

A Thermodynamic Model for Extended Complexes of Cholesterol and Phospholipid

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ABSTRACT Studies of monolayer mixtures of certain phospholipids with cholesterol by epifluorescence microscopy and measurement of cholesterol desorption show evidence for the formation of “condensed complexes.” A thermodynamic model of these complexes has been developed and has been shown to be generally consistent with observed phase diagrams, cholesterol desorption rates, and electric field susceptibility. Previous work has shown that complexes comprising 10–50 molecules provide good agreement with experimental results. The present study examines the calculated properties of complexes containing very large numbers of molecules and extends the condensed complex model to incorporate the formation of complexes of variable size. Trends in equilibrium composition are similar to those calculated for small complexes. Thermal transitions are continuous, with a strong composition dependence of the breadth of the transition. The average number of molecules in a large complex shows a pronounced dependence on the composition of the reaction mixture. Large complexes have properties of a separate thermodynamic phase.

INTRODUCTION

Binary mixtures of cholesterol and phospholipids have been studied extensively as model systems for biological membranes (Finegold, 1993). The nonideality of such mixtures has been demonstrated by diverse measurements, including subadditivity of molecular areas (Leathes, 1925), deviation from ideal melting point depression as measured by differential scanning calorimetry (DSC) (Hinz and Sturtevant, 1972), and phase separation in monolayers (McConnell, 1991). Various thermodynamic models have been advanced to account for the properties of cholesterol/phospholipid mixtures (Ipsen et al., 1987; Thewalt and Bloom, 1992).

In recent work, a model of “condensed complexes” of cholesterol and phospholipids has been proposed to account for unusual properties of mixtures of these molecules in monolayers at the air-water interface (Radhakrishnan and McConnell, 1999a, b). This model has recently been extended (Anderson and McConnell, 2001) to describe DSC results in bilayer mixtures (McMullen et al., 1993). There the condensed complex model was found to describe many of the unusual features of the DSC data, although a spurious calculated heat absorption at low temperatures was a persistent discrepancy.

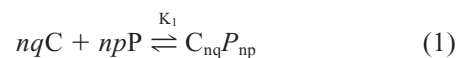
As originally articulated, the condensed complex model involves a reversible reaction between cholesterol (C) and phospholipid (P) to form a supramolecular unit, $C_{nq}P_{np}$, in which q and p are stoichiometry integers and n is a measure of the size of the complex. The parameter n also reflects the cooperativity of complex formation, and in previous studies was taken to be of the order of 3 to 12. This thermodynamic model for complexes has been extremely helpful for inter-

preting the results of experiments involving mixtures of cholesterol and phospholipid. We have found the model to be equally valuable for planning future experiments. The present study was undertaken to explore the consequences of certain extensions of the model. The design in this case has not been to attempt to explain or account for specific experimental results, but rather to examine quantitatively properties of the model for cholesterol-phospholipid interactions not previously considered, in the event that these properties may be useful in guiding future experimental studies.

The present work examines the properties of phospholipid-cholesterol mixtures containing condensed complexes of very large size ($n \gg 10$). Then the condensed complex model is extended to allow for variability of the size parameter n . To keep the analysis as transparent as possible, we make the highly simplifying assumption of ideal mixing of cholesterol, phospholipid, and complex.

THE CONDENSED COMPLEX MODEL

The formation of condensed complexes of cholesterol and phospholipid is represented by the formal chemical reaction



where p and q are stoichiometry integers and n is a measure of complex size and the cooperativity of complex formation. The use of a size parameter distinct from the stoichiometry integers allows the size of the complex in the model to be adjusted independently of the proportion of cholesterol and phospholipid in the complex. The equilibrium constant for complex formation, K_1 , varies with temperature according to

$$K_1(T) = K_1^\circ \exp \left[\frac{\Delta H_1}{k_B} \left(\frac{1}{T^\circ} - \frac{1}{T} \right) \right] \quad (2)$$

where K_1° is the value of the equilibrium constant at the reference temperature $T^\circ = 298$ K and ΔH_1 is the heat of

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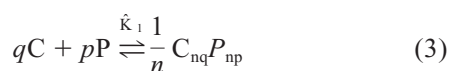
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reaction. In general, the composition of the reaction mixture is expressed in terms of the mole fraction of cholesterol in the mixture before complex formation; this is designated by $x_{C,0}$.

To facilitate comparison between complexes of different sizes, it is convenient to express the reaction (Eq. 1) in a normalized form (Anderson and McConnell, 2001),



The normalized reaction can be thought of as representing the incorporation of a single formula unit “ $C_q P_p$ ” into the complex. The equilibrium constant and heat of reaction for the normalized reaction are $\hat{K}_1 = K_1^{1/n}$ and $\Delta\hat{H}_1 = \Delta H_1/n$, respectively.

The mixture of cholesterol, phospholipid, and condensed complexes is modeled as a regular solution (Hildebrand, 1929), with a free energy G and enthalpy H of

$$G = \sum_i N_i \mu_i^\theta + N k_B T \sum_i x_i \ln x_i + N \sum_{i < j} \alpha_{ij} x_i x_j \quad (4)$$

$$H = \sum_i N_i H_i^\theta + N \sum_{i < j} \alpha_{ij} x_i x_j \quad (5)$$

Here the N_i are the number of moles of cholesterol, phospholipid, and complex; the x_i are the corresponding mole fractions, and N is the total number of moles of all three species. The μ_i^θ and H_i^θ are standard chemical potentials and molar enthalpies of the species, and the α_{ij} are pairwise mean-field interaction terms. The use of $(\ln x)$ terms to express the mixing entropy is a dubious approximation, particularly when the complexes are large. As an alternative, the solution entropy might be expressed in terms of the area fractions of the species (Flory, 1941, 1942; Huggins, 1941, 1942). Without knowledge of the structure of the complexes, however, a more detailed treatment of the entropy of mixing is not warranted. Corrales and Wheeler have shown that the chemical potential of linear structures in a three-dimensional lattice may be expressed in $(\ln x)$ terms (Corrales and Wheeler, 1989).

