Phase Behavior of Multicomponent Phospholipid Mixtures with Cholesterol

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Previous work has shown that mixtures of multiple phospholipids with cholesterol exhibit phase behavior which may be approximately modeled by a hypothetical binary mixture of cholesterol and an "average"-phospholipid.[1] This study presents a detailed thermodynamic model of such multicomponent mixtures based on regular-solution theory. When the individual phospholipids are treated independently, calculated phase behavior shows systematic deviations from the average-phospholipid model. These deviations may be expressed concisely in terms of the distribution of binary critical parameters of the individual phospholipids, and the deviations are small for mixtures in which the individual phospholipids exhibit similar phase behavior in binary mixtures with cholesterol.

Introduction

The membranes of mammalian cells are based on lipid bilayers containing cholesterol and numerous phospholipid species;[2,3] extensive experimental and theoretical studies of lipid bilayers containing cholesterol up until 1993 are summarized in a book edited by Feingold.[4] One approach to the problem of lipid membrane properties has been to study well-defined mixtures of lipids in monolayers. This approach has several advantages from both experimental and theoretical points of view. Most significantly, the molecular density in a monolayer is easily changed by changing the two-dimensional pressure; this provides a convenient, quantitative method to study changes in molecular interactions. Another advantage of this approach is that the phase behavior of lipids and cholesterol in monolayers can be observed by epifluorescence microscopy. Many model mixtures of phospholipids and cholesterol exhibit liquid−liquid immiscibility at low pressures or high areas per molecule; the addition of a small amount of fluorescently labeled phospholipid provides sharp contrast between cholesterol-rich and -poor domains.[5,6] As the area per molecule is decreased in the monolayer, the surface pressure rises and eventually the domains merge into a single uniform phase. If the membrane composition is such that this transition occurs near a critical point, on increasing the pressure the domains form a "stripe phase" just below the transition to one uniform phase.[5,7,8] This behavior is observed in binary mixtures of phospholipid and cholesterol, as well as more complicated mixtures, including monolayers that approximate the inner and outer leaflet compositions of a red blood cell.[8]

Regular-solution theory and regular-solution theory allowing for complex formation have proven remarkably effective in accounting for the phase behavior of binary mixtures of phospholipids and cholesterol. From both physical−chemical and biological perspectives, it has been important to extend this work both experimentally *and* theoretically to more complex mixtures of phospholipids and cholesterol. One theoretical crutch that has been used in earlier work is the pseudo-binary-mixture picture, where complicated mixtures are regarded as binary mixtures of cholesterol and an "average phospholipid."[1] Experimental work has suggested that the deviations from the average-phospholipid approximations are often significant, but not severe.[1,9] The purpose of the present work has been to investigate this subject theoretically, continuing to use regular-solution theory, but including explicitly the individual phospholipids in complicated mixtures.

For the purposes of the present study, all phospholipids were assumed to show a single critical point in mixtures with cholesterol, without formation of a "condensed complex". Such complexes have been proposed to account for observed cholesterol−phospholipid phase diagrams showing two upper miscibility critical points.[10,11] The contributions of such complexes to the phase behavior of multicomponent phospholipid−cholesterol mixtures will be examined in a forthcoming study.[12]

Results and Discussion

The Average-Phospholipid Model. Monolayer mixtures of cholesterol (C) and a phospholipid (P) sometimes exhibit liquid−liquid immiscibility.[5] This behavior has been effectively modeled by treating the mixture as a regular-solution whose free energy is described by[13]

$$\bar{G} = X_C\mu_C^\circ + X_P\mu_P^\circ + kT(X_C \ln X_C + X_P \ln X_P) + \xi_{CP}X_CX_P \quad (1)$$

The last term is the excess free energy of mixing; its coefficient, the interaction parameter ξ_{CP}, is equal to $2kT_c$, where T_c is the miscibility critical temperature.[14] It is convenient to normalize the free energy expression in eq 1 by dividing by kT to give

$$\tilde{G} = \frac{\bar{G}}{kT} = X_C\tilde{\mu}_C^\circ + X_P\tilde{\mu}_P^\circ + X_C \ln X_C + X_P \ln X_P + a_{CP}X_CX_P \quad (2)$$

Here the interaction parameter a_{CP} is equal to ξ_{CP}/kT. This normalization removes the temperature dependence of the entropy terms $X_i \ln X_i$ and has the merit of encapsulating all of the miscibility of the mixture in the interaction parameter a_{CP}; phase separation occurs when $a_{CP} > 2$, and the mixture is fully miscible when $a_{CP} < 2$. This is discussed in more detail below.

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For experiments conducted at a fixed temperature, in which the surface pressure of the mixture is varied, a_{CP} may be approximately expressed as a function of the surface pressure π, as

$$a_{CP} = 2 - a'_{CP}(\pi - \pi_c) \tag{3}$$

Here π_c is the miscibility critical pressure of the binary mixture and a'_{CP}, which represents the pressure dependence of the interaction energy, is related to the nonideality of the area of mixing.[15,16] Note that at the miscibility critical pressure π_c, $a_{CP} = 2$. Temperature may also be varied; in terms of the miscibility critical temperature T_c, the interaction term is

$$a_{CP} = 2T_c/T \tag{4}$$

As discussed by Hagen and McConnell,[1] mixtures of multiple phospholipids with cholesterol sometimes exhibit phase behavior that resembles a binary mixture of cholesterol with a single "average" phospholipid. Following eq 2, a regular-solution model of such an n-component mixture has a molar Gibbs free energy of

$$\tilde{G} = \sum_{i=0}^{n-1}(X_i \tilde{\mu}_i^\circ + X_i \ln X_i) + \sum_{i<j} a_{ij} X_i X_j \tag{5}$$

where μ_i° is the chemical potential of pure component i, normalized with respect to kT, and

$$a_{ij} = 2T_{c(ij)}/T \tag{6}$$

is the (normalized) interaction energy coefficient for components i and j, for which the binary critical temperature is $T_{c(ij)}$. As above, if pressure is varied instead of temperature, this interaction term may be approximated by the expression

$$a_{ij} = 2 - a'_{ij}(\pi - \pi_{c(ij)}) \tag{7}$$

where $\pi_{c(ij)}$ is the binary critical pressure for components i and j and a'_{ij} reflects the pressure-dependence of the interaction energy. Component 0 is cholesterol and components 1, 2, ..., $n - 1$ are phospholipids. Binary mixtures of phospholipids with similar headgroups and chain lengths exhibit near-ideal mixing,[9,17] although deviations from ideality in mixtures of dissimilar phospholipids are sometimes significant.[9,17] For simplicity, the binary interactions between the phospholipids are assumed to be ideal. Under this assumption, all of the interaction terms a_{ij} are equal to zero for $i, j > 0$.

