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# Miscibility Critical Pressures in Monolayers of Ternary Lipid Mixtures

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ABSTRACT When phospholipids are mixed with cholesterol in a monolayer at an air–water interface, coexisting 2-dimensional liquid phases can be observed if the surface pressure, $\pi$, is lower than the miscibility critical pressure, $\pi_c$. Ternary mixtures of two phospholipid species with dihydrocholesterol have been reported to have critical pressures that are linearly proportional to the relative composition of the phospholipids. However, we report here that, if the acyl chains of the two phospholipids differ significantly in length or unsaturation, the behavior is markedly different. In this case, the critical pressure of the ternary mixture can be remarkably high, exceeding the critical pressures of the corresponding binary mixtures. High critical pressures are also seen in binary mixtures of phospholipid and dihydrocholesterol when the two acyl chains of the phospholipid differ sufficiently in length. Using regular solution theory, we interpret the elevated critical pressures of these mixtures as an attractive interaction between the phospholipid components.

## INTRODUCTION

Biological membranes are formed from lipid bilayers and membrane proteins. The bilayers are composed of hundreds of different lipid species (e.g., over 250 in an erythrocyte membrane [Myher et al., 1989]). One experimental approach to the question of how the membrane lipid composition is set in a biological membrane is to study simple lipid mixtures in monolayers. For example, the miscibility critical point behavior observed in binary mixtures of phospholipid and cholesterol is also observed in complicated mixtures of lipids approximating the outer and inner leaflets of a red blood cell (Keller et al., 1998).

Several mixtures of cholesterol and phospholipids form immiscible liquid phases when spread as a monolayer at an air–water interface (Subramaniam and McConnell, 1987; Hirshfeld and Seul, 1990; Keller et al., 1998). By epifluorescence microscopy, domains rich in cholesterol appear black, and those rich in phospholipid appear white (Benvegnu and McConnell, 1993; Seul and Chen, 1993). As the area per molecule is decreased and the surface pressure increases, the domains eventually mix into one uniform liquid phase. If this transition occurs near the miscibility critical point, both in pressure and in composition, the domains form a *stripe phase* before mixing as in Fig. 1 *A* (Seul and Chen, 1993; Keller et al., 1998).

Miscibility critical pressure is a sensitive function of the lipids in the monolayer. It is known that, in binary mixtures of phospholipids and dihydrocholesterol, the critical pressure increases with decreasing chain length and with unsaturation of the phospholipid's acyl chains (Fig. 1 *B*) (Hagen and McConnell, 1997). Dihydrocholesterol (Dchol) is used rather than cholesterol because it is more resistant to air oxidation yet produces very similar phase behavior when mixed with phospholipids (Benvegnu and McConnell, 1993; Radhakrishnan and McConnell, 2000). Two sketches of typical phase diagrams for binary mixtures of saturated phospholipids (called P1 and P2) and Dchol are marked by arrows in Fig. 2. The critical points of the binary mixtures, marked A and B, lie at the highest pressure at which coexisting phases occur. The areas of black and white domains are equal at these points. As shown in Fig. 2, the two binary phase diagrams for P1-Dchol and P2-Dchol form axes of the ternary phase diagram. Mixtures of Dchol and two phospholipids were investigated to ascertain whether the critical pressure of the ternary system is simply related to the critical pressures of the binary P1-Dchol and P2-Dchol mixtures.

In this manuscript, all lipid mixtures are treated as if they have only one critical point, with no formation of condensed complexes as discussed in (Radhakrishnan and McConnell, 1999). This simplification does not alter the conclusions of this work. The effect of lipid chain length on formation of phospholipid–cholesterol complexes is discussed elsewhere (Keller et al., 2000).

## METHODS

Miscibility critical points were determined by epifluorescence microscopy of lipid mixtures in a monolayer at an air–water interface as described previously (Subramaniam and McConnell, 1987; Hirshfeld and Seul, 1990). All transitions were from two liquid phases to one liquid phase at room temperature (23 ± 0.5°C) and are reported in dyne/cm (mN/m). Phospholipids (from Avanti Polar Lipids, Alabaster, AL) and Dchol (Sigma, St. Louis, MO) were used without further purification. The lipids had phosphatidylcholine (PC) or phosphatidylethanolamine (PE) headgroups and two acyl chains, which are described as (chain length:unsaturation). A minimal amount of the dye Texas red dimyristoyl-phosphatidylethanolamine (0.4%, Molecular Probes, Eugene, OR) was used to provide contrast between black and white domains. Except where noted, Dchol comprises 33 mol % of the sample as shown in Fig. 2 and the amount of phospholipid 2 (fraction *f*) quoted refers to the proportion (0% to 100%) of the remaining 67 mol %. Experiments involving unsaturated

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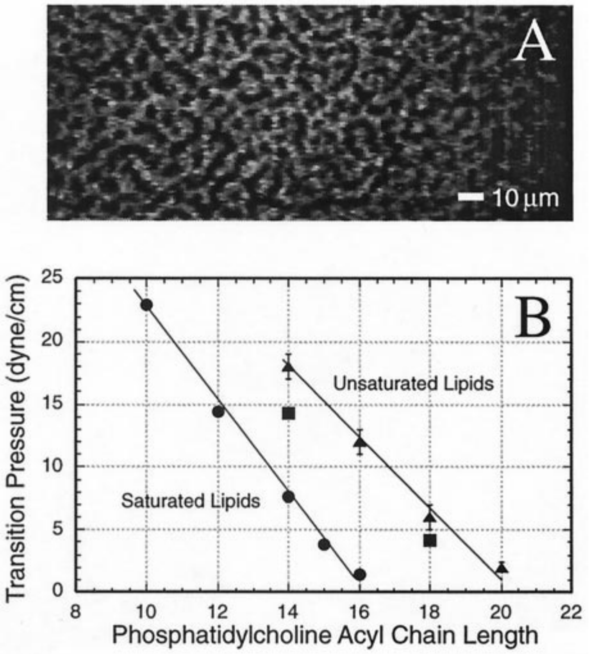

FIGURE 1 (*A*) "Stripe phase" observed in a mixture of 67 mol % di(14:0)PC and 33% dihydrocholesterol at 7.14 dyne/cm. (*B*) Critical pressure of binary mixtures of dihydrocholesterol with phosphatidylcholine lipids of different acyl chain lengths. The acyl chains are symmetric and are either saturated (*circles*) or singly *cis*-unsaturated at the ninth position (*squares* and *triangles*). The data were taken at Dchol concentrations of either 33% (*circles* and *squares*) or 50% (*triangles*; data from Hagen and McConnell, 1997). The decrease in critical pressure for each additional carbon in the acyl chains is 3.6 (*circles*), 2.5 (*squares*), and 2.7 (*triangles*) dyne/cm.

lipids were conducted under argon, with argon-degassed aqueous subphases.