The standard chemical potentials and molar enthalpies of cholesterol and phospholipid are arbitrarily set equal to zero; the corresponding values for the condensed complex ($C_{nq} P_{np}$, denoted by the subscript X) are then

$$\mu_X^\theta = -k_B T \ln K_1 \quad (6)$$

$$H_X^\theta = \Delta H_1 \quad (7)$$

The composition of a reaction mixture described by Eq. 1 at chemical equilibrium may be found by minimizing the free energy in Eq. 4 with respect to the extent of reaction (Radhakrishnan and McConnell, 1999b). The mole fractions of cholesterol, phospholipid, and complex present at equilibrium vary with the overall composition of the mixture, as

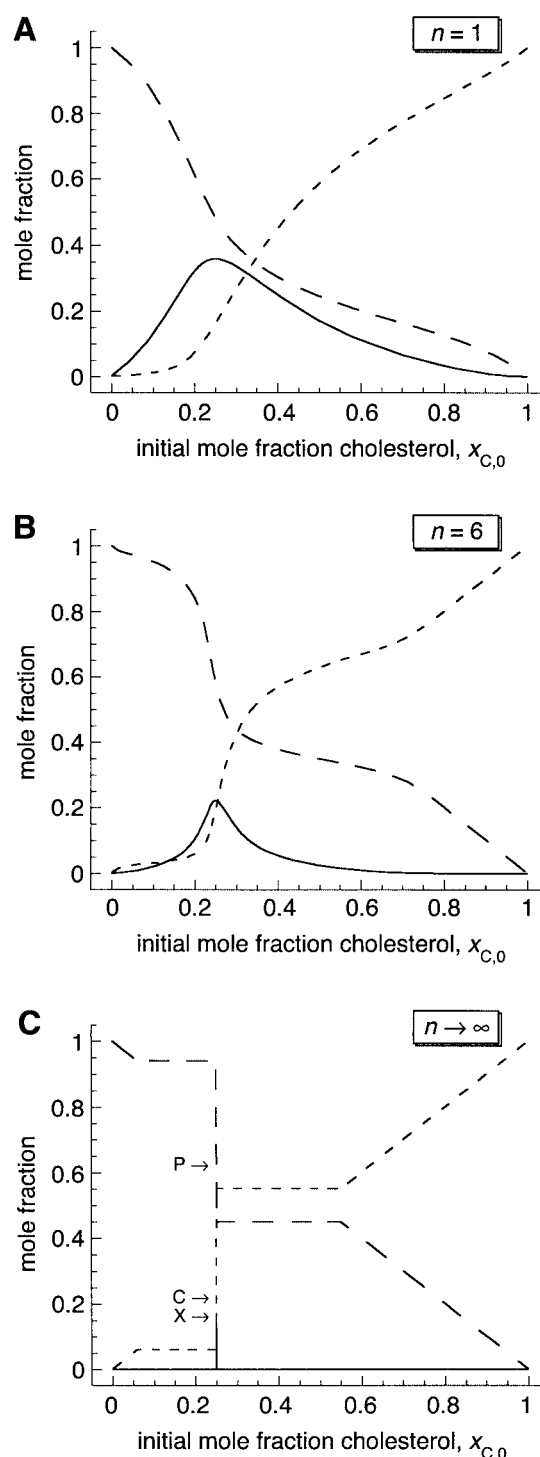


FIGURE 1 Equilibrium mole fractions of free cholesterol (dotted lines), free phospholipid (dashed lines), and condensed complex (solid lines) for a reaction with 3:1 stoichiometry, a normalized equilibrium constant of $\hat{K}_1 = 20$, and a size parameter of (A) $n = 1$, (B) $n = 6$, and (C) the limit of $n \rightarrow \infty$. In C, the mole fractions in a stoichiometric mixture of cholesterol and phospholipid are indicated by arrows.

shown in Fig. 1. The variation is particularly striking in the case of very large complexes (Fig. 1 C); this will be dis-

cussed later. Knowledge of the equilibrium mole fractions is useful for some purposes: for example, the chemical activity of a species is to a first approximation equal to its mole fraction in solution. However, the use of mole fractions tends to be misleading with respect to the relative two-dimensional area occupied by the complexes in a membrane, particularly when n is large. A large complex may be present at a small mole fraction even though it accounts for a large area fraction of the membrane, because the number of complexes is small relative to the number of free cholesterol and phospholipid molecules.

In terms of membrane area, the extent of complex formation is better conveyed by the fraction of cholesterol and phospholipid molecules in the mixture that have reacted to form complex. We designate this the *reactant fraction* of the complex, r_X :

$$r_X \equiv \frac{n(q+p)N_X}{N_0} \quad (8)$$

where N_X is the number of moles of complex and N_0 is the total number of moles of cholesterol and phospholipid before reaction. The reactant fraction of complex is proportional to the extent of the reaction in Eq. 1 (Anderson and McConnell, 2001). Reactant fractions of free cholesterol and phospholipid are similarly defined: $r_C \equiv N_C/N_0$, $r_P \equiv N_P/N_0$. The reactant fractions of cholesterol, phospholipid, and complex are approximately equal to the corresponding area fractions in a two-dimensional membrane; reactant and area fractions are identical in the idealized case of equal molecular areas for cholesterol and phospholipid and no area change upon complex formation.

To express the reactant fraction of complex in terms of the mole fractions of the reacting species, we note that the number of moles of cholesterol and phospholipid consumed to form complex are related by the reaction stoichiometry,

$$\frac{N_{C,0} - N_C}{N_{P,0} - N_P} = \frac{r_{C,0} - r_C}{r_{P,0} - r_P} = \frac{q}{p} \quad (9)$$

The initial reactant fractions $r_{C,0}$ and $r_{P,0}$ are equal to the respective initial mole fractions. Additionally, the ratios of the mole fractions and reactant fractions of cholesterol and phospholipid are equal,

$$\frac{r_C}{r_P} = \frac{x_C}{x_P} \quad (10)$$

Equations 9 and 10 may be solved for r_C and r_P . In conjunction with the relations $x_{C,0} + x_{P,0} = 1$ and $r_C + r_P + r_X = 1$, this leads to the following expression for the equilibrium mole fraction of complex:

$$r_X = \frac{(p+q)(x_{P,0}x_C - x_{C,0}x_P)}{px_C + qx_P} \quad (11)$$

Representative plots of the reactant fractions of cholesterol, phospholipid, and complex at chemical equilibrium are