In the average-phospholipid model, it is additionally assumed that phase separation of the mixture takes place along the cholesterol composition axis so that the fraction of each phospholipid $f_i = X_i/(1 - X_0)$ is the same in both phases. Under this assumption, the normalized free energy is

$$\tilde{G} = X_0\tilde{\mu}_0^\circ + X_0 \ln X_0 + (1 - X_0)\ln(1 - X_0) + (1 - X_0)\sum_{i=0}^{n-1}(f_i \tilde{\mu}_i^\circ + f_i \ln f_i) + X_0(1 - X_0)\sum_{i=0}^{n-1} f_0 a_{0i} \tag{8}$$

The stability of the mixture with respect to phase separation along the cholesterol composition axis is determined by the second derivative of the free energy with respect to X_0,

$$\frac{\partial^2 \tilde{G}}{\partial {X_0}^2} = \frac{1}{X_0} + \frac{1}{1 - X_0} - 2a^{avg} \tag{9}$$

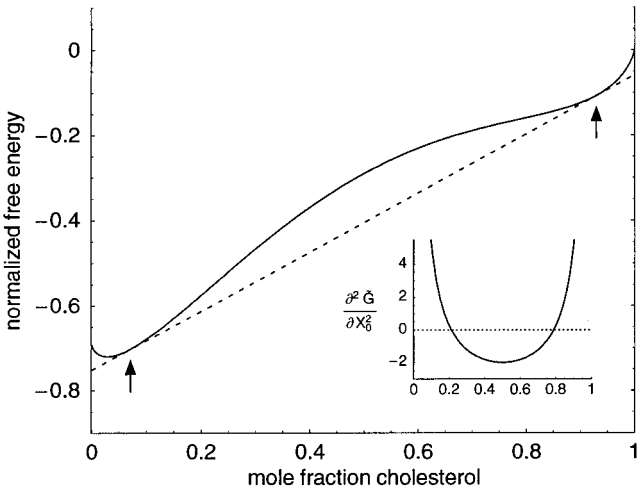

Figure 1. Normalized free energy of a ternary (two phospholipid) mixture with $f_1 = f_2 = 1/2$ and $a_{avg} = 3$. For simplicity, the chemical potentials of the pure components, $\tilde{\mu}_i^\circ$, were all set equal to zero; the free energy at $X_0 = 0$ arises from the entropy of mixing of the two phospholipids. The dotted line is tangent to the curve at two points, indicated by arrows, which represent the compositions of the coexisting phases. The endpoints of the dotted line indicate the chemical potentials of the (average) phospholipid and cholesterol for these phases: $\tilde{\mu}_P = -0.751$, $\tilde{\mu}_C = -0.058$. Inset: the second derivative of the free energy along the cholesterol axis with respect to the mole fraction of cholesterol.

where

$$a^{avg} = \sum_{i=1}^{n-1} f_i\, a_{0i} \tag{10}$$

is the average-phospholipid−cholesterol interaction energy, weighted by the fractions of each phospholipid. The superscript "avg" refers to the average-phospholipid model. A positive value for the expression in eq 9 indicates that the mixture is stable (or possibly metastable) with respect to phase separation; a negative value indicates instability. The relationship between phase stability and the second derivative of the free energy is illustrated in Figure 1.

At a critical point the mixture is at the boundary of stability, such that the second derivative of the free energy along the separation axis is zero. On either side of the critical point, the mixture is stable; that is, the second derivative of the free energy is positive. Consequently, the critical point occupies a minimum in the second derivative of the free energy, which means that at the critical point, the third derivative of the free energy is also zero and the fourth derivative is positive. The third derivative of the free energy (eq 8) along the cholesterol composition axis is

$$\frac{\partial^3 \tilde{G}}{\partial {X_0}^3} = \frac{1}{{X_0}^2} + \frac{1}{(1 - X_0)^2} \tag{11}$$

For a binary regular solution of a phospholipid with cholesterol, whose free energy is described by eq 2, the critical composition is $X_0 = 1/2$. Similarly, from eq 11 it is evident that in a multicomponent mixture the third derivative of the free energy *along the cholesterol composition axis* is zero when $X_0 = 1/2$.

From eq 9, the second derivative of the free energy along X_0 is also zero at this composition if the average-phospholipid−cholesterol interaction energy is

$$a^{avg} = 2 \tag{12}$$

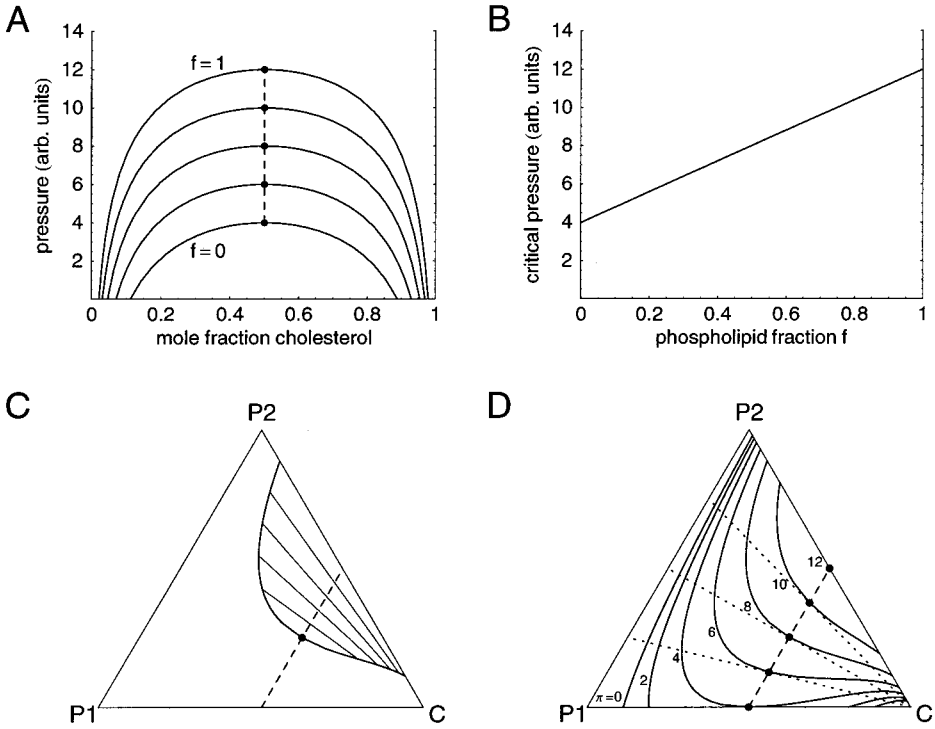

Figure 2. Schematic representations of hypothetical average-phospholipid model phase behavior for a ternary mixture of cholesterol with two phospholipids with (binary) critical pressures of $\pi_c = 4$ and 8. (A) Phase diagrams predicted by the average-phospholipid assumption for mixtures with phospholipid compositions of $f = 0$, 0.25, 0.50, 0.75, and 1. Dots indicate the critical points, all of which have the cholesterol composition $X_0 = 0.5$; the dashed line serves to guide the eye. (B) A plot of the average-phospholipid model critical pressure as a function of the phospholipid fraction $f = X_{P2}/(X_{P1} + X_{P2})$. (C) The predicted binodal curve for a pressure $\pi = 8$. A dot shows the critical point at $f = 0.5$; representative tie lines are shown for $f = 0.6$, 0.7, 0.8, and 0.9. The dashed line indicates the cholesterol composition $X_0 = 0.5$. (D) A contour plot of the predicted binodal surface for this mixture, showing the binodal curves for $\pi = 0$, 2, 4, ..., 12. The curves in part A correspond to vertical slices through this surface at $f = 0$, 0.25, etc., indicated by the dotted lines that converge at the cholesterol vertex. The line in part B shows a vertical slice through the surface at $X_0 = {}^1/_2$, indicated in parts A, C, and D by the dashed line. The curve in part C corresponds to a horizontal slice through this surface at $\pi = 8$. The parameters used for the calculations shown here are $\pi_c(1) = 4$, $\pi_c(2) = 12$, $a'_{01} = a'_{02} = 1/6$, $a_{12} = 0$.

If eq 12 is satisfied, then the second and third derivatives of the free energy along the X_0 axis are both zero at $X_0 = {}^1/_2$. This means that if the average interaction energy is equal to 2 *and phase separation occurs along the cholesterol composition axis*, the critical composition of the mixture is $X_0 = {}^1/_2$, the same as for the binary P−C mixtures.