## EXPERIMENTAL RESULTS

In the experiments described below, transition pressures of ternary mixtures of phospholipids (P1 and P2) with 33% Dchol were found, following the dashed line in Fig. 2. The concentration 33% Dchol was chosen to be near the critical composition of Dchol with saturated phospholipids. The binary phase diagrams of short-chain saturated lipids tend to be slightly asymmetric with a critical point near 33% Dchol (Benvegnu and McConnell, 1993; Keller et al., 2000). In contrast, reported phase diagrams of unsaturated lipids are symmetric, around 50% Dchol (Hagen and McConnell, 1997). Figure 3 shows transition pressures for a series of three different ternary mixtures where P1 and P2 are saturated. The solid lines show what the transition pressures would be if the phospholipids acted as one average lipid such that the transition pressure of the mixture was linearly proportional to the amount of P2. The difference in chain lengths of the phospholipids, ΔC, increases from ΔC = 0 (experiment 1), to ΔC = 2 (experiment 2) and ΔC = 4 (experiment 3). As ΔC increases, the deviation of the transition pressures from the average-lipid line increases. The deviation is largest near a 1/1 mixture of P1 and P2. In experiment 3, the highest transition pressure (at 60% di(14:0)PC) is nearly twice that of di(10:0)PC with Dchol and four times that of di(14:0)PC with Dchol!

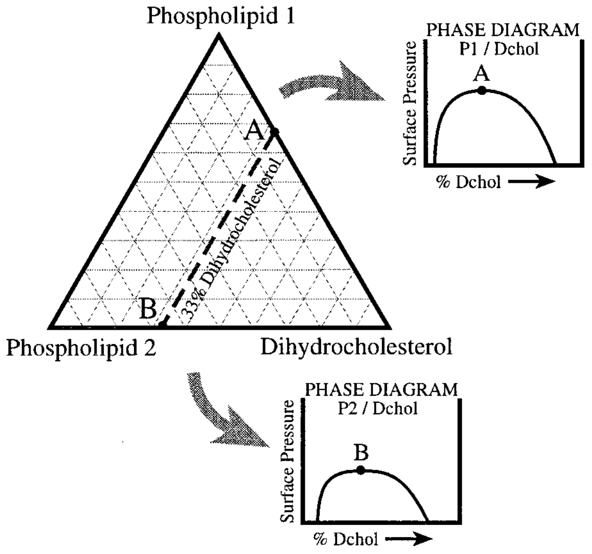

FIGURE 2 A ternary diagram for phospholipid 1 (P1) phospholipid 2 (P2) and dihydrocholesterol (Dchol). Interaction parameters, $a_{ij}$, are between components *i* and *j*. Phase diagrams of the binary mixtures along the P1–Dchol and P2–Dchol axes are shown at the arrows. Both diagrams show a 2-phase liquid region at low pressures and a 1-phase liquid region at high pressures with miscibility critical points (A and B) at 33% Dchol. Experiments to find transition pressures were done at compositions along the dashed line from point A to B at 33% Dchol.

Although stripes were seen at all the transitions in Fig. 3, the areas of black and white phases were not always equal. In other words, in some experiments, stripes of one phase were always seen against a continuous background of the other phase. This implies that the experimental compositions were always near, but not always at, a critical composition. Using the same lipids as in experiment 3 of Fig. 3, the miscibility phase transition pressures were found over more of the composition space, this time keeping the 1/1 ratio of the phospholipids di(10:0)PC/di(14:0)PC constant while the amount of Dchol was varied (point C to point D, Fig. 4). The phase diagram for this mixture does not differ significantly from the shape of common binary phase diagrams of phospholipid and Dchol (Hagen and McConnell, 1996; Keller et al., 2000). At transition points far from the critical point, stripes are not seen (*open symbols,* Fig. 4). The inset records whether there is a majority of black or white phase at the transition (*square* = white, *star* = equal white and black, *triangle* = black). Because critical compositions are identified by equal areas of black and white phases, a putative path of critical points can be drawn (*dashed line,* Fig. 4, *inset*).

As discussed above, ternary mixtures of Dchol with two saturated, symmetric-chain phospholipids of different chain

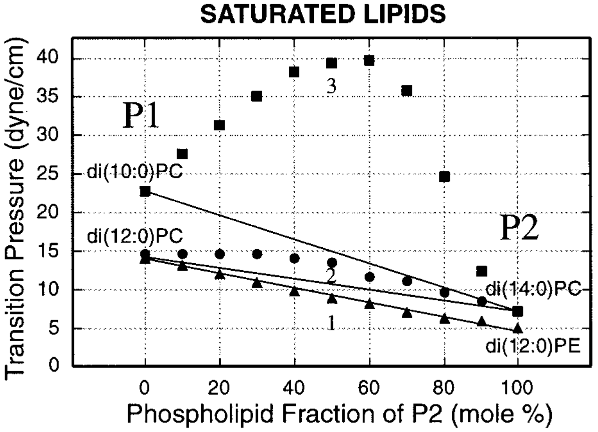

FIGURE 3 Transition pressures for ternary mixtures of 33% Dchol with phospholipids with saturated, symmetric acyl chains. Stripe phases were observed at all transitions. Experiment 1 used di(12:0)PC, di(12:0)PE, and Dchol. Experiment 2 used di(12:0)PC, di(14:0)PC, and Dchol, and a majority of black phase was seen at all concentrations. Experiment 3 used di(10:0)PC, di(14:0)PC, and Dchol. Solid lines show what the pressures would be if the phospholipids acted as one average lipid. Deviations from average-lipid lines increase with the difference in phospholipid chain length. Errors are ±0.5 dyne/cm and ±1 mol %.

lengths can produce surprisingly high transition pressures. Table 1 demonstrates that similar high transition pressures can be produced in a binary mixture of 33% Dchol and a single phospholipid molecule with asymmetric chains. The sn-1 chain is thought to protrude farther than the sn-2 chain (Ali et al., 1998). As the difference in the number of carbons in the lipid chains, ΔC, increases, the transition pressure increases. Transition pressures for mixtures with average phospholipid chain length of 15 are taken from Keller et al. (2000).

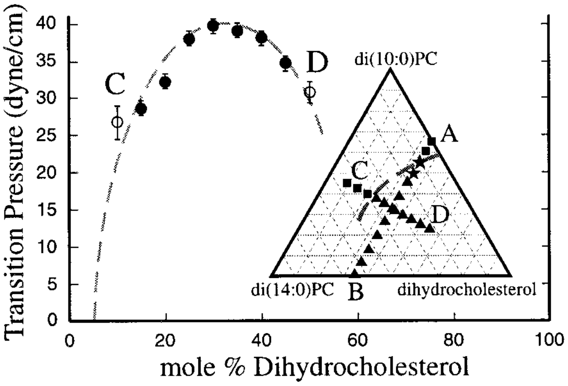

FIGURE 4 Miscibility phase transition pressures for 1/1 di(10:0)PC/di(14:0)PC with increasing Dchol (point C to point D). At points far from the critical point, stripes are not seen at the transition (*open symbols* versus *filled*). The inset shows whether there is a majority of black or white phase at the transition (*square,* white; *star,* equal white and black; *triangle,* black). Equal areas of black and white phases are found at the critical compositions. One of many possible lines of critical points is drawn in the inset (*dashed line*).