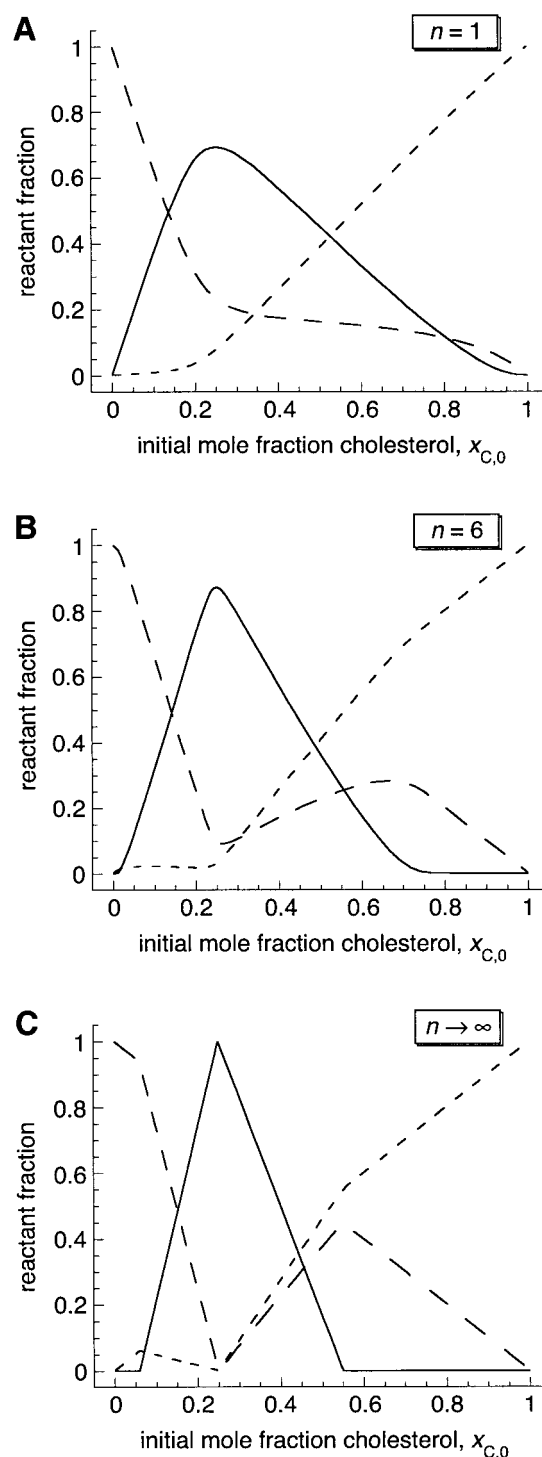


FIGURE 2 Equilibrium reactant fractions of free cholesterol (dotted lines), free phospholipid (dashed lines), and condensed complex (solid lines) corresponding to the reaction mixtures shown in Fig. 1.

shown in Fig. 2. These curves are very different in appearance from the corresponding mole fraction plots in Fig. 1, particularly for large values of n . In both figures, the fraction of complex is peaked at the stoichiometric composition,

$x_{C,0} = q/(q + p)$. The mole fraction of complex falls off much more rapidly to either side of this composition than does the reactant fraction of complex. In the cases of free cholesterol and phospholipid, the equilibrium mole fractions vary monotonically across the range of compositions, whereas for moderate to large n their reactant fractions have local minima at the stoichiometric composition.

In DSC experiments, the absorption of heat by a mixture of cholesterol and phospholipid is measured over a range of temperatures. This heat absorption corresponds to changes in the mixture's excess enthalpy, H^E , which is the difference between the equilibrium enthalpy of the reaction mixture (Eq. 5) and the enthalpy of the separate (unreacted) components. Expressed per mole of reactant, this is in the condensed complex model

$$H_m^E = \frac{1}{N_0} [(N_C H_C^0 + N_P H_P^0 + N_X H_X^0 + H_\alpha) - (N_{C,0} H_C^0 + N_{P,0} H_P^0)] \quad (12)$$

$$= \frac{r_X}{q + p} \Delta \hat{H}_1 + H_\alpha$$

where H_α is the regular-solution enthalpy term (N/N_0) $\sum_{i < j} \alpha_{ij} x_i x_j$. Apart from regular-solution interactions, the excess enthalpy of the reaction mixture is proportional to the reactant fraction of complex.

Assumption of ideal mixing

In the calculations presented here we make the simplifying assumption of ideal mixing of the cholesterol, phospholipid, and complex. This forecloses the possibility of describing phase separation on the basis of regular-solution repulsions. Nevertheless, it affords the great advantage of allowing the thermodynamic properties of the reaction mixture to be expressed in analytical form.

Under the assumption of ideal mixing, the chemical activity of each species is equal to its mole fraction. As a result, the equilibrium mole fraction of complex x_X may be expressed in terms of the reaction equilibrium constant K_1 and the mole fractions of free cholesterol, x_C , and phospholipid, x_P ,

$$x_X = K_1 x_C^{nq} x_P^{np} = (\hat{K}_1 x_C^q x_P^p)^n \quad (13)$$

The equilibrium mole fractions of cholesterol, phospholipid, and complex are also related through the overall composition of the reaction mixture,

$$\frac{x_{C,0}}{x_{P,0}} = \frac{x_C + nq x_X}{x_P + np x_X} \quad (14)$$

Provided that n is finite, Eqs. 13 and 14 may be solved in conjunction with the relation $x_C + x_P + x_X = 1$ to find the equilibrium composition of the reaction mixture.

Formation of very large complexes

To investigate the properties of large complexes ($n \gg 10$), we consider the reaction in the limit of $n \rightarrow \infty$. The equilibrium composition of the mixture is again described by Eq. 13, which may be rearranged to give $\hat{K}_1 x_C^q x_P^p = x_X^{1/n}$. Since the mole fraction of complex must be < 1 , in the limit of $n \rightarrow \infty$ this becomes

$$\hat{K}_1 x_C^q x_P^p = 1 \quad (n \rightarrow \infty) \quad (15)$$

If any excess cholesterol or phospholipid is present in the reaction mixture, the mole fraction of a very large complex will be essentially zero (Fig. 1 C), because the number of complexes present will be very small relative to the excess reactant molecules. As a result, x_P can be replaced by $1 - x_C$ in Eq. 15. The result is a polynomial of order $(p + q)$ with two physically relevant solutions; these correspond to the cases of excess cholesterol and excess phospholipid. The two mixture compositions bound a "reaction zone," outside which no complex is formed (Fig. 2 C). Importantly, the equilibrium mole fractions of cholesterol and phospholipid inside the reaction zone are constant on either side of the stoichiometric composition (Fig. 1 C). For any mixture having an initial composition inside the reaction zone, the complex formation reaction proceeds, changing the mole fractions of cholesterol and phospholipid until Eq. 15 is satisfied.