Equation 12 corresponds closely to the case of binary mixtures, in which $a_{0i} = 2$ at the critical point. In a binary mixture, the critical temperature is $T_{c(0i)}$; similarly, it follows from eq 12 that in the average-phospholipid model, in which phase separation is restricted as described above, the critical temperature of a multicomponent mixture is the weighted average of the binary critical temperatures,

$$T_c^{\,avg} = \sum_{i=1}^{n-1} f_i\, T_{c(0i)} \tag{13}$$

Likewise, in constant-temperature systems in which the pressure is varied, the average-phospholipid model critical pressure of the mixture with respect to separation along the cholesterol axis is the average of the binary critical pressures,

$$\pi_c^{avg} = \sum_{i=1}^{n-1} f_i\, \pi_{c(0i)} \tag{14}$$

provided that the pressure dependences of each of the a_{0i} are the same. This result was derived by Hagen and McConnell;[1] Figure 2a illustrates this result for a ternary mixture having two phospholipids.

"Average-Phospholipid" Phase Behavior. Below the critical temperature or pressure, a binary phospholipid−cholesterol mixture described by eq 2 separates into two coexisting phases, in which the chemical potential of each component is the same. The chemical potential of cholesterol in a phase is

$$\tilde{\mu}_0 = \tilde{G} + (1 - X_0)\frac{\partial \tilde{G}}{\partial X_0} = \tilde{\mu}_0^\circ + a^{avg}(1 - X_0)^2 + \ln X_0 \tag{15}$$

Graphically, this is the intercept of the $X_0 = 1$ axis with the tangent to the free energy curve (see Figure 1). Likewise, the chemical potential for the "average-phospholipid" in a phase can be expressed as

$$\tilde{\mu}_P = \tilde{G} - X_0\frac{\partial \tilde{G}}{\partial X_0} = \tilde{\mu}_P^\circ + a^{avg}X_0^2 + \ln(1 - X_0) \tag{16}$$

where the average-phospholipid chemical potential $\tilde{\mu}_P^\circ$ is defined as

$$\tilde{\mu}_P^\circ \equiv \sum_{i=1}^{n-1}(f_i\,\tilde{\mu}_i^\circ + f_i \ln f_i) \tag{17}$$

At equilibrium, the chemical potential of cholesterol is the same in both phases; the same is true of the phospholipids.

Setting the chemical potentials equal in both phases gives

$$a^{\text{avg}}(1 - X_{0(1)})^2 + \ln X_{0(1)} = a^{\text{avg}}(1 - X_{0(2)})^2 + \ln X_{0(2)}$$
$$a^{\text{avg}}X_{0(1)}^2 + \ln(1 - X_{0(1)}) = a^{\text{avg}}X_{0(2)}^2 + \ln(1 - X_{0(2)}) \quad (18)$$

where the subscripts 1 and 2 designate the two phases. The two eqs 18 may be solved to give the compositions $X_{0(1)}$ and $X_{0(2)}$ of the coexisting phases for a given value of a^{avg}.

Phase coexistence curves calculated using eqs 12 and 18 for a ternary "average-phospholipid" system are shown in Figure 2. Here, the phospholipid composition may be specified by a single variable, $f = X_2/(X_1 + X_2)$. For all values of f, the phase diagrams in Figure 2A have the same symmetric shape, centered at the critical composition $X_0 = {}^1/_2$. Figure 2B shows a linear interpolation of the critical pressure discussed above, and Figure 2C illustrates the assumption that demixing results in two phases having the same phospholipid composition. The tie lines shown radiate out from the $X_0 = 1$ corner of the composition triangle, each line corresponding to a particular phospholipid composition. All of these views together comprise the *binodal surface* shown as a contour plot in Figure 2D.

Instability of the "Average-Phospholipid" Critical Point. The average-phospholipid model results derived above are based on the assumption that the proportions of the phospholipids are the same in both phases. In general, however, this is not the case: the phospholipid compositions of the two phases are usually different. To determine whether the "average-phospholipid" critical points described above are genuine critical points, it is necessary to consider such asymmetric phase separations.

Consider the case of a ternary mixture of cholesterol and two phospholipids where the phospholipids interact ideally with one another. Ternary regular solutions were analyzed in detail by Meijering,[18] who provided a useful mathematical framework for discussing the phase stability of such mixtures. For convenience, designate the mole fractions of the two phospholipids as x and y; the cholesterol mole fraction is then $z = 1 - x - y$.

For phase separation along a general coordinate direction **v** in composition space defined by the slope $n = \mathrm{d}y/\mathrm{d}x$, the second derivative of the free energy may be expressed in terms of the two independent composition variables x and y:

$$\frac{\partial^2\tilde{G}}{\partial v^2} = \frac{\partial^2\tilde{G}}{\partial x^2} + 2n\frac{\partial^2\tilde{G}}{\partial x\partial y} + n^2\frac{\partial^2\tilde{G}}{\partial y^2} \quad (19)$$

where v is the distance along **v**. This equation is quadratic in n; hence, the second derivative of $\tilde{G}$ is equal to zero in the direction **v** when the slope n satisfies

$$n = \frac{-2\tilde{G}_{xy} \pm (4\tilde{G}_{xy}^2 - 4\tilde{G}_{xx}\tilde{G}_{yy})^{1/2}}{2\tilde{G}_{yy}} \quad (20)$$

where $\tilde{G}_{xx}$ is the partial derivative $\partial^2\tilde{G}/\partial x^2$, and so on. At the critical point, there is only one direction along which the second derivative of the free energy is zero, the direction of phase separation; all other directions have positive second derivatives.

At a particular composition (x, y), the slope n is unique if the discriminant of eq 20 is zero, that is,

$$d = \tilde{G}_{xy}^2 - \tilde{G}_{xx}\tilde{G}_{yy} = 0 \quad (21)$$

For a regular solution with a free energy of the form shown in eq 8, the relevant partial derivatives of the free energy are

$$\frac{\partial^2\tilde{G}}{\partial x^2} = \frac{1}{x} + \frac{1}{1 - x - y} - 2a_x \quad (22a)$$

$$\frac{\partial^2\tilde{G}}{\partial x\partial y} = \frac{1}{1 - x - y} - a_x - a_y \quad (22b)$$

$$\frac{\partial^2\tilde{G}}{\partial y^2} = \frac{1}{y} + \frac{1}{1 - x - y} - 2a_y \quad (22c)$$

At the average-phospholipid model critical point discussed above, the interaction terms a_x and a_y satisfy eq 12 above, which may be expressed here as

$$\left(\frac{x}{x + y}\right)a_x + \left(\frac{y}{x + y}\right)a_y = 2 \quad (23)$$

The average-phospholipid model critical composition is $X_0 = {}^1/_2$; hence at the average-phospholipid critical point, the compositions of the two phospholipids are related by $x + y = {}^1/_2$. Using these relations, the second derivatives of the free energy at the average-phospholipid model critical point are

$$\frac{\partial^2\tilde{G}}{\partial x^2} = \frac{1}{x} + 2(1 - a_x) \quad (24a)$$

$$\frac{\partial^2\tilde{G}}{\partial x\partial y} = \frac{4x(1 - a_x) + a_x}{2x - 1} \quad (24b)$$

$$\frac{\partial^2\tilde{G}}{\partial y^2} = \frac{4x(1 - a_x)}{2x - 1} \quad (24c)$$

and the discriminant of eq 20 is

$$d = \left(\frac{a_x - 2}{2y}\right)^2 = \left(\frac{a_y - 2}{2x}\right)^2 \quad (25)$$

If the two phospholipids have identical properties, the condition $a_x = a_y = 2$ describes a critical point at the composition $X_0 = {}^1/_2$ for any mixture of the phospholipids.[9] In this case, the discriminant d is equal to zero, so there is a unique direction v along which the second derivative of the free energy is zero and the average-phospholipid critical point is a genuine critical point. However, for all other cases, $a_x \neq a_y$ and the "average-phospholipid" interaction term a^{avg} is between them. Since $a^{\text{avg}} = 2$ at the average-phospholipid critical point (eq 12), a_x and a_y must be unequal to 2 at this point. Consequently, the discriminant d must be positive, and there are two directions in composition space, $\mathbf{v}_1$ and $\mathbf{v}_2$, along which the second derivative of the free energy is zero. Furthermore, because eq 19 is quadratic in n, the second derivative of $\tilde{G}$ must be negative along directions *between* $\mathbf{v}_1$ and $\mathbf{v}_2$, indicating that the mixture is unstable with respect to separation along these directions. That is, the average-phospholipid model critical point is unstable with respect to phase separation and is *not* a genuine critical point.