**TABLE 1 Transition pressure and acyl chain asymmetryu**

| Lipid + 33% Dchol | Average Chain Length | Number of Components | ΔC | Transition Pressure (dyne/cm) |
|---|---|---|---|---|
| di(14:0)PC | 14 | binary | 0 | 7.60 ± 0.5 |
| (18:0-10:0)PC | 14 | binary | 8 | 12.9 ± 0.5 |
| 1/1 di(18:0)PC/di(10:0)PC | 14 | ternary | 8 | 39.7 ± 0.5 |
| di(15:0)PC | 15 | binary | 0 | 3.60 ± 0.5 |
| (14:0-16:0)PC | 15 | binary | 2 | 4.30 ± 0.5 |
| (16:0-14:0)PC | 15 | binary | 2 | 5.60 ± 0.5 |
| (18:0-12:0)PC | 15 | binary | 6 | 9.15 ± 0.5 |
| 1/1 di(14:0)PC/di(16:0)PC | 15 | ternary | 2 | 5.00 ± 0.5 |

Compared with the data in Fig. 3, the effect of chain-length differences on transition pressure is not as pronounced when the phospholipids are unsaturated. Within error, the lipids di(14:1)PC and di(18:1)PC, with ΔC = 4, behave as an average lipid when mixed with 33% Dchol (Fig. 5). Deviations from average lipid behavior only appear with larger changes in chain length, as with ΔC = 10 for di(14:1)PC and di(24:1)PC. The critical pressure of di(24:1)PC with 33% Dchol is not directly known and is extrapolated at −11.5 dyne/cm from Fig. 1 to construct the average lipid line.

Sketches of possible 3-dimensional ternary phase diagrams for saturated versus unsaturated lipids with ΔC = 4 are shown in Fig. 6. On the left, the critical pressures for ternary mixtures of the saturated lipids di(10:0)PC and di(14:0)PC with Dchol are often higher than for the binary mixtures on the axes. In contrast, the unsaturated lipids di(14:1)PC and di(18:1)PC act as an average lipid in the presence of 33% Dchol. The phase diagrams were made by recording data along the P1-Dchol and P2-Dchol axes, along points A–B, and along C–D. The gray surfaces were

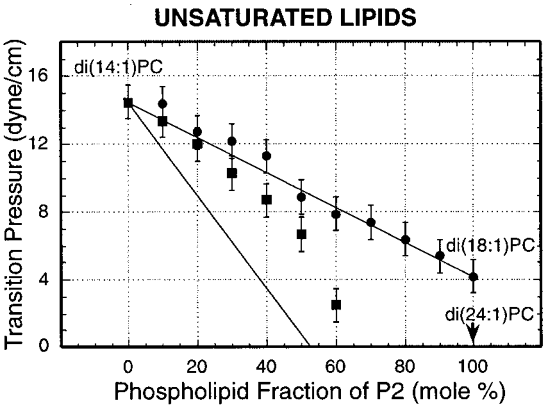

FIGURE 5 Transition pressures for ternary mixtures of singly-unsaturated, symmetric acyl chain phospholipids with 33% Dchol. Stripe phases were observed at all transitions. Solid lines show what the pressures would be if the phospholipids acted as one average lipid. The critical pressure of 67% di(24:1)PC and 33% Dchol is extrapolated from Fig. 1 at −11.5 dyne/cm to construct the average lipid line. Errors are ±1 mol %.

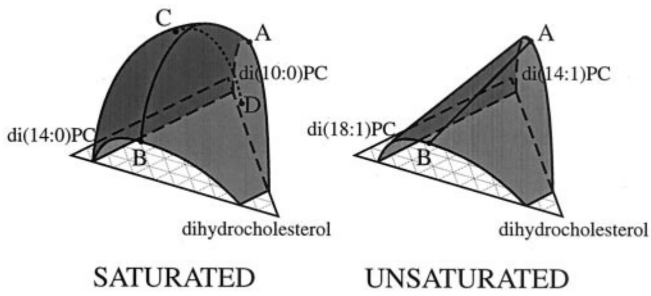

FIGURE 6 Sketches of possible 3-dimensional ternary phase diagrams for saturated versus unsaturated lipids with $\Delta C = 4$. On the left, the transition pressures for intermediate mixtures of the saturated lipids di(10:0)PC and di(14:0)PC with Dchol are higher than for the binary mixtures on the axes. In contrast, the unsaturated lipids di(14:1)PC and di(18:1) PC act as an average lipid in the presence of 33% Dchol. The phase diagrams were made by plotting data along the P1–Dchol and P2–Dchol axes (data from Hagen and McConnell, 1996; Hagen and McConnell, 1997; Keller et al., in press), along points A–B (data from Fig. 3), and along C–D (data from Fig. 4). The gray surfaces in the phase diagrams were sketched to connect these known data.

sketched to connect these known data. Although all of the lipid mixtures with 33% Dchol exhibited stripe phases, the line from A to B does not necessarily denote a line of critical points.

Large deviations from average lipid behavior are also observed when lipids of different unsaturation are mixed as shown in Fig. 7 for the ternary mixture of di(14:1)PC and di(14:0)PC with Dchol. The most relevant parameter that describes deviation from average lipid behavior may not be chain length per se, but perhaps an effective chain length that accounts for varying degrees of unsaturation. That is, some physical properties of monolayers and bilayers may be similar for phospholipids with short saturated phospholipid chains as compared to longer unsaturated phospholipid chains.

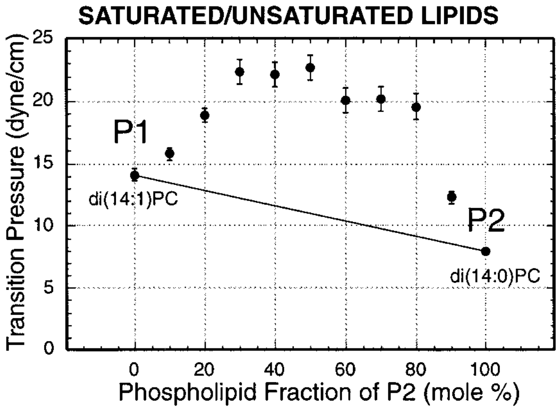

FIGURE 7 Transition pressures for mixtures of di(14:1)PC and di(14:0)PC with 33% Dchol. The phospholipids have symmetric acyl chains that differ in a single *cis* unsaturation at position 9. Stripe phases were observed at all transitions. The solid line shows what the pressures would be if the phospholipids acted as one average lipid. White phase predominated from 0% to 30% di(14:0)PC, equal areas of black and white phase were observed at 40%, and black phase predominated from 45% to 100%. Errors are ±1 mol%.