The equilibrium mole fractions of cholesterol and phospholipid in the $n \rightarrow \infty$ reaction mixture show a discontinuity at the stoichiometric composition, $x_{C,0} = q/(p + q)$ (Fig. 1 C). In practice, it is difficult to achieve an exactly stoichiometric composition. Nevertheless, it is useful to consider such a mixture. For a mixture precisely at the stoichiometric composition, neither reactant is present in excess, which means that the equilibrium mole fraction of complex need not be infinitesimal. In this case, the mole ratio of cholesterol to phospholipid, q/p , is unchanged by the reaction, and we may make the substitutions $x_C = q/p + q(1 - x_{X,\text{stoich}})$ and $x_P = p/p + q(1 - x_{X,\text{stoich}})$. Then the equilibrium condition (Eq. 15) becomes

$$\frac{\hat{K}_1}{J} (1 - x_{X,\text{stoich}})^{p+q} = 1 \quad (16)$$

where J is the numerical factor

$$J \equiv \frac{(p + q)^{p+q}}{p^p q^q} \quad (17)$$

For a complex with 3:1 stoichiometry, $J = 256/27 \approx 9.5$. When the normalized equilibrium constant is less than J , no reaction will occur at the stoichiometric composition (or any other composition). When $\hat{K}_1 > J$, complex will form; its equilibrium mole fraction can be found using Eq. 16. As \hat{K}_1 is increased from below to above J (by changing the tem-

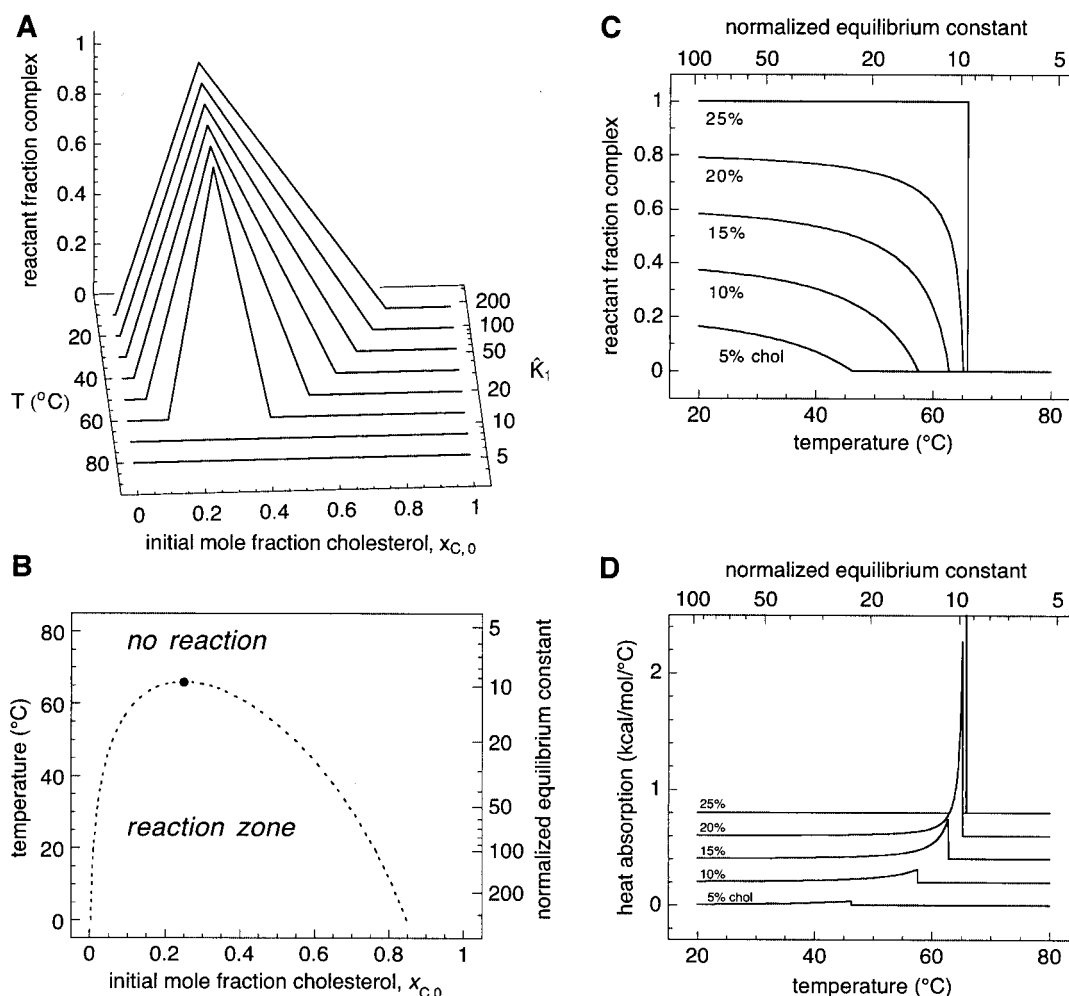


FIGURE 3 Thermal transitions of condensed complex reaction mixtures, for an exothermic reaction with 3:1 stoichiometry and $n \rightarrow \infty$. (A) Equilibrium reactant fraction of complex as a function of mixture composition and temperature. (B) Temperature of the onset of complex formation versus composition. Complex is formed below the dotted line. The black dot indicates a first-order transition at the stoichiometric composition. (C) Extent of complex formation as a function of temperature for mixtures containing 5–25% cholesterol. (D) Heat absorption curves corresponding to the extents of reaction shown in C. The plots have been shifted on the y-axis for clarity. Reaction parameters: $\Delta\hat{H} = -10$ kcal/mol, $\hat{K}_1^\circ = 71.5$.

perature, for example), the mole fraction of complex abruptly increases from zero.

As noted above, in the limit of $n \rightarrow \infty$, the equilibrium mole fraction of complex is zero, so that $x_C + x_P = 1$ (provided that the mixture is not precisely at the stoichiometric composition). In this case, the expression for the equilibrium reactant fraction of complex (Eq. 11) may be simplified to give

$$r_X = \frac{x_C - x_{C,0}}{x_C - \frac{q}{p+q}} \quad (n \rightarrow \infty) \quad (18)$$

As before, $q/(p+q)$ is the stoichiometric composition. Since the equilibrium mole fraction of cholesterol x_C is constant inside the reaction zone when n is infinite, Eq. 18 implies that the reactant fraction of complex varies linearly

with the composition $x_{C,0}$ inside the reaction zone and reaches unity at the stoichiometric composition (Fig. 2 C, solid line).