Determining the True Critical Points. For a regular solution, the miscibility of the system increases as the temperature (or pressure) of the system is raised. As discussed above, a mixture of phospholipids and cholesterol at the "average-phospholipid" critical point is unstable with respect to phase separation, so it may be expected that temperature or pressure of the system must be raised above this level to reach the true critical point of the mixture. It should also be noted that the true critical composition may differ from the average-phospholipid model value of $X_0 = {}^1/_2$. These deviations may be expressed

more quantitatively by deriving expressions for the critical temperature/pressure and the critical composition, allowing for phase separation in any direction, not just along the cholesterol composition axis.

A method for computing critical points in a ternary regular solution is described by Meijering.[18] Essentially, for a given set of interaction energies, one finds compositions where there is a unique direction along which the second derivative of the free energy is zero *and* along which the third derivative of the free energy is also zero. This method is illustrated below for a general ternary regular solution; we later introduce the ideal interaction between the two phospholipids.

The compositions at which there is a unique direction where $\partial^2\tilde{G}/\partial v^2 = 0$ lie on a *spinodal curve*, which separates regions which are unstable with respect to phase separation in some directions from regions which are fully stable (or possibly metastable). Put another way, the spinodal curve is the locus of points which satisfy eq 21. For ternary mixtures with free energies described by eq 5, the second derivatives of $\tilde{G}$ are

$$\frac{\partial^2\tilde{G}}{\partial x^2} = \frac{1}{x} + \frac{1}{1-x-y} - 2a_x \tag{26a}$$

$$\frac{\partial^2\tilde{G}}{\partial x\partial y} = \frac{1}{1-x-y} - a_x - a_y + a_{12} \tag{26b}$$

$$\frac{\partial^2\tilde{G}}{\partial y^2} = \frac{1}{y} + \frac{1}{1-x-y} - 2a_y \tag{26c}$$

Inserting these expressions into eq 21 gives the spinodal equation

$$\left(\frac{1}{1-x-y} - a_x - a_y + a_{12}\right)^2 - \left(\frac{1}{x} + \frac{1}{1-x-y} - 2a_x\right)\left(\frac{1}{y} + \frac{1}{1-x-y} - 2a_y\right) = 0 \tag{27}$$

which may be rearranged into

$$2(a_x x(1-x-y) + a_y y(1-x-y) + a_{12}xy) + (a_x^2 + a_y^2 + a_{12}^2 - 2(a_x a_y + a_x a_{12} + a_y a_{12}))xy(1-x-y) = 1 \tag{28}$$

To locate the critical point(s), it is necessary to find the point(s) on the spinodal curve where the second and third derivatives are both zero. For any point (x, y) on the spinodal curve, the direction **v** along which the second derivative of the free energy is zero has a slope of, from eq 20,

$$n = -\frac{\partial^2\tilde{G}/\partial x\partial y}{\partial^2\tilde{G}/\partial y^2} = -\frac{\partial^2\tilde{G}/\partial x^2}{\partial^2\tilde{G}/\partial x\partial y} = -\frac{y - (a_x + a_y - a_{12})y(1-x-y)}{1 - x - 2a_y y(1-x-y)} \tag{29}$$

The third derivative of the free energy along this direction **v** is given by

$$\frac{\partial^3\tilde{G}}{\partial v^3} = \frac{\partial^3\tilde{G}}{\partial x^3} + 3n\frac{\partial^3\tilde{G}}{\partial x^2\partial y} + 3n^2\frac{\partial^3\tilde{G}}{\partial x\partial y^2} + n^3\frac{\partial^3\tilde{G}}{\partial y^3} \tag{30}$$

where the constituent derivatives of the free energy are

$$\frac{\partial^3\tilde{G}}{\partial x^3} = -\frac{1}{x^2} + \frac{1}{(1-x-y)^2} \tag{31a}$$

$$\frac{\partial^3\tilde{G}}{\partial x^2\partial y} = \frac{\partial^3\tilde{G}}{\partial x\partial y^2} = \frac{1}{(1-x-y)^2} \tag{31b}$$

$$\frac{\partial^3\tilde{G}}{\partial y^3} = -\frac{1}{y^2} + \frac{1}{(1-x-y)^2} \tag{31c}$$

Combining eqs 29 through 31 gives, after some rearrangement, the "critical equation"

$$\{x^2 z(1 + (a_x - a_y - a_{12})y)^3 - (y + z - 2a_y yz)^3 + x^2 y(1 + (a_{12} - a_x - a_y)z)^3\}/\{x^2(y + z - 2a_y yz)^3\} = 0 \tag{32}$$

As before, $z = 1 - x - y$ represents the mole fraction of cholesterol. Equations 28 and 32 may be combined and solved numerically to give the composition (x, y) of the critical point(s) if the interaction energies a_{ij} are known.

We are more interested in the inverse problem: finding the temperature/pressure of the critical point for a particular phospholipid composition. In this case, the values of the a_{ij} at the critical point (which depend on the temperature/pressure) are not known a priori; it is necessary solve the above equations to obtain them. Consider the case of determining the critical pressure for a mixture of two phospholipids with cholesterol in which the pressure dependence of the interaction energy is the same for both binary P−C mixtures. From eq 7, the difference of the interaction parameters is

$$\Delta a = a_y - a_x = a'_{\mathrm{P}}(\pi_y - \pi_x) \tag{33}$$

The composition of the mixture may be specified by the mole fraction of cholesterol, z, and the composition of phospholipid P2 may be specified as a fraction of the total phospholipid mole fraction,

$$f \equiv \frac{y}{x+y} \tag{34}$$

Making the substitutions shown by eqs 33 and 34, the spinodal and critical equations may be expressed as

$$z(1-z)(2a_x + f\Delta a(2 + (1-f)(1-z)\Delta a)) = 1 \tag{35}$$

$$\frac{z(1 - f(1-z)\Delta a)^3 + f^3(1-z)^3(1 - z(2a_x + \Delta a))^3}{(z + f(1-z)(1 - 2z(a_x + \Delta a)))^3} - \frac{1}{(1-f)^2(1-z)^2} = 0 \tag{36}$$

From eq 35, the interaction energy a_x for a point on the spinodal curve is

$$a_x = \frac{1 - f(1-z)z\Delta a(2 + (1-f)(1-z)\Delta a)}{2(1-z)z} \tag{37}$$

Inserting this expression into the critical equation (eq 36) gives, after some simplification,

$$1 - z(2 + (1-f)f(1-z)^2 z\Delta a^2(3 + (1-2f)(1-z)\Delta a)) = 0 \tag{38}$$

For a particular value of f, this equation may be solved to give the cholesterol composition z of the critical point. Inserting this value of z into eq 37 then gives the interaction term a_x of the critical point, from which the critical pressure (temperature) may be determined using eq 6 or 7, as appropriate. Calculated critical