## CALCULATIONS

The problem of determining the phase behavior of a three-component regular solution was analyzed by Meijering (1950). Although Meijering's discussion concerned critical temperatures rather than pressures, as in the present study, much of his analysis still applies. Below, we describe our thermodynamic model and then summarize Meijering's method for calculating critical compositions in a ternary regular solution, using our free energy formulation. Subsequently, Meijering's examination of the relationship between the thermodynamic parameters and the calculated phase behavior is discussed in terms of the experimental results reported here.

The ternary P1/P2/Dchol mixture is modeled as a regular solution, with a molar free energy of

$$\begin{aligned} G/RT^\circ = {} & (X_1 \ln X_1 + X_2 \ln X_2 + X_C \ln X_C) \\ & + (a_{1C} X_1 X_C + a_{2C} X_2 X_C + a_{12} X_1 X_2)\,, \end{aligned} \tag{1}$$

where $X_1$, $X_2$, and $X_C$ are the mole fractions of P1, P2, and Dchol such that

$$X_1 + X_2 + X_C = 1\,, \tag{2}$$

and the interaction parameters $a_{ij}$ are normalized with respect to $RT^\circ$. $T^\circ$ is a reference temperature that is set equal to room temperature in the experiments described here. Strictly speaking, the term *regular solution* applies when the parameters $a_{ij}$ express a nonideal enthalpy of mixing, but not entropy (Hildebrand, 1929). We use the term more generally, to include both nonideal enthalpy and entropy effects; such a system is sometimes called a *quadratic solution* (Rowlinson and Swinton, 1982). In binary systems, a critical point is observed when $a_{ij} = 2$ (Hildebrand, 1929). When $a_{ij} < 2$, the two components are fully miscible and do not separate into coexisting phases. The formulation above is identical to the one used by Meijering, except that here the temperature is fixed at room temperature and the interaction energies are taken to be linear functions of the surface pressure $\pi$,

$$a_{ij} = 2 - a'_{ij}(\pi - \pi_{ij})\,, \tag{3}$$

where $\pi_{ij}$ is the (binary) critical pressure of a mixture of components $i$ and $j$ and $a'_{ij}$ is an area-contraction parameter. Nonideal mixing of components $i$ and $j$ results in a change of molecular area of $a'_{ij}X_iX_j$ where $X_i$ and $X_j$ are the mole factions of components $i$ and $j$. Measured area contraction values of phospholipid–cholesterol pairs are $-10$ to $-40$ Å$^2$ (Phillips, 1972); in terms of the units used here, this translates to $a'_{ij}$ values of $-0.025$ to $-0.1$ cm/dyne. Within error, there is no change in molecular area for a 1/1 mixture of saturated phosphatidylcholines (di(10:0)PC and di(14:0)PC, data not shown). Values of $a'_{ij}$ less than zero mean that $T_{crit}$ increases with increasing pressure (Keller et al., 1998).

At a critical point, the compositions of the coexisting phases in a mixture become identical; the mixture is on the verge of instability with respect to phase separation. Expressed mathematically, a critical point occurs when the second and third derivatives of the free energy with respect to composition are simultaneously zero. In a binary regular solution of phospholipid and cholesterol, this condition is met when the composition is $X_c = 0.5$ and the interaction parameter $a_{P\text{-}C} = 2$ (i.e., $\pi = \pi_{crit}$). In a ternary mixture, the analysis is more complicated, but still essentially involves finding the composition and pressure at which the second and third composition derivatives of Eq. 1 are zero. We are interested in determining the critical pressure for a particular phospholipid composition. This is the inverse of the problem of finding the *composition* of the critical point at a particular pressure at which the interaction parameters $a_{ij}$ are known. Meijering solved this latter problem in the context of critical temperatures rather than critical pressures, but much of the analysis applies here and is summarized below.

As shown in Fig. 2, the composition of a ternary mixture is defined by its position in a two-dimensional composition triangle, and may be conveniently expressed in terms of the independent mole fractions $X_1$ and $X_2$ of the phospholipids. Compositions that are unstable with respect to phase separation are bounded by a *spinodal curve,* along which the second derivative of $G$ with respect to composition is positive in all directions save one, along which it is zero. That is, the spinodal curve consists of the set of inflection points in the free energy surface. The spinodal curve for a regular ternary solution may be expressed parametrically in the form (Meijering, 1950)

$$LX_1X_2X_C + 2a_{1C}X_1X_C + 2a_{2C}X_2X_C + 2a_{12}X_1X_2 = 1 \tag{4}$$

where

$$L = a_{1C}^2 + a_{2C}^2 + a_{12}^2 - 2a_{1C}a_{2C} - 2a_{1C}a_{12} - 2a_{2C}a_{12}\,. \tag{5}$$

At a point on the spinodal curve, the direction $v$, along which the second derivative of $G$ is zero, is described by its slope in the composition plane, $n = \mathrm{d}X_2/\mathrm{d}X_1$, which may be expressed as

$$n = -\frac{\partial^2 G/\partial X_1\partial X_2}{\partial^2 G/\partial X_2^{\,2}}\,. \tag{6}$$

Outside the spinodal curve is a *binodal curve,* which connects the tie lines of coexisting phases. When the compositions of the coexisting phases converge at a critical point, the binodal curve coincides with the spinodal curve. The spinodal curve can thus be used as a guide in locating the compositions of critical points.

At a critical point, the third derivative of the free energy is zero, along with the second derivative, in the direction $v$. This condition may be expressed by Eq. 7, the derivation of which is made easier by setting $X_1 = v\cos\varphi$ and $X_2 = v\sin\varphi$ and then using the chain rule,

$$\frac{\partial^3 G}{\partial v^3} = \frac{\partial^3 G}{\partial X_1^{\,3}} + \frac{3n\partial^3 G}{\partial X_1^{\,2}\partial X_2} + \frac{3n^2\partial^3 G}{\partial X_1\partial X_2^{\,2}} + \frac{n^3\partial^3 G}{\partial X_2^{\,3}} = 0\,. \tag{7}$$

As indicated above, a critical point must satisfy both Eqs. 4 and 7. To find a critical point for a particular pressure $\pi$ given that the interaction parameters are $a_{1C}(\pi)$, $a_{2C}(\pi)$, and $a_{12}(\pi)$, Eqs. 4 and 7 may be solved simultaneously for the composition variables $(X_1, X_2)$ at the critical point. Equivalently, Eqs. 4 and 7 may be reparameterized in terms of the phospholipid fraction $f$ of P2, the mole fraction of cholesterol, $X_C$, and the surface pressure $\pi$. Given the phospholipid composition $f$ of a mixture, these reparameterized equations may be solved to obtain the critical pressure $\pi_{crit}$ and the critical cholesterol composition $X_C$(crit). This method will be discussed in depth in a forthcoming publication (Anderson and McConnell, 2000).