The size of the reaction zone depends upon the value of the normalized equilibrium constant \hat{K}_1 . When $\hat{K}_1 = J$, the reaction zone is confined to the stoichiometric composition. As \hat{K}_1 is increased beyond J , the reaction zone grows outward, eventually spanning the entire composition range in the limit of $\hat{K}_1 \rightarrow \infty$. This is illustrated for exothermic complex formation in Fig. 3, A and B. As a nonstoichiometric reaction mixture at a high temperature is cooled, the reactant fraction of complex is zero until \hat{K}_1 reaches a transition value, beyond which the reactant fraction of complex increases gradually (Fig. 3 C). This is not a second-order phase transition, however; we return to this point later. The transition is sharper for reaction mixtures that are closer

to the stoichiometric composition. At the stoichiometric composition itself, the transition is first-order: as soon as \hat{K}_1 reaches the value of J , all of the molecules of cholesterol and phospholipid react and the reactant fraction of complex jumps abruptly from 0 to 1. The formation of an infinitely large complex from a stoichiometric mixture of cholesterol and phospholipid is analogous to a crystallization process, in a thermodynamic sense.

Because the excess enthalpy of the ideal-solution reaction mixture is proportional to the reactant fraction of complex (Eq. 12), the variation of the excess enthalpy of a condensed complex reaction mixture with temperature resembles the r_X curves in Fig. 3 C. For exothermic complex formation, this leads to heat absorption curves with sharp peaks that tail toward low temperatures (Fig. 3 D). At the stoichiometric composition, the heat absorption is contained in an infinitely narrow peak. The reaction of cholesterol and phospholipid to form complex thus appears as a thermal transition of the mixture. Integration of the heat capacity over the entire temperature range gives the transition enthalpy, H^\ddagger . This is equal to the normalized heat of reaction multiplied by the maximum reaction extent. For stoichiometric mixtures, the transition enthalpy is $\Delta\hat{H}_1/(p + q)$ per mole of reactant. For nonstoichiometric mixtures, the transition enthalpy is $\Delta\hat{H}_1x_{C,0}/q$ when cholesterol is the limiting reactant and $\Delta\hat{H}_1x_{P,0}/p$ when phospholipid is limiting. In the literature, transition enthalpy values in DSC experiments are often reported per mole of *phospholipid*; this is obtained from the above expressions by dividing by $x_{P,0}$.

Free energy of the condensed complex reaction mixture

The free energy of a mixture of cholesterol, phospholipid, and complex is given by Eq. 4. Using the standard chemical potential for the complex given in Eq. 6 and the equilibrium mole fraction of complex in Eq. 13, the equilibrium free energy of the reaction mixture is

$$G = k_B T [N_C \ln x_C + N_P \ln x_P + N_X (-\ln \hat{K}_1^n + \ln(\hat{K}_1 x_C^q x_P^p))] \quad (19)$$

This expression may be rearranged into

$$G = k_B T [(N_C + nqN_X) \ln x_C + (N_P + npN_X) \ln x_P] \quad (20)$$

The terms in parentheses are equal to the initial number of moles of cholesterol, $N_{C,0}$, and phospholipid, $N_{P,0}$, respectively. Making these substitutions and dividing through by the total initial number of moles N_0 gives the free energy per mole of reactant,

$$G_m = k_B T (x_{C,0} \ln x_C + x_{P,0} \ln x_P) \quad (21)$$

Free energy plots for representative condensed complex reaction mixtures are shown in Fig. 4. The free energy of a

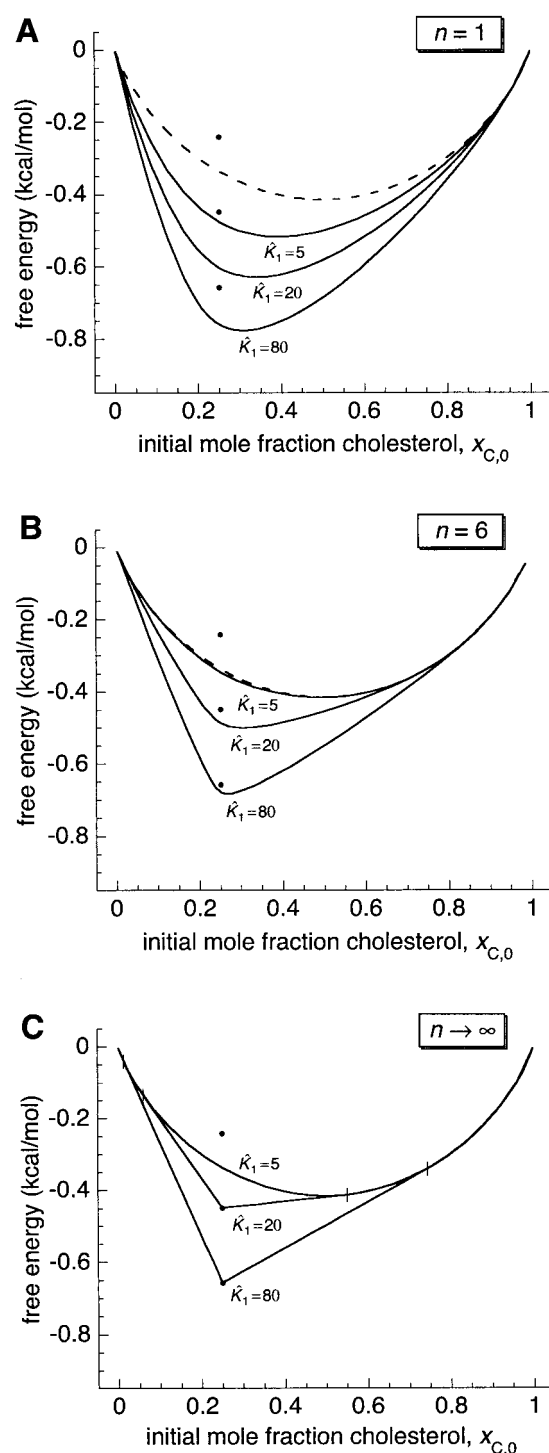


FIGURE 4 Free energy at T° for 3:1 complex reaction mixtures with size parameters of (A) $n = 1$, (B) $n = 6$, and (C) the limit of $n \rightarrow \infty$. Three curves are shown in each case, corresponding to normalized equilibrium constants of $\hat{K}_1 = 5, 20$, and 80 . Dots indicate the free energy of hypothetical pure complex phases for the three values of \hat{K}_1 . The free energy of the mixture in the absence of complex formation is shown by a dotted line. In the case of $n \rightarrow \infty$, no complex is formed when $\hat{K}_1 = 5$ (see text) and the equilibrium and no-reaction lines coincide; complex formation is restricted to a reaction zone for $\hat{K}_1 = 20$ and 80 (delineated by short vertical lines). The curves for $\hat{K}_1 = 20$ correspond to the mixtures shown in Figs. 1 and 2.