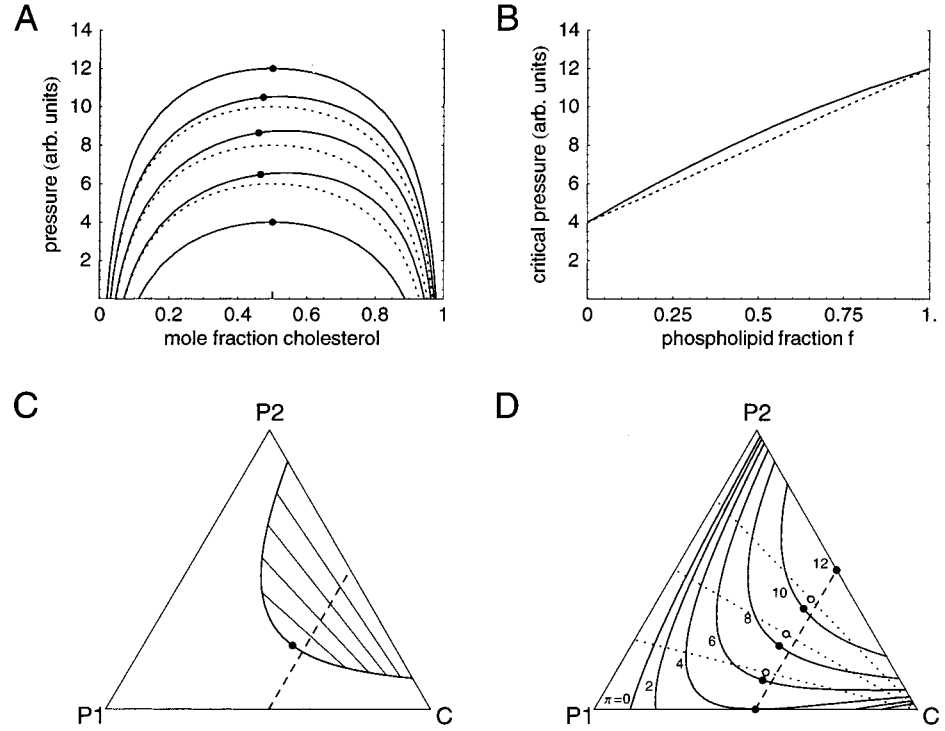

Figure 3. Calculated phase behavior for the system described schematically in Figure 2, allowing for phases with dissimilar phospholipid compositions. (A) Calculated phase diagrams for phospholipid fractions $f = 0$, 0.25, 0.5, 0.75, and 1. Dots indicate critical points; the dashed line connects points having a cholesterol composition of $X_0 = 0.5$. The dotted curves show the "average-phospholipid" phase diagrams from Figure 2A. (B) A plot of the calculated critical pressure as a function of *f*. The dotted line shows the "average-phospholipid" critical pressures from Figure 2B. (C) The calculated binodal curve for $\pi = 8$. A dot shows the critical point; four representative tie lines are shown. The dashed line indicates the cholesterol composition $X_0 = 0.5$. (D) A contour plot of the calculated binodal surface for this mixture, showing the binodal curves for pressures of $\pi = 0$, 2, 4, ..., 12. The dotted lines indicate phospholipid compositions of $f = 0.25$, 0.5, and 0.75, corresponding to the middle three curves in part A; open circles indicate the critical points for these phospholipid compositions. As in Figure 2, the curves in parts A through C represent cross sections through this surface. The parameters used are the same as in Figure 2.

points for a P1−P2−C ternary mixture are shown in Figure 3, which is further discussed below.

Computation of Tie Lines. To determine the compositions of coexisting phases below the critical temperature/pressure, a procedure similar to that used to calculate the average-phospholipid model phase diagrams in Figure 2 may be followed. Here, however, the phospholipid composition is not assumed to be the same in both phases. Consequently, there are *two* independent composition variables, *x* and *y*, in a ternary mixture, rather than a single "average-phospholipid" composition. The chemical potentials of the two phospholipids and cholesterol are

$$\mu_x = \mu_x^\circ + a_{12}y + a_x z - (a_{12}xy + a_x xz + a_y yz) + \ln x \quad (39a)$$

$$\mu_y = \mu_y^\circ + a_{12}x + a_y z - (a_{12}xy + a_x xz + a_y yz) + \ln y \quad (39b)$$

$$\mu_z = \mu_z^\circ + a_x x + a_y y - (a_{12}xy + a_x xz + a_y yz) + \ln z \quad (39c)$$

In graphical terms, the chemical potential of component *i* for a ternary mixture of composition (x, y) is equal to the intercept of the tangent *plane* to the free energy surface at (x, y) with the $X_i = 1$ axis. For a mixture with overall composition (x_0, y_0) that separates into two coexisting phases, the compositions of the phases may be determined by equating the chemical potentials of each of the components. This leads, after some rearrangement, to the equations

$$a_{12}(y_2 - y_1) + a_x(z_2 - z_1) + \ln(x_2/x_1) = A \quad (40a)$$

$$a_{12}(x_2 - x_1) + a_y(z_2 - z_1) + \ln(y_2/y_1) = A \quad (40b)$$

$$a_x(x_2 - x_1) + a_y(y_2 - y_1) + \ln(z_2/z_1) = A \quad (40c)$$

where

$$A = a_{12}(x_2y_2 - x_1y_1) + a_x(x_2z_2 - x_1z_1) + a_y(y_2z_2 - y_1z_1) \quad (41)$$

This is a system of three equations in four variables because z_1 and z_2 are not independent. A fourth equation arises from the constraint that the compositions of the two phases must be collinear with the initial composition (x_0, y_0), that is, the tie line that must pass through (x_0, y_0). This condition may be expressed as

$$\frac{y_1 - y_0}{x_1 - x_0} = \frac{y_2 - y_0}{x_2 - x_0} \quad (42)$$

Equations 40 and 42 may be solved numerically to give tie lines for the ternary P1−P2−Chol system, where a_{12} is taken to be zero. The calculated phases for the ternary mixture discussed above are shown in Figure 3. These computations were performed for the same mixture used for Figure 2, but here the coexisting phases are not assumed to have identical phospholipid compositions. Comparison of the curves in Figures 2 and 3 shows that the phase separations that occur in a ternary P1−P2−Chol system differ systematically from those obtained using the average-phospholipid model. In Figure 3A, the calculated phase diagrams for the mixtures with $f = 0$ and $f = 1$ (the binary P−C mixtures) are the same as those in Figure 2A. However, the three-phase diagrams for mixtures in which both phospholipids are present with cholesterol, with $f = 0.25$,

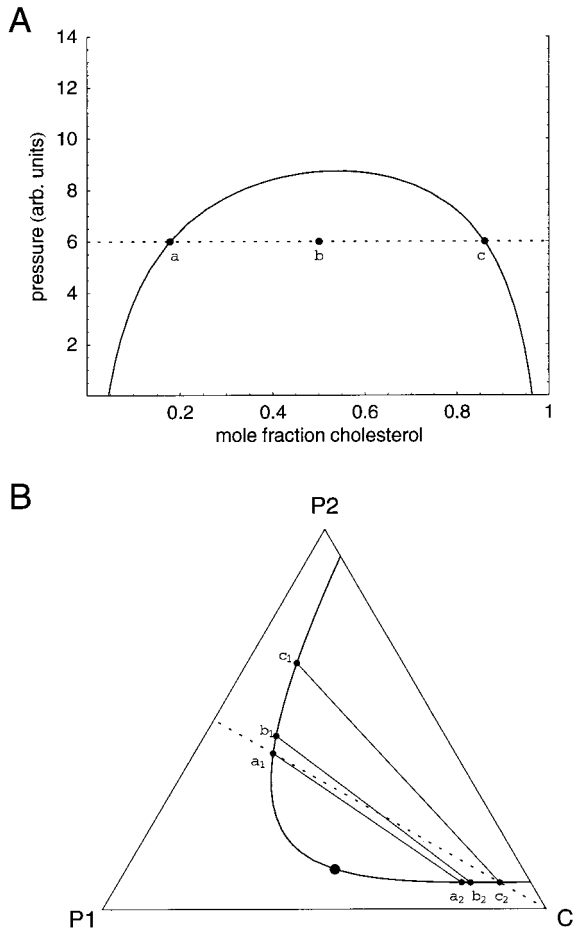

Figure 4. Compositions of coexisting phases for the ternary mixture shown in Figures 2 and 3 for a phospholipid composition of $f = 0.5$ and a pressure of $\pi = 6$. (A) Calculated phase diagram for a mixture with $f = 0.5$. The broken line indicates a pressure of $\pi = 6$. Points a, b, and c indicate compositions at the onset of two-phase coexistence (a and c) and inside the two-phase region (b). (B) Calculated binodal curve for $\pi = 6$. The broken line indicates compositions for which $f = 0.5$. The three tie lines shown indicate the compositions of the coexisting phases present in part A: phases a_1 and a_2 coexist at point a; phases b_1 and b_2 coexist at point b; phases c_1 and c_2 coexist at point c.