Plots of the critical values of $\pi$ and $X_C$ as functions of the phospholipid P2 fraction $f$ are shown in Fig. 8. Fig. 8 *A* shows calculations for a regular P1/P2/Dchol solution in which the two binary mixtures P1–Dchol and P2–Dchol have identical critical pressures; in Fig. 8 *B,* the two binary mixtures have very different critical pressures. The thick solid lines represent mixtures in which the two phospholipids interact ideally, that is, $a_{12} = 0$. In Fig. 8 *A,* the thick solid line falls on top of a straight line connecting the two binary critical points, corresponding to average phospholipid behavior. In Fig. 8 *B,* the thick solid line deviates from the average phospholipid behavior, which is indicated by the thin solid line. A detailed discussion of average phospholipid phase behavior is presented in (Anderson and Connell, 2000).

The dotted and dashed lines correspond to systems wherein the two phospholipids exhibit attractive ($a_{12} < 0$) and repulsive ($a_{12} > 0$) interactions, respectively. In all cases, the phospholipids are taken to be fully miscible with one another, so $a_{12}$ is never greater than 2. The phospholipid interaction parameter $a_{12}$ was taken to be independent of pressure, with the exception of the system shown by the dashed-dotted line in Fig. 8 *B,* in which the interaction parameter $a_{12}$ has a strong pressure dependence. Note that, in all cases in which the P1–P2 interaction is nonideal such that $a_{12} \neq 0$, the critical pressures deviate from the average phospholipid line.

The plots on the right-hand side of Fig. 8 show the critical compositions. In general, the critical cholesterol compositions in Fig. 8 *A* are all close to 50 mol% Dchol, which is taken to be the composition at the binary critical points. In Fig. 8 *B,* significant deviations from this critical composition are seen.

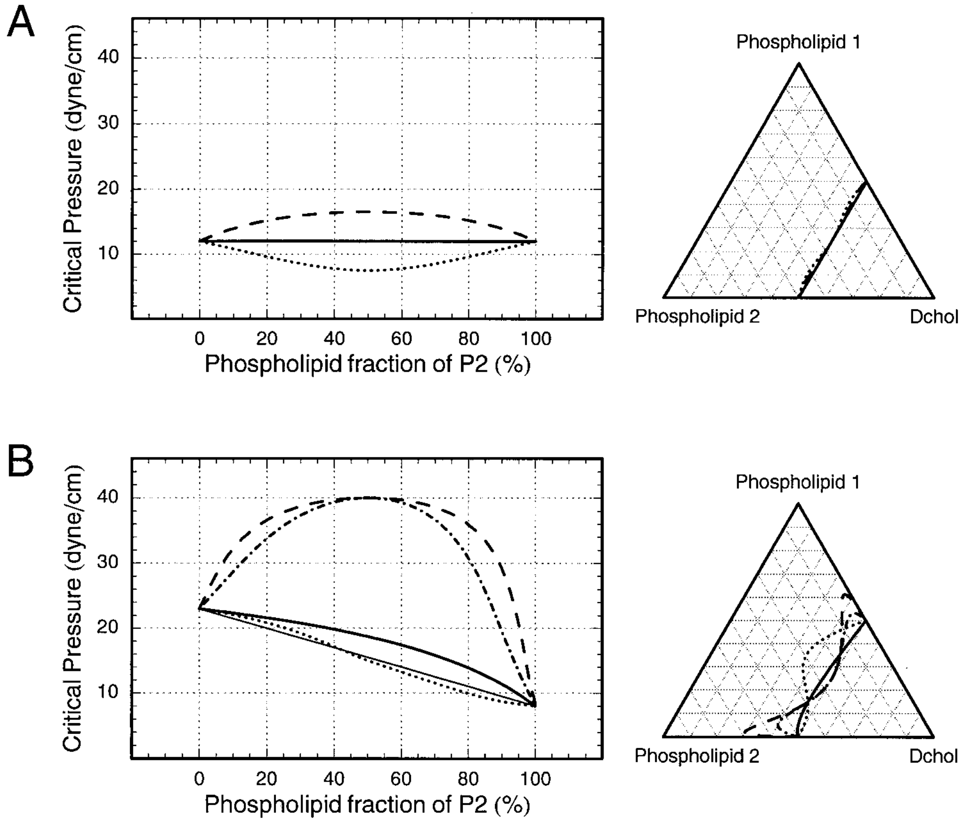

FIGURE 8 Calculated transition pressures (*left*) and ternary composition diagrams (*right*) for mixtures of Dchol and two phospholipids in which the binary P1–Dchol and P2–Dchol mixtures have (*A*) equal and (*B*) unequal critical pressures. Calculations were done for cases in which the two phospholipids interact ideally (*heavy solid line*), repulsively (*dotted line*), and attractively (*dashed* and *dot-dashed lines*). The thin line indicates the average phospholipid critical pressure. Interactions between phospholipids were taken to be pressure independent except in the case shown by the dot-dashed line in (*B*), in which a strong pressure dependence in the phospholipid interaction energy was introduced. Parameters used: (*A*) $a'_{1C} = a'_{2C} = 1/12$; $\pi_{1C} = \pi_{2C} = 12$; $a_{12} = 0$ (*solid line*), 1.5 (*dotted line*), $-1.5$ (*dashed line*); (*B*) $a'_{1C} = 1/8.5$; $a'_{2C} = 1/16$; $\pi_{1C} = 23$; $\pi_{2C} = 8$; $a_{12} = 0$ (*solid line*), 1.5 (*dotted line*), $-8$ (*dashed line*), $2 - 1/5\,(\pi + 10)$ (*dot-dashed line*).

## APPLICATIONS

In addition to quantitative calculations of the critical points of ternary regular solutions, the equations presented in the previous section may be used to provide a qualitative understanding of the relationship between the interaction parameters $a_{ij}$ and the critical behavior of P1/P2/Dchol mixtures. Using the results of the experiments and calculations discussed above along with the analysis presented by Meijering, we can uncover the magnitude and sign of phospholipid–phospholipid interactions in monolayers, which would be difficult to probe in another way. Our conclusion is that, in mixtures of two phospholipids with cholesterol, a ternary critical point that is elevated in pressure with respect to the binary critical points indicates that the interaction parameter $a_{12}$ is less than zero.

### Addition of a third component to a binary mixture

We begin by considering the phase behavior at the edges of the composition triangle, where the mixture consists mostly of P1 and Dchol. The question of how the addition of a third component (P2) affects the critical parameters of a regular binary mixture (P1 and Dchol) was addressed by Meijering, again in the context of critical temperatures. We review the relevant part of Meijering's discussion below in terms of critical pressures, using our notation.