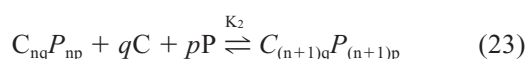
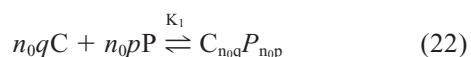
pure complex phase is $\mu_X^\theta/(p + q)$; this is illustrated by the black dots in Fig. 4.

In the absence of mean-field interactions, the shape of the free energy curve is largely determined by the entropy of mixing. When the size of the complexes is large, there is less curvature in the free energy because the number of molecules present at equilibrium—and hence the entropy of mixing—is small. In the limit of $n \rightarrow \infty$, the curvature vanishes and the free energy is linear inside the reaction zone (bounded by vertical bars in Fig. 4 C). This follows from Eq. 21 and the flat shape of the cholesterol and phospholipid mole fraction curves (Fig. 1 C). In this respect, the $n \rightarrow \infty$ reaction mixture is equivalent to a mixture of two coexisting thermodynamic phases: a solution phase having a composition at the edge of the reaction zone, and a pure complex phase.

The linearity of the free energy curve for large values of n suggests that such reaction mixtures may be particularly susceptible to phase separation if repulsive mean-field interactions are present. This point will be developed in future work.

Complexes of variable size: nucleation and growth

In the preceding analysis, the size parameter n was taken to be fixed. Now we extend the condensed complex model to encompass mixtures of various complex sizes. Letting n be variable, the reaction of free cholesterol and phospholipid to form complex is represented by the series of reactions



The first reaction (Eq. 22) represents a nucleation step in which a small complex of size n_0 is formed. The second reaction (Eq. 23) represents incremental growth of the complex by addition of a “ $C_q P_p$ ” unit. The stoichiometric ratio q/p is held to be fixed for all values of n . Complexes of a size smaller than n_0 are not permitted in this model; the role of smaller complexes is encapsulated in the values of n_0 and K_1 . To simplify the notation, $X(n)$ is used to denote the complex $C_{nq} P_{np}$ containing n formula units “ $C_q P_p$.”

The nucleation step (Eq. 22) may be written in normalized form, in analogy with Eq. 3. The reaction mixture is again taken to be a regular solution with free energy and enthalpy described by Eqs. 4 and 5. The standard chemical potentials and molar enthalpies of the complexes are

$$\mu_{X(n)}^\theta = -k_B T (n_0 \ln \hat{K}_1 + (n - n_0) \ln K_2) \quad (24)$$

$$H_{X(n)}^\theta = n_0 \Delta \hat{H}_1 + (n - n_0) \Delta H_2 \quad (25)$$

The complexes in this version of the model span a range of sizes at equilibrium. The population of complexes may

usefully be described by two parameters, the average size of the complexes,

$$\langle n \rangle \equiv \frac{\sum_{n=n_0}^{\infty} n X_{X(n)}}{\sum_{n=n_0}^{\infty} X_{X(n)}} \quad (26)$$

and the total reactant fraction of all complexes,

$$r_{X(\text{tot})} = \sum_{n=n_0}^{\infty} r_{X(n)} \quad (27)$$

The total reactant fraction of complex may be considered as an overall extent of complex formation. It has the same form as in the fixed- n model (Eq. 11).

Retaining the ideal-mixing simplification, the equilibrium mole fraction of a particular complex of size n is related to the mole fractions of free cholesterol and phospholipid by

$$X_{X(n)} = \hat{K}_1 K_2^{n-n_0} x_C^{nq} x_P^{np} \quad (28)$$

Eq. 28 places an important constraint on the composition of the mixture, because the ratio of the mole fractions of successive complexes is

$$\frac{X_{X(n+1)}}{X_{X(n)}} = K_2 x_C^q x_P^p \quad (29)$$

This ratio cannot be greater than unity, because that would lead to mole fractions >1 for sufficiently large values of n . Taking the ratio in Eq. 29 to be ≤ 1 , the total mole fraction of all complexes may be found by summing Eq. 28 from $n = n_0$ to infinity. This geometric series is equal to

$$X_{X(\text{tot})} = \frac{(\hat{K}_1 x_C^q x_P^p)^{n_0}}{1 - K_2 x_C^q x_P^p} \quad (30)$$

Using the relation $x_C + x_P + X_{X(\text{tot})} = 1$, the total mole fraction of complex can be eliminated from Eq. 30 to give

$$(\hat{K}_1 x_C^q x_P^p)^{n_0} = (1 - x_C - x_P)(1 - K_2 x_C^q x_P^p) \quad (31)$$

Furthermore, the average value of n in the reaction mixture is found by combining Eqs. 26 and 28, which leads to

$$\langle n \rangle = n_0 + \frac{K_2 x_C^q x_P^p}{1 - K_2 x_C^q x_P^p} \quad (32)$$

The equilibrium composition of the reaction mixture is again constrained by stoichiometry according to Eq. 14, but with $\langle n \rangle$ in place of n . Equations 14 and 31 may be solved simultaneously to find the equilibrium composition of the reaction mixture. Representative composition plots for reaction mixtures with variable complex size are shown in Fig. 5. The mole fraction and reactant fraction curves are broadly similar to those in the fixed- n model (Figs. 1 and 2). In analogy with the fixed- n model, the features of the composition curves become sharper when n_0 is increased (not shown). The curves also