0.5, and 0.75, all lie outside the corresponding "average-phospholipid" phase diagrams in Figure 2A. Note that the calculated critical pressure is elevated with respect to that calculated using the average-phospholipid model; this is may be clearly seen in Figure 3B. Furthermore, the computed critical points for these mixtures all lie at cholesterol compositions less than $^1/_2$, the critical composition obtained using the average-phospholipid model.

Figure 3C shows the ternary binodal curve for an intermediate pressure between the two binary P−C critical pressures with some representative tie lines to indicate the compositions of coexisting phases. The tie lines are roughly parallel with the P2−C side of the composition triangle, which means that P1 is about equally miscible with the P2-rich and C-rich phases. Figure 3D shows the binodal surface for this ternary mixture. The deviation of the critical cholesterol composition from the average-phospholipid value of $^1/_2$ is clearly visible.

Figure 4 illustrates the calculated phase behavior that would be observed on adding cholesterol to a 1:1 mixture of phospholipids P1 and P2 at a pressure of $\pi = 6$. The phase diagram that would be observed for this ternary mixture is shown in Figure 4A, and the ternary binodal curve for $\pi = 6$ is shown in Figure 4B. In both parts, the process described here follows the dotted line shown. When the amount of cholesterol present is small ($X_0 < 0.17$), the mixture exists as a single stable phase. When the cholesterol composition reaches 17% (Figure 4A, point a, and Figure 4B, point a_1), a second, cholesterol-rich phase begins to form, which is enriched in P1 (Figure 4B, point a_2). As more cholesterol is added, the composition of the coexisting phases changes continuously. For example, at $X_0 = 0.5$ (point b in Figure 4A), the coexisting phases have compositions indicated by points b_1 and b_2 in Figure 4B. When the cholesterol composition reaches 87% (Figure 4A, point c), the phospholipid-rich phase has the composition indicated by c_1 and is present in an infinitesimal amount, in equilibrium with a cholesterol-rich phase of composition c_2. Upon further addition of cholesterol, the entire mixture is present as a single cholesterol-rich phase.

Although the phase diagram shown in Figure 4A resembles the phase diagram for a binary mixture, care must be taken in its interpretation. Because the tie lines of the ternary mixture are "rotated" out of the line of uniform phospholipid composition, the boundaries of the two-phase region of the phase diagram in Figure 4A do *not* represent the exact compositions of the coexisting phases inside the region. Furthermore, the precise amounts of the coexisting phases cannot be determined by applying the lever rule to this phase diagram. A binodal curve of the type shown in Figure 4B must be constructed if the exact compositions and amounts of the coexisting phases are to be determined for a particular cholesterol composition. Another interesting consequence of the tie line rotation described here is that for a ternary mixture with a fixed phospholipid ratio, the critical point does not lie at the top of the phase diagram, but rather at a somewhat lower pressure; this can be seen in Figure 3A.

The average-phospholipid model assumes that coexisting phases in a mixture of several phospholipids with cholesterol will have the same phospholipid compositions. As demonstrated by the tie line rotation discussed above, this assumption is generally not valid: in general, two average-phospholipid model phases can lower their energy by enriching the cholesterol-rich phase with the phospholipid(s) which have *lower* P−C interaction energies and by enriching the phospholipid-rich phase with the phospholipid(s) which have *higher* P−C interaction energies. The enhanced stability of the resulting phases leads to elevation of the critical pressure (temperature) of the mixture with respect to the average-phospholipid model value, as illustrated in Figure 3.

The deviation of the critical composition from the average-phospholipid model value of $X_0 = ^1/_2$ also arises from the difference in the binary P−C interaction energies of the phospholipids. This effect can be most clearly seen in a hypothetical ternary mixture in which $a_x = 0$; that is, the phospholipid P1 interacts ideally with cholesterol as well as with the other phospholipid, P2 (Figure 5). In this case, the tie lines of the ternary P1−P2−C mixture run parallel to the P2−C side of the composition triangle because P1 mixes equally well with both phases. Note that the critical point in this mixture has a cholesterol composition less than $^1/_2$; in this case, $X_{0(\mathrm{crit})} = ^1/_3$. When the binary interaction energy a_x is greater than zero, this effect is reduced. As a_x is increased, the critical point moves toward the P1−C binary critical point, where the critical composition is $X_0 = ^1/_2$. For the ternary mixture shown in Figure 3, the P1−C interaction energy a_x is 0.67 at the P2−C binary critical pressure, $\pi_{c(02)} = 12$, and gradually increases as the pressure is lowered, until reaching 2 at the P1−C binary critical pressure, $\pi_{c(01)} = 4$.

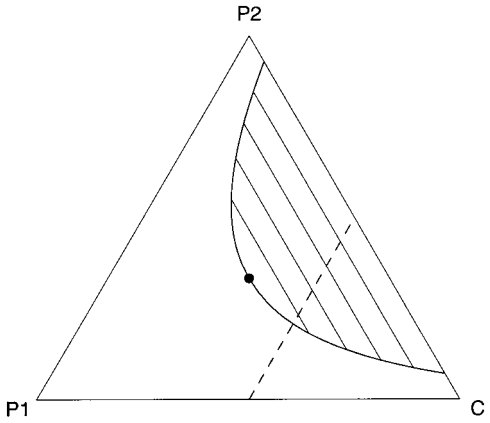

Figure 5. Binodal curve and representative tie lines for a ternary mixture of cholesterol with P2, a phospholipid with a binary P−C critical pressure of $\pi_c = 12$, and P1, a hypothetical phospholipid which interacts ideally with both P2 and cholesterol, at a pressure of $\pi = 6$. The critical point is indicated by a dot; the dashed line shows the points for which the cholesterol composition is $X_0 = {}^1/_2$.

Quantitative Aspects of Deviations from Average-Phospholipid Model Behavior. As discussed above, deviations from average-phospholipid behavior arise from differences between the binary interaction energies of the phospholipids with cholesterol, and the size of the deviation depends on the magnitude of the difference in P−C interaction energies. This relationship may be described more precisely in ternary P1−P2−Chol mixtures by finding general expressions for the critical pressure and composition of such mixtures.