As discussed in the previous section, we take the critical composition of a binary P1–Dchol mixture to be 50% Dchol (rather than 33%); this means that near the P1–Dchol critical point, $X_1 \approx X_C \approx 1/2$. Consider the addition of a very small amount of P2 to the P1–Dchol mixture (such that $X_2 \ll 1$), at the P1–Dchol critical pressure (such that $a_{1C} = 2$). Setting $a_{1C} = 2$, the spinodal curve in Eq. 4 may be rearranged to give

$$(X_C - X_1)^2 = X_2(2a_{2C}X_C + 2a_{12}X_1 + (4 + (a_{2C} - a_{12})^2 - 4a_{2C} - 4a_{12})X_1X_C) - 1 + (X_C + X_1)^2 . \tag{8}$$

Because $X_1 + X_2 + X_C = 1$, Eq. 8 may be simplified by replacing the last term by $(1 - X_2)^2$, or $1 - 2X_2 + X_2{}^2$,

$$(X_C - X_1)^2 = X_2(2a_{2C}X_C + 2a_{12}X_1 + (4 + (a_{2C} - a_{12})^2 - 4a_{2C} - 4a_{12})X_1X_C) - 2X_2 - X_2{}^2 . \tag{9}$$

Because $X_2 \ll 1$, the last term is negligible and may be dropped. Finally, because $X_1 \approx X_C \approx 1/2$, we can approximate Eq. 9 as

$$4(X_C - X_1)^2 = X_2((a_{2C} - a_{12})^2 - 4) . \tag{10}$$

This equation describes a parabola, which is illustrated schematically in Fig. 9. The orientation of the parabola described by Eq. 10 depends on the magnitude of ($a_{2C} - a_{12}$). When the absolute difference between $a_{2C}$ and $a_{12}$ is $>2$, the right side of Eq. 10 is positive for $X_2 > 0$, which means that the parabola curves inside the composition triangle, touching the binary P1–Dchol critical point at $X_1 = X_C = ½$ (Fig. 9 *A*).

Now consider a mixture at the P1–Dchol critical composition with a small perturbation of the pressure from the P1–Dchol critical pressure. When the pressure is lowered, phase separation begins to occur between P1 and Dchol; when the pressure is raised, P1 and Dchol become more miscible. For small changes in the pressure, the shape of the spinodal curve remains essentially the same. Recall that composition points inside the spinodal curve are unstable with respect to phase separation. As the pressure is raised, these compositions become more stable, and the spinodal curve moves away from the P1–Dchol edge, into the composition triangle (Fig. 9 *A*). The critical point is now found at a point for which $X_2 > 0$. Further raising the pressure shifts the critical point to compositions with larger $X_2$. Turned around, this is equivalent to saying that, when the absolute difference of $a_{2C}$ and $a_{12}$ is $>2$, adding P2 to a binary mixture of P1 and Dchol raises the critical pressure of the mixture.

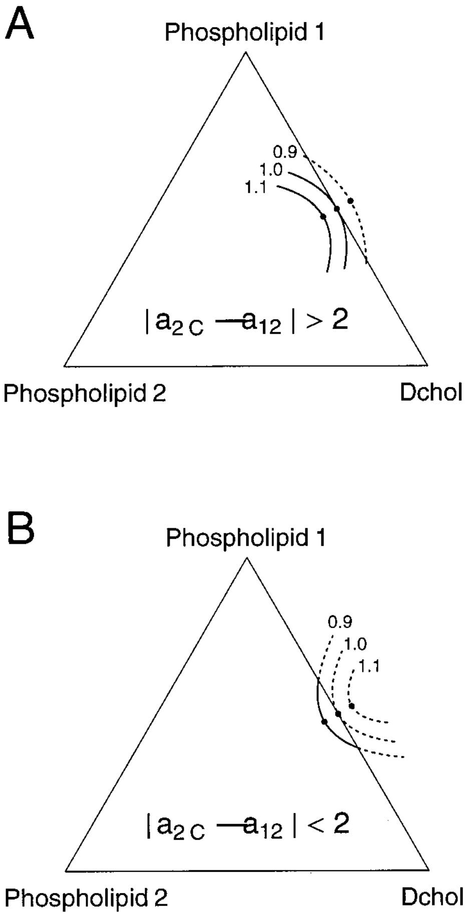

FIGURE 9 Schematic representations of the spinodal curves in a P1–P2–Dchol ternary mixture near the binary P1–Dchol miscibility critical point, when the absolute difference between the interaction parameters $a_{2C}$ and $a_{12}$ is (*A*) greater than 2 and (*B*) less than 2. The numbers indicate the pressure relative to the binary P1–Dchol critical pressure.

If the absolute difference between $a_{2C}$ and $a_{12}$ is $<2$, the situation is different. In this case, Eq. 10 describes a parabola that lies outside the composition triangle, touching the P1–Dchol edge at the P1–Dchol critical point when $\pi = \pi_{1C}$ (Fig. 9 *B*). As above, when the critical pressure is lowered, phase separation occurs in a binary P1–Dchol mixture. The spinodal curve emerges from the P1–Dchol side of the composition triangle, surrounding the now-unstable region. The critical point is now found at a point where $X_2 > 0$. Further lowering the pressure shifts the critical point to compositions with larger $X_2$. Equivalently, when the absolute difference of $a_{2C}$ and $a_{12}$ is $<2$, adding P2 to a binary mixture of P1 and Dchol lowers the critical pressure of the mixture.

These statements may be summarized as follows: upon adding a small amount of P2 to a mixture of P1 and Dchol, the critical pressure is raised if the absolute difference between the interaction parameters $a_{2C}$ and $a_{12}$ is $>2$ and lowered if the absolute difference is $<2$.

## Implications of a critical point maximum

We can learn more about the magnitude of the various lipid interactions by considering mixtures in which the critical pressure is elevated (or lowered) with respect to both of the binary critical points, as is the case for the observed results shown in Figs. 3 and 7 and the calculated results in Fig. 8. For simplicity, consider a binary mixture in which the binary miscibility critical pressures of P1–Dchol and P2–Dchol are similar. In this case, the phospholipid–cholesterol interaction parameters $a_{1C}$ and $a_{2C}$ will both be close to 2 (the binary critical-point value) over a range of pressures near their critical points, the phospholipid with the higher binary critical pressure with Dchol having a somewhat higher value of $a_{1C}$. If we stipulate that the two phospholipids are fully miscible, so that $a_{12} < 2$, the above rule implies that, when a small amount of the phospholipid with the lower P–C critical point is added to a binary mixture of the other phospholipid and Dchol, the critical pressure of the resulting ternary mixture is raised if $a_{12}$ is negative (attractive P1–P2 interactions) and lowered if $a_{12}$ is positive (repulsive P1–P2 interactions). When $a_{12}$ is zero (ideal P1–P2 interactions) the critical pressure moves in a fairly straight line from one binary critical point to the other. These trends are illustrated in Fig. 8 *A*.

