membrane can be completely specified by measuring the activity of one component at all compositions $\{x\}$.

APPENDIX

Let us briefly review the thermodynamics of a multicomponent solution with respect to its stability (35, 37). We assume a closed system (I) composed of r different components, which has two subsystems called A and B. There are n_A moles of A and n_B moles of B. The boundary between the subsystems is permeable to mass and heat transfer and it is expandable. The subsystems A and B are in equilibrium so that the mole fraction (x_i) and chemical potential (μ_i) of the ith component in A and B are the same for all components. Now we perturb the subsystem A from its equilibrium composition $\{x_i\}$ to $\{x_i + dx_i\}$ keeping the temperature T, pressure P, and n_A constant. The accompanying change in composition of B will be $\{x_i - (n_A/n_B)dx_i\}$. If the subsystems A and B are in a stable equilibrium, then the system I will return to its original equilibrium. However, if they were not, then a perturbation would cause subsystem A to go to a new state of lower free energy. In the case of a stable equilibrium the total free energy change of system I should be positive:

$$\Delta G^I = \Delta G^A + \Delta G^B > 0. \tag{15}$$

By definition the molar free energy is written

$$g(T, P, x_1, \ldots, x_r) = \sum_{i=1}^{r} x_i\mu_i. \tag{16}$$

At equilibrium the molar free energy of the subsystem A, g^A is equal to that of subsystem B, $g^B[g = g^A = g^B]$. We can rewrite the stable equilibrium condition (cf. Eq. 15) in terms of g^A and g^B as:

$$n_A\Delta g^A + n_B\Delta g^B > 0, \tag{17}$$

where $\Delta g^A = g(T, P, \{x_i + dx_i\}) - g(T, P, \{x_i\})$ and $\Delta g^B = g(T, P, \{x_i - (n_A/n_B)dx_i\}) - g(T, P, \{x_i\})$. Let us expand g^A and g^B in Taylor's series around the equilibrium composition $\{x_i\}$ and neglect the higher order terms beyond second order. The first order term will cancel and only the second order term will remain:

$$n(1 + n_A/n_B) \sum_{i,j=2}^{r} \left(\frac{\partial^2 g}{\partial x_i \partial x_j}\right)_{T,P,n} dx_i dx_j > 0, \tag{18}$$

where $n = n_A + n_B$.

It is well known that the $r \times r$ determinant formed from the $\partial^2 g/\partial x_i \partial x_j$, as well as its minors, must be positive to satisfy the inequality 18 everywhere.

For a binary solution this will reduce to a single independent inequality:

$$\left(\frac{\partial^2 g}{\partial x_2^2}\right)_{T,P,n} > 0. \tag{19}$$

Using the Gibbs-Duhem equation, which may be written as

$$(1 - x_2)\frac{\partial \mu_1}{\partial x_2} + x_2\frac{\partial \mu_2}{\partial x_2} = 0, \tag{20}$$

and the inequality 19 we will have a sufficient condition for stable binary mixtures:

$$\left(\frac{\partial \mu_2}{\partial x_2}\right)_{T,P} > 0. \tag{3}$$

On the other hand the binary mixture will be unstable if

$$\left(\frac{\partial \mu_2}{\partial x_2}\right)_{T,P} < 0. \tag{4}$$

Inequality 4 implies that the free energy change of the system will be negative if a certain part of the binary mixture (e.g., subsystem A) undergoes an infinitesimal displacement from its equilibrium composition. Consequently, such a displacement will occur spontaneously and it will result in a spatial inhomogeneity in the solution, i.e., "a phase separation." However, insofar as the inequality 3 holds, the binary solution will remain a stable mixture. The limit of stability of the binary solution will be at the composition where

$$\left(\frac{\partial \mu_2}{\partial x_2}\right)_{T,P} = 0. \tag{5}$$

This composition may be considered as the point of a phase boundary which separates the region where the phases separate from the region of a stable binary mixture in a temperature-composition phase diagram.

For a ternary solution there are three inequalities which must be satisfied simultaneously to maintain a stable solution. This is a consequence of Eq. 18 for $r = 3$. However, this can be simplified to the equivalent of the case of binary mixtures by examining the chemical potential of one component as a function of its mole fraction keeping the molar ratio of the other two fixed. On these so called pseudobinary lines the molar free energy is written

$$g = x_1\mu_1 + x_2'\{k\mu_2 + (1 - k)\mu_3\}, \tag{21}$$

where $x_2' = x_2 + x_3$ and $x_2/x_3 = k/(1 - k) = \text{const}$. This is equivalent to Eq. 16 when r equals 2, with $\mu' = k\mu_2 + (1 - k)\mu_3$. Thus, the three-component mixture will be stable as long as $(\partial\mu_1/\partial x_1)_{T,P}$ is positive.

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