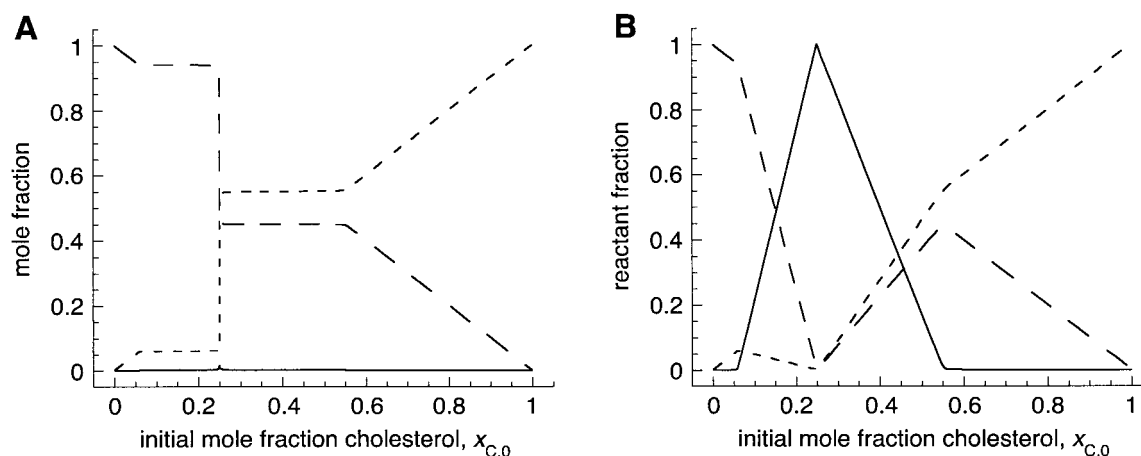


FIGURE 5 Composition of a 3:1 reaction mixture with variable complex size. (A) Equilibrium mole fractions of free cholesterol (dotted lines), free phospholipid (dashed lines), and condensed complexes of all sizes (solid lines). (B) Equilibrium reactant fractions corresponding to part A. Reaction parameters: $n_0 = 6$, $\hat{K}_1 = 2$, $K_2 = 20$.

sharpen when the normalized nucleation equilibrium constant \hat{K}_1 is made smaller (not shown).

It is instructive to consider the case of the initial nucleation step (Eq. 22) being highly unfavorable. In the limit of $\hat{K}_1 \rightarrow 0$, Eq. 31 has two solutions: $x_C + x_P = 1$ (no reaction occurs) and $K_2 x_C^q x_P^p = 1$. The second solution corresponds to a situation in which the mole fractions of successively larger complexes are equal; this provides the driving force to overcome the unfavorable nucleation step. In this case, the average size of the complexes (Eq. 32) is infinite, and the reaction is equivalent to the fixed- n model in the limit of $n \rightarrow \infty$.

As an alternative to the average size parameter $\langle n \rangle$, the ensemble of complexes may be described by a growth parameter g ,

$$g \equiv \frac{\langle n \rangle - n_0}{\langle n \rangle} \quad (33)$$

which represents the extent to which the average complex size exceeds the minimum size n_0 . This parameter ranges from 0 (when $\langle n \rangle = n_0$) to 1 (when $\langle n \rangle = \infty$).

For nonzero values of \hat{K}_1 , the average complex size is finite and displays a strong composition dependence (Fig. 6). Outside the reaction zone, very little complex is formed, i.e., $r_{X(\text{tot})} \approx 0$ (Fig. 5 B, solid line) and the small amount of complex that is formed is of minimal size ($\langle n \rangle \approx n_0$; $g \approx 0$). On entering the reaction zone, the average size of the complexes increases dramatically (Fig. 6, top), and the growth parameter g rises close to 1 (Fig. 6, bottom). Inside the reaction zone, as the stoichiometric composition is approached the average size of the complexes increases rapidly and g remains close to 1. The plot of $\langle n \rangle$ versus mixture composition has a characteristic shape that resembles an ogee arch, with a sharp peak at the stoichiometric composition flanked by sloping shoulders that extend to the edges of the reaction zone. This pattern is a consequence of

dilution of the condensed complex upon addition of excess cholesterol or phospholipid. This dilution leads to a reversal of the growth reactions (Eq. 23) by Le Chatelier's principle.

The excess enthalpy of the mixture, described by Eq. 12 in the case of fixed n , is now

$$H_m^E = \frac{r_{X(\text{tot})}}{(p + q)\langle n \rangle} [n_0 \Delta \hat{H}_1 + (\langle n \rangle - n_0) \Delta H_2] \quad (34)$$

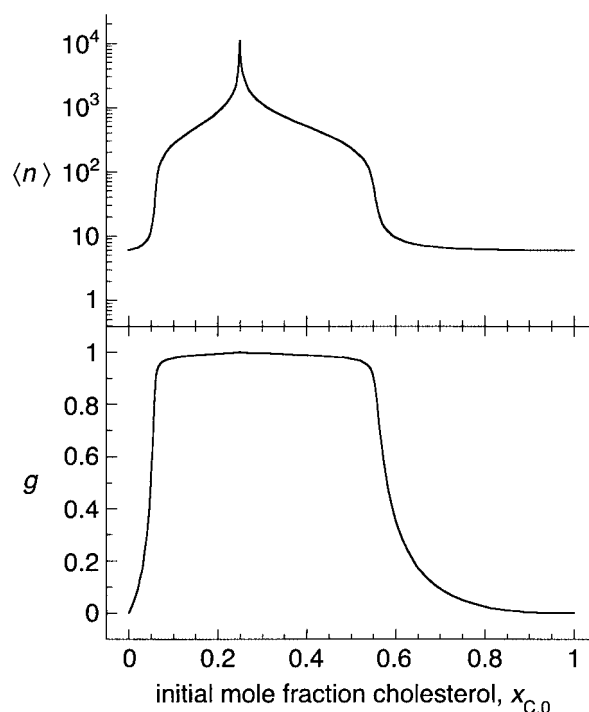


FIGURE 6 Calculated average size $\langle n \rangle$ (top) and growth function g (bottom) for a representative mixture of 3:1 variable-size complexes as functions of the composition of the reaction mixture. For this calculation, $n_0 = 6$ and the equilibrium constants are $\hat{K}_1 = 2$ and $K_2 = 20$.