This behavior can be understood qualitatively by noting that, when both phospholipids are present, an attractive interaction between the phospholipids will enhance the tendency of the phospholipids to separate from cholesterol—and hence raise the critical pressure—because the energy of the resulting phospholipid-rich phase is lowered to a larger extent than in the fully mixed solution: in the fully mixed solution, the attractive interactions between the phospholipids are diluted by cholesterol. In contrast, repulsive phospholipid interactions favor mixing because the resulting dilution of the phospholipids by cholesterol reduces the energetic cost of their repulsive interactions.

In principle, these qualitative rules may be violated in mixtures in which the two binary critical points are very different. Particularly if the phospholipid–Dchol interactions are highly pressure dependent, the values of $a_{1C}$ and $a_{2C}$ may vary considerably over the range of critical pressures. For example, if the value of $a_{2C}$ at the P1–C binary critical point is significantly less than zero, a positive $a_{12}$ may differ from $a_{2C}$ by more than 2 and lead to an increase in the critical pressure upon adding P2 to a P1–C binary mixture. However, the critical cholesterol composition of such ternary mixtures differs significantly from the binary mixture value of $X_{\mathrm{chol}} = 0.5$ (calculations not shown), so this scenario does not apply to the results reported in this study.

## Interaction parameters at a ternary critical pressure maximum (or minimum)

Further understanding of the phenomenon of elevated (or lowered) critical pressures inside the composition triangle of a regular solution may be gained by analyzing the free energy expression of the mixture. Such an analysis was described by Meijering, again in the context of critical temperatures. The key points of his discussion are outlined below, in terms of critical pressures and using our notation.

The extremum in the critical pressure arises from the merging of two first-order critical points, and, as such, is a second-order, or ternary, critical point. (This should not be confused with the term tricritical point, which is used to describe a different phenomenon.) As shown by Meijering (1950), in a regular solution, a ternary critical point must lie at a composition at which the mole fraction of one of the components is ½. In the results shown in Fig. 8, this component is cholesterol. At the ternary critical point the two phospholipids are in a 1/1 ratio, so each has a mole fraction of 1/4 at this point.

Meijering (1950) further showed that, for such a ternary critical point, the interaction terms of the ternary regular solution are related by

$$-L = 8a_{12}, \tag{11}$$

$$X_1 = (a_{12} - a_{1C} + a_{2C})/4a_{12}, \tag{12}$$

$$X_2 = (a_{12} + a_{1C} - a_{2C})/4a_{12}, \tag{13}$$

where $L$ is defined by Eq. 5. In the context of the present study, where the observed ternary critical point is close to a 1/1 mixture of the two phospholipids, it is convenient to express the mole fractions of the phospholipids as $X_1 = \frac{1}{4} + \delta$ and $X_2 = \frac{1}{4} - \delta$. Inserting these expressions into Eqs. 12 and 13 and combining them with Eqs. 5 and 11 gives

$$a_{12} = \frac{4(a_{1C} - 2)}{(1 - 4\delta)^2} = \frac{4(a_{2C} - 2)}{(1 + 4\delta)^2}. \tag{14}$$

If the ternary critical point is elevated in pressure with respect to the two binary critical points, the values of the binary interaction terms $a_{1C}$ and $a_{2C}$ are both $<2$ at this pressure (recall that the interaction terms decrease with increasing pressure, and are equal to 2 at their respective binary critical pressures.) Hence, by Eq. 14, the P1–P2 interaction term $a_{12}$ must be negative at an elevated ternary critical point. Conversely, a ternary critical point that is depressed with respect to the two binary critical points would indicate a positive value of $a_{12}$.

Based on these considerations, the elevated critical pressure of the di(10:0)PC–di(14:0)PC-Dchol mixture shown in Fig. 3 implies that, at the ternary critical point, there is a significant attractive interaction between the two phospholipids. The attraction need not be as strong at low pressures. This is illustrated at the left in Fig. 8 *B* in which the P1–P2 interactions are constant (*dashed lines*) or pressure dependent (*dot-dashed line*). The calculated critical pressure trajectory of the pressure-dependent system has a narrower peak, which more closely resembles the results shown in Fig. 3.

In terms of critical cholesterol compositions, the calculated critical trajectories shown at right in Fig. 8 *A* show that, when the binary critical pressures of P1–Dchol and P2–Dchol are similar, the critical cholesterol composition of ternary mixtures generally lies very close to the binary-mixture value of $X_C = \frac{1}{2}$, regardless of whether the interaction between the two phospholipids is attractive or repulsive. However, when the binary critical pressures differ significantly, as shown in Fig. 8 *B,* the critical composition of a ternary mixture may show large deviations from $X_C = \frac{1}{2}$ if the phospholipids have a nonzero interaction parameter. For repulsive P1–P2 interactions, the composition deviation is largest at the center of the composition space, near 50 mol % P2. For attractive P1–P2 interactions, however, the deviations are confined to near the edges of the composition space. As discussed above, the cholesterol composition at the critical pressure maximum, which is at a phospholipid fraction of 50% P2, must be $X_C = \frac{1}{2}$. Hence, the fact that the observed shape of the 1:1 di(10:0)PC–di(14:0)PC/Dchol phase diagram shown in Fig. 4 is similar to the binary phospholipid–Dchol phase diagrams is fully consistent with this model.

## DISCUSSION

Previous work has reported that the miscibility transition pressure of monolayers containing phospholipid and 20 mol% Dchol is linearly, systematically altered by the addition of a second phospholipid (Hagen and McConnell, 1996). This conclusion is true for the cases previously studied. However, when observed over a wider range of lipid compositions, the transition pressures can be strikingly nonlinear functions of the amount of second phospholipid.

For experiments reported here, the critical Dchol concentration of the ternary mixture never strays far from 33% because stripes are observed for all ternary mixtures with 33 mol % Dchol. In this sense, previous conclusions are correct that the ternary mixtures behave as a quasi-binary mixture with the critical Dchol concentration roughly constant (Hagen and McConnell, 1996). The surprisingly high transition pressures observed here are not an artifact of straying too far from the critical composition.

As discussed in the previous section, the elevated critical pressure of the di(10:0)PC–di(14:0)PC–Dchol mixture shown in Fig. 3 implies that, at the ternary critical point, there is a significant attractive interaction between the two phospholipids. The largest increase in critical pressure is seen when P1 and P2 are saturated and have a large difference in chain lengths. We speculate that the attraction arises because the terminal carbons of the long-chain phospholipids gain entropy by moving in the voids of neighboring short-chain lipids. The order of magnitude of this entropy $S$ may be estimated by considering that a protruding carbon unit of each chain of P2 may assume three conformations (*trans* plus two *gauche*), for which the molar conformational entropy for $n$ protruding carbon units is approximately

$$S \approx 2R \ln 3^{n}. \tag{15}$$

For the di(10:0)PC–di(14:0)PC mixture, $n = 4$. In terms of the free energy of a mixture for which $X_{P1} \approx X_{P2} \approx 1/4$, the equivalent quadratic energy is of the order $\sim 2.2RT$. The fitted value of $a_{12}$ ($-8$, cf. Fig. 8 caption) translates into free energy $RTa_{12}X_{P1}X_{P2} = -0.5RT$ that is of the same order of magnitude. In the case of P1 = di(14:0)PC and P2 = di(14:1)PC, the large increase in the critical pressure for the ternary mixture (Fig. 7) implies an attraction between the two phospholipids, although in this case this effect is not due to a large discrepancy in chain lengths.