For later convenience, we express the cholesterol composition at the critical point in terms of its deviation from the average-phospholipid value of $X_0 = {}^1/_2$:

$$z = {}^1/_2 + \epsilon \tag{43}$$

Inclusion of this expression in the combined spinodal-critical eq 38 gives, after some simplification,

$$64\epsilon + f(1-f)(1-4\epsilon^2)^2\Delta a^2(6 + (1-2f)(1-2\epsilon)\Delta a) = 0 \tag{44}$$

This equation is quintic in ϵ and has no simple analytical solution. However, the magnitude of ϵ must be less than ${}^1/_2$, so eq 44 may be closely approximated by expanding the left-hand side and dropping terms which are quadratic and higher in ϵ. Doing this and solving for ϵ as a function of the phospholipid composition f gives

$$\epsilon_{\text{crit}} \doteq -\frac{f(1-f)\Delta a^2(6-(1-2f)\Delta a)}{64 - 2f(1-f)(1-2f)\Delta a^3} \tag{45}$$

For small values of Δa ($\lesssim 3$), this is very close to

$$\epsilon_{\text{crit}} \doteq -\frac{3}{32} f(1-f)\Delta a^2 - \frac{1}{64} f(1-f)(1-2f)\Delta a^3 \tag{46}$$

The same result is obtained when the quadratic term in eq 44 is retained. The magnitude of this deviation is greatest at the phospholipid composition

$$f \doteq \frac{1}{2} - \frac{\Delta a}{24} \tag{47}$$

The term Δa was defined above as $a_y - a_x$. Equation 47 indicates that the maximum deviation of the cholesterol deviation lies close to $f = {}^1/_2$, shifted slightly toward the composition edge with a smaller binary P−C interaction energy. The magnitude of the deviation of the critical cholesterol composition at this phospholipid composition is approximately

$$\epsilon_{\text{max}} \doteq -\frac{3}{128}(\Delta a)^2 \tag{48}$$

Recall that ϵ was defined as the deviation of the critical cholesterol composition from the average-phospholipid model value of $X_0 = {}^1/_2$. Consequently, eq 48 indicates that the critical cholesterol composition of a ternary mixture of two phospholipids with cholesterol *must be less than* ${}^1/_2$.

The deviation of the critical pressure/temperature from the average-phospholipid value can be determined indirectly by finding the value of the interaction energy a_x at the critical point. From eq 35, the value of a_x for a point on the spinodal curve, on which the critical point must lie, is

$$a_x^{\text{rs}} = \frac{1 - fz(1-z)\Delta a(2 + (1-f)(1-z)\Delta a)}{2z(1-z)} \tag{49}$$

The superscript "rs" denotes the unconstrained regular-solution model. For comparison, the average-phospholipid model value of a_x at the critical point for a phospholipid composition of f is, from eqs 23 and 24,

$$a_x^{\text{avg}} = 2 - f\Delta a \tag{50}$$

If the cholesterol composition z is expressed in terms of its deviation ϵ from the average-phospholipid model critical cholesterol composition of ${}^1/_2$, eqs 49 and 50 can be combined to give the deviation of the critical a_x value from the average-phospholipid model value:

$$a_x^{\text{rs}} - a_x^{\text{avg}} = \frac{32\epsilon^2 - f(1-f)(1-2\epsilon)^2(1+2\epsilon)(\Delta a)^2}{4(1-2\epsilon)(1+2\epsilon)} \tag{51}$$

The critical-point value of ϵ in the above equation may be expressed in terms of f by using eq 45. Maximizing the expression in eq 51 shows that the magnitude of the deviation of the critical value of a_x from its average-phospholipid value reaches a maximum at a phospholipid composition of

$$f \doteq \frac{1}{2} + \frac{(\Delta a)^3}{256} \tag{52}$$

Interestingly, this is not the same as the phospholipid composition that gives a maximal deviation in the critical cholesterol composition: the maximum deviation of the critical a_x from the average-phospholipid model value again lies at a phospholipid composition near ${}^1/_2$, but shifted slightly *away* from the edge with a smaller binary P−C interaction energy. At this phospholipid composition, the deviation of a_x is

$$a_x^{\text{rs}} - a_x^{\text{avg}} \doteq -\frac{(\Delta a)^2}{16} \tag{53}$$

If the pressure dependence a' is the same for both binary interaction energies a_x and a_y this translates (using eq 7) into a maximum deviation in the critical pressure from the average-phospholipid model value of

$$\pi_x^{\text{rs}} - \pi_x^{\text{avg}} = \frac{a'(\Delta\pi_c)^2}{16} \tag{54}$$

For the ternary mixture shown in Figure 3, where $a' = 1/6$ and

$\Delta a = 4/3$, these equations give a maximum critical pressure deviation of 0.667 (arbitrary units) at a phospholipid composition of $f = 0.509$. The actual maximum deviation is very close to $\pi_c^{rs} - \pi_c^{avg} = 0.640$ at $f = 0.510$. For comparison, the difference between the binary critical pressures used in this example is $\Delta\pi_c = \pi_{c(02)} - \pi_{c(01)} = 8$, so the critical pressure deviation is roughly an order of magnitude smaller than the difference between the binary critical pressures. Equations 47 and 52 show that the maximum deviations of the critical point parameters from their average-phospholipid model values occur when the phospholipids are present in approximately equal amounts.

Deviations from Average-Phospholipid Model Behavior in Multicomponent Mixtures. The preceding discussion considered a ternary mixture of two phospholipids with cholesterol. In such mixtures, there are small but meaningful discrepancies between the true regular-solution phase behavior and the predicted average-phospholipid model phase behavior. For mixtures containing more than two phospholipids, one might expect these deviations to be enhanced because there is more "room" in the composition space for the tie lines to rotate with respect to the cholesterol axis. Alternatively, adding more phospholipids might dilute the effects described above, leading to more nearly average behavior.

To address this issue, the method described by Meijering[18] of computing a parametric spinodal curve (surface) and finding the critical point on it must be adapted for a multidimensional composition space rather than the relatively simple composition triangle of a ternary mixture. This process is outlined below.

Consider a mixture of cholesterol and l phospholipids so that the total number of components is $n = l + 1$. If the interactions among the phospholipids are ideal, the free energy of the mixture may be expressed as

$$\tilde{G} = X_0 \ln X_0 + \sum_{i=0}^{l} X_i \ln X_i + X_0 \sum_{i=0}^{l} a_{0i} X_i \qquad (55)$$

For the two-phospholipid example analyzed above, the largest deviations from average-phospholipid model behavior were observed when the two phospholipids were present in nearly equal amounts. There is little reason to believe that this should not also be the case for mixtures of more than two phospholipids, so the following analysis focuses on mixtures in which the phospholipids are present in approximately equal amounts.

If all of the phospholipids are present in equal amounts *prior to any phase separation*, the initial composition of each phospholipid in the mixture is

$$X_i(\text{init}) = \frac{1 - z_0}{l} \qquad (56)$$

where z_0 is the initial composition of cholesterol. Let the direction along which the (potential) phase separation occurs be defined by the vector

$$\mathbf{v} = (n_1, n_2, ..., n_l) \qquad (57)$$

such that the compositions of the phospholipids can be expressed in terms of the direction numbers n_i and a generic composition variable υ as

$$X_i = \frac{1 - z_0}{l} + n_i \upsilon \qquad (58)$$

For later convenience, set $n_i = 1$. Using these expressions and expressing the interaction energies a_{0i} in terms of their difference from a_x, which is taken as a reference point for the interaction energies in the mixture, the free energy (eq 55) becomes

$$\tilde{G} = (z_0 - \upsilon \sum_i n_i) \ln(z_0 - \upsilon \sum_i n_i) + \sum_i \left(\frac{1 - z_0}{l} + \upsilon n_i\right) \ln\left(\frac{1 - z_0}{l} + \upsilon n_i\right) + (z_0 - \upsilon \sum_i n_i) \sum_i (a_x + \Delta a_i) \left(\frac{1 - z_0}{l} + \upsilon n_i\right) \qquad (59)$$

where all of the sums are from $i = 1$ to l.

At a critical point, the second and third derivatives of the free energy along **v** are both zero. Taking the second derivative of eq 59 and setting υ equal to zero gives

$$\frac{\partial^2 \tilde{G}}{\partial \upsilon^2} = \frac{1}{z_0}(1 - 2a_x z_0)(\sum_i n_i)^2 + \frac{l}{1 - z_0} \sum_i n_i^2 - 2(\sum_i n_i)(\sum_i n_i \Delta a_i) \qquad (60)$$

As noted earlier, a critical point must lie on a spinodal curve. This means that at the critical point, there is a unique direction **v** along which the second derivative of the free energy is zero; in all other directions, the second derivative is positive. This unique direction **v** is in turn associated with a unique set of direction numbers n_i. Equivalently, for each of the direction numbers n_1, n_2, ..., n_l, there is a single value that makes the right-hand side of eq 60 equal to zero at the critical point.