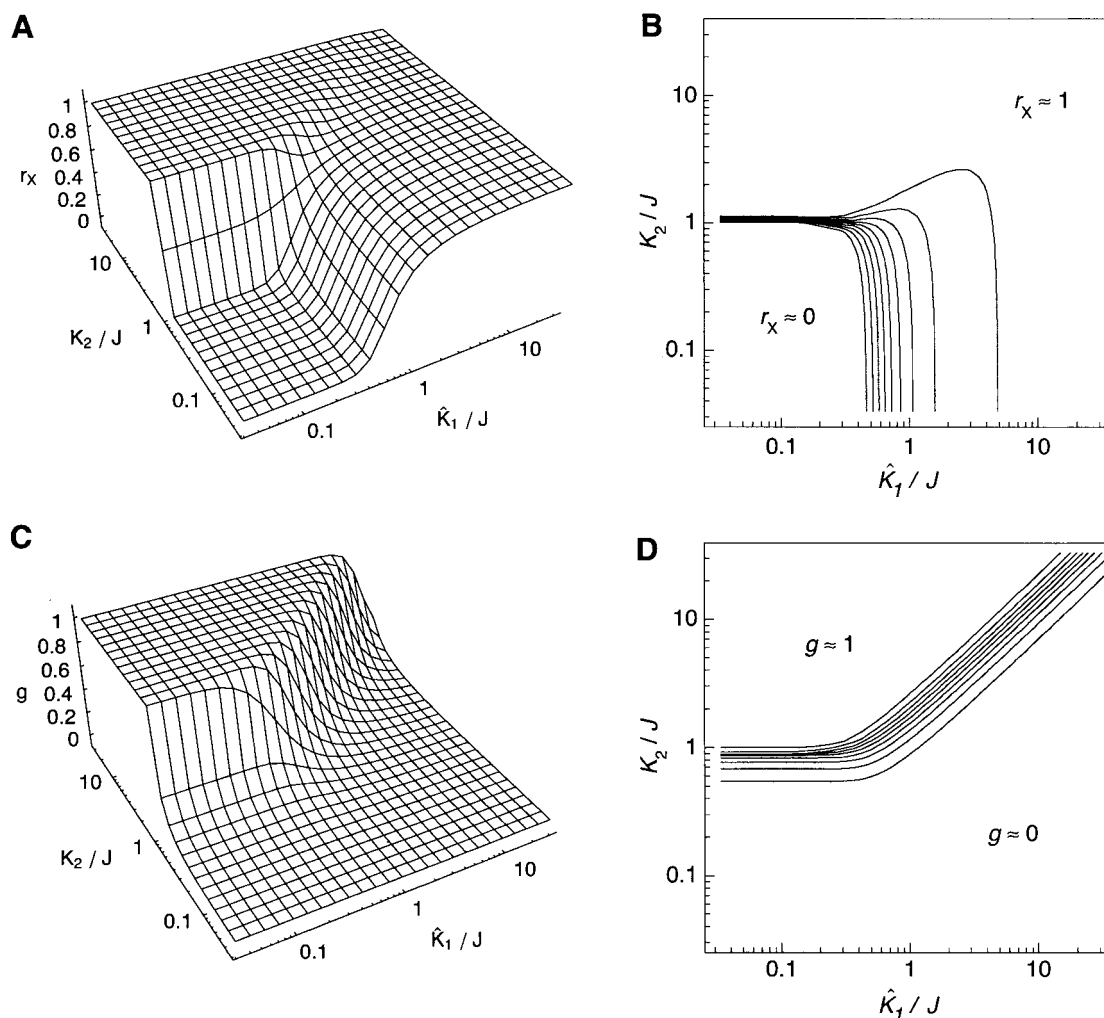


FIGURE 7 Surface and contour plots of the extents of variable-size complex formation and growth as a function of the equilibrium constants \hat{K}_1 and K_2 . Calculations are for a 3:1 stoichiometric mixture of phospholipid and cholesterol with a minimum size of $n_0 = 6$. The reference value J is ~ 9.48 . (A and B) Total reactant fraction of all complexes, $r_{X(\text{tot})}$. (C and D) Complex growth function, g . Note the logarithmic scales for the equilibrium constants.

In terms of the growth parameter g , the excess enthalpy of the reaction mixture is

$$H_m^E = \frac{r_{X(\text{tot})}}{p + q} [(1 - g)\Delta\hat{H}_1 + g\Delta H_2] \quad (35)$$

From Eq. 35 it is evident that the excess enthalpy of the reaction mixture is determined by both the total reactant fraction of the complexes $r_{X(\text{tot})}$ and the extent of complex growth g (provided that $\Delta\hat{H}_1 \neq \Delta H_2$; if the two heats of reaction are equal, the excess enthalpy reduces to Eq. 12).

The reactant fraction of complex and the extent of complex growth are both functions of the equilibrium constants \hat{K}_1 and K_2 ; this is illustrated in Fig. 7 for a mixture at the stoichiometric composition. For small values of \hat{K}_1 and K_2 , very little complex forms ($r_X \approx 0$) and what little complex is present is of minimal size ($g \approx 0$). When one or both

equilibrium constants is large, the extent of complex formation is high ($r_X \approx 1$), and if K_2 is also larger than \hat{K}_1 , then the complexes are of large size ($g \approx 1$). This pattern is summarized schematically in Fig. 8. The regions of essentially no reaction, the presence of small complexes, and the presence of large complexes are labeled NR, S, and L, respectively. At a particular temperature, the values of \hat{K}_1 and K_2 correspond to a point on this plot. If the temperature is changed, both equilibrium constants will change, moving to a new point on the plot. Provided that the equilibrium constants depend upon the temperature according to Eq. 2, this motion is along a straight line, the slope of which is $\Delta H_2/\Delta\hat{H}_1$.

If the equilibrium constants move from one region of Fig. 8 into another, a transition will occur in the reaction mixture. This is illustrated for six representative examples in Fig. 9. Depending upon the values of the reaction parameters, the mixture may undergo either one (Fig. 9 A, C, E) or

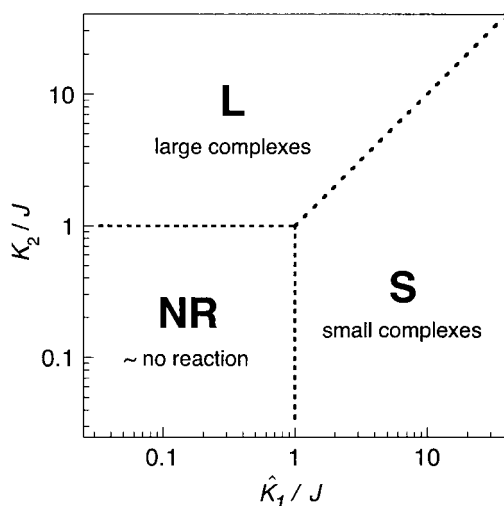


FIGURE 8 Schematic representation of the equilibrium constant parameter space, showing regions in which essentially no reaction occurs (NR), small complexes are present (S), and large complexes are present (L).

two (Fig. 9 *B, D, F*) thermal transitions, and each transition corresponds to one of three types.

In the first type of thermal transition ($\text{NR} \leftrightarrow \text{S}$), small complexes form from or dissociate into a mixture of free phospholipid and