For cases in which the binary critical pressures of P1–Dchol and P2–Dchol are nearly equal, the attraction (or repulsion) between phospholipids can be small and still result in an increased (or decreased) critical pressure, as shown in Fig. 8 *A*. Hence, it is possible to probe weak interactions between phospholipids in monolayers by investigating the ternary phase diagrams of the phospholipids with Dchol. These weak interactions are difficult to probe by other means. Good candidates for ternary mixtures with similar binary P1–Dchol and P2–Dchol critical pressures are (di(12:0)PC, di(14:1)PC, and Dchol) and (di($n$:0)PE, di($n$ + 2:0)PC, and Dchol, where $n \leq 12$).

The lipids in biological membranes have a wide array of symmetric and asymmetric acyl chain lengths (e.g., see Marsh, 1990). The mismatch in acyl chain length, which creates an attraction between symmetric lipids in a monolayer may have different effects in a bilayer due to constraints on hydrophobic mismatch. There is indirect evidence that some symmetric lipids with different chain lengths mix inhomogeneously in bilayers, even in fluid phases (Maer et al., 1998). Cholesterol may affect this demixing (Silvius et al., 1996 and references therein).

Within bilayers, asymmetric lipids are best known for interdigitation in the gel phase. However, when the lipids are above their melting temperature, interdigitation is highly dynamic. For example, asymmetric lipids diffuse at the same rate as symmetric lipids (Schram and Thompson, 1995). Chain length asymmetry in lipids mimics many of the attributes of unsaturation (Ali et al., 1998). The current work shows that lipid asymmetry and unsaturation both result in high critical pressures in monolayers. Given that lipids in bilayers are thought to be at an area per molecule corresponding to a high surface pressure in a monolayer (Demel et al., 1975; Nagle, 1986; Marsh, 1996), it will be very interesting to discover which lipid mixtures result in high miscibility critical points. The present work demonstrates that critical pressures for phospholipids and dihydrocholesterol mixtures can be used to gain insight into the magnitude of the interaction between the phospholipids alone.

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## REFERENCES

Ali, S., J. M. Smaby, M. M. Momsen, H. L. Brockman, and R. E. Brown. 1998. Acyl chain-length asymmetry alters the interfacial elastic interactions of phosphatidylcholines. *Biophys. J.* 74:338–348.

Anderson, T. G., and H. M. McConnell. 2000. Phase behavior of multicomponent phospholipid mixtures with cholesterol. *J. Phys. Chem. B.* In press.

Benvegnu, D. J., and H. M. McConnell. 1993. Surface dipole densities in lipid monolayers. *J. Phys. Chem.* 97:6686–6691.

Demel, R. A., W. S. M. Geurts Van Kessel, R. F. A. Zwaal, B. Roelofsen, and L. L. M. Van Deenen. 1975. Relation between various phospholipase actions on human red cell membranes and the interfacial phospholipid pressure in monolayers. *Biochim. Biophys. Acta.* 406:97–107.

Hagen, J. P., and H. M. McConnell. 1996. Critical pressures in multicomponent lipid monolayers. *Biochim. Biophys. Acta.* 1280:169–172.

Hagen, J. P., and H. M. McConnell. 1997. Liquid–liquid immiscibility in lipid monolayers. *Biochim. Biophys. Acta.* 1329:7–11.

Hildebrand, J. H. 1929. Solubility. XII. Regular solutions. *J. Am. Chem. Soc.* 51:66–80.

Hirshfeld, C. L., and M. Seul. 1990. Critical mixing in monomolecular films: pressure-composition phase diagram of a two-dimensional binary mixture. *J. Phys. France.* 51:1537–1552.

Keller, S. L., W. H. Pitcher, III, W. H. Huestis, and H. M. McConnell. 1998. Red blood cell lipids form immiscible phases. *Phys. Rev. Lett.* 81:5019–5022.

Keller, S. L., A. Radhakrishnan, and H. M. McConnell. 2000. Saturated phospholipids with high melting temperature form complexes with cholesterol in monolayers. *J. Phys. Chem. B.* 104:7522–7527.

Maer, A. M., L. L. Providence, C. Nielsen, and O. S. Andersen. 1998. Gramicidin A channel's behavior in bilayers made of lipid mixtures. *Biophys. J.* 74:A203.

Marsh, D. (Ed.). 1990. *CRC Handbook of Lipid Bilayers.* CRC Press, Boca Raton, FL.

Marsh, D. 1996. Lateral pressure in membranes. *Biochim. Biophys. Acta.* 1286:183–223.

Meijering, J. L. 1950. Segregation in regular ternary solutions. *Philips Res. Rep.* 5:333–356.

Myher, J. J., A. Kuksis, and S. Pind. 1989. Molecular species of glycerophospholipids and sphingomyelins of human erythrocytes: improved method of analysis. *Lipids.* 24:396–407.

Nagle, J. F. 1986. Theory of lipid monolayer and bilayer chain-melting phase transitions. *Faraday Discuss. Chem. Soc.* 81:151–162.

Phillips, M. C. 1972. The physical state of phospholipids and cholesterol in monolayers, bilayers and membrane. *In* Progress in Surface and Membrane Science. Academic Press, New York. 139–221.

Radhakrishnan, A., and H. M. McConnell. 1999. Cholesterol-phospholipid complexes in membranes. *J. Amer. Chem. Soc.* 121:486–487.

Radhakrishnan, A., and H. M. McConnell. 2000. Chemical activity of cholesterol in membranes. *Biochemistry.* 39:8119–8124.

Rowlinson, J. S., and F. L. Swinton. 1982. Liquids and Liquid Mixtures. 3rd Ed. Butterworth Scientific, London. 135–137.

Schram, V., and T. E. Thompson. 1995. Interdigitation does not affect translational diffusion of lipids in liquid crystalline bilayers. *Biophys. J.* 69:2517–2520.

Seul, M., and V. S. Chen. 1993. Isotropic and aligned stripe phases in a monomolecular organic film. *Phys. Rev. Lett.* 70:1658–1661.

Silvius, J. R., D. del Giudice, and M. Lafleur. 1996. Cholesterol at different bilayer concentrations can promote or antagonize lateral segregation of differing acyl chain length. *Biochemistry.* 35:15198–15208.

Subramaniam, S., and H. M. McConnell. 1987. Critical mixing in monolayer mixtures of phospholipid and cholesterol. *J. Phys. Chem.* 91: 1715–1718.