The expression for the second derivative in eq 60 is quadratic in each of the direction numbers n_i. Setting this expression equal to zero and solving for any one of the n_i give a solution of the form

$$n_{i(\partial^2 \tilde{G}/\partial \upsilon^2 = 0)} = k_i \pm d_i^{1/2} \qquad (61)$$

where k_i lies between the two (possibly identical) solutions and d_i is the discriminant expression associated with solution for the particular n_i. Since each of the n_i must be unique at the critical point, all of the d_i must equal zero, and the value of each n_i is k_i.

In our earlier discussion of the two-phospholipid mixture, it was noted that the spinodal curve is the locus of composition points for which the discriminant of n is zero. This spinodal curve was then used to obtain an expression for the "reference" interaction energy a_x, which in turn led to expressions for the critical temperature and pressure. A similar strategy is followed here for multicomponent mixtures. Unfortunately, in the multicomponent case, the discriminant expressions cannot be solved directly. Each of the d_i in eq 61 is a complicated function that contains all of the as-yet-unknown n_i. Before the set of discriminants can be set equal to zero and solved for a_x, these expressions must be disentangled.

Each d_i must equal zero; this constraint gives $(l - 1)$ equations in the $(l - 1)$ unknown direction numbers n_i. (Recall that the first direction number, n_i, was defined to be 1, so it is not a variable.) Solving this system of equations for n_i gives

$$n_{i(d=0)} = \{(1 - z_0)(z_0(1 - z_0)(\sum a^2 - \Delta a_i \sum a) + l(-1 + z_0(2a_x + \Delta a_i)))\}/\{l(l - 1 + z_0) + z_0(1 - z_0)(-2l(\sum a + a_x(l - 1)) + (1 - z_0)((\sum a)^2 - (l - 1)\sum a^2))\} \quad (62)$$

where the summation terms are

$$\sum a = \sum_{i=1}^{l} \Delta a_i \quad (63a)$$

$$\sum a^2 = \sum_{i=1}^{l} \Delta a_i^2 \quad (63b)$$

Inserting eq 62 into the general expression for the discriminant d_i (which is not shown) gives the deconvoluted discriminant expression

$$d_i = \{(l^2 + z_0(1 - z_0)((1 - z_0)(\sum a)^2 + l(2(a_x l + \sum a) - (1 - z_0)(\sum a)^2)))(2z_0(1 - z_0)(a_x + \Delta a_i) - z_0(l - 1) - 1)\}/\{(l(1 - z_0 - l) + z_0(1 - z_0)(2l(a_x(l - 1) + \sum a) + (1 - z_0)((l - 1)\sum a^2 - (\sum a)^2))\} \quad (64)$$

The right-hand side of eq 64 can now be set equal to zero to solve for a_x. Doing so gives

$$a_x = \frac{1}{2z_0(1 - z_0)} + \frac{(1 - z_0)(\sum a)^2 - l(2\sum a(1 - z_0)\sum a^2)}{2l^2} \quad (65)$$

Finally, inserting this expression for a_x back into the direction number expression (eq 62) above gives the expression

$$n_i = \frac{(1 - z_0)(\sum a - l\Delta a_i) - l}{(1 - z_0)\sum a - l} \quad (66)$$

For a mixture with equal initial phospholipid compositions, the expression above corresponds to the direction **v** along which the second derivative of the free energy is zero. Because this direction was derived so as to be unique, this composition point lies on the spinodal surface.

We wish to find the parameters of the critical point, where the *third* derivative of the free energy, in addition to the second derivative, is zero. Along the direction **v**, this derivative is

$$\frac{\partial^3 \tilde{G}}{\partial v^3} = \frac{1}{z_0^2}(\sum_{i=1}^{l} n_i)^3 + \frac{l^2}{(1 - z_0)^2}\sum_{i=1}^{l} n_i^3 \quad (67)$$

Inserting the expression (eq 66) derived for the spinodal curve direction numbers n_i into eq 67 and expressing the cholesterol composition as $^1/_2 + \epsilon$ gives an equation which may be solved for ϵ, the deviation of the critical composition from its average-phospholipid model value of $^1/_2$. This equation (not shown) is quintic in ϵ, but may be approximated in linear form as

$$\epsilon \doteq -\frac{3}{32}\left(\frac{1}{l}\sum_{i=1}^{l}(\Delta a_i)^2 - \frac{1}{l^2}(\sum_{i=1}^{l}\Delta a_i)^2\right) \quad (68)$$

This is equivalent to

$$\epsilon \doteq -\frac{3}{32}\sigma^2(a_{0i}) \quad (69)$$

where $\sigma^2(a_{0i})$ is the variance of the binary interaction energies a_{0i},

$$\sigma^2(a_{0i}) = \frac{1}{l}\sum_{i=1}^{l}(a_i - \langle a_i \rangle) \quad (70)$$

As in the case of the ternary mixtures described above, the deviation of the critical cholesterol composition shown in eq 69 from the average-phospholipid model value is negative. That is, the true critical cholesterol composition must be less than $^1/_2$, regardless of the identity of the lipids present.

By insertion of eq 69 into the expression for a_x in eq 65, the value of this reference interaction energy at the critical point may be determined. Doing this and subtracting the average-phospholipid model expression for a_x gives a deviation from the average-phospholipid model value of

$$a_x^{rs} - a_x^{avg} \doteq -\frac{1}{4}\sigma^2(a_{0i}) \quad (71)$$

For systems in which pressure is the variable of interest, this translates into a deviation of the true critical pressure from the average-phospholipid model value of

$$\pi_c^{rs} - \pi_c^{avg} \doteq \frac{a'}{4}\sigma^2(\pi_c) \quad (72)$$

where $\sigma^2(\pi_c)$ is the variance of the binary critical pressures $\pi_{c(0i)}$ of the constituent phospholipids with cholesterol. In the ternary mixture considered earlier, $l = 2$ and the variance $\sigma^2(\pi_c)$ is equal to $(\Delta\pi_c)^2/4$, which leads to the same result, eq 54, obtained in the previous section.

The foregoing analysis makes the assumption that the pressure dependences of the binary interaction energies of all of the phospholipids with cholesterol, a'_{0i}, are identical. Under this assumption, the difference between (or variance of) the interaction energies is constant over the pressure range of interest. If the a'_{0i} are not equal, or if critical temperatures rather than pressures are of interest, then the variance is not constant. In such cases, it is possible in principle to express the a_{0i} as functions of pressure or temperature and solve for the critical pressure/temperature directly. However, for mixtures in which the binary critical parameters are not widely separated, the variance of the interaction energies does not change greatly over the relevant range of pressure or temperature, and the expressions derived above are therefore still appropriate. In particular, the trends described here, namely, an elevated critical pressure/temperature relative to the average value, a critical cholesterol composition of less than $^1/_2$, and "twisting" of the tie lines of the phase diagram, should apply to most mixtures of cholesterol with multiple phospholipids.

It should be noted that some of the effects described here are small, of the order of experimental errors. Experimental phospholipid−cholesterol phase diagrams have low critical exponents[16] rather than the critical exponent of $^1/_2$ which corresponds to the mean-field approximation used here. This means that the observed phase diagrams have a somewhat flatter shape than the ones shown here. As a result, accurate experimental measurement of the critical cholesterol composition is difficult. Nonetheless, the overall shapes of the phase diagrams calculated using this approximation describe the observed phase diagrams semiquantitatively quite well.[16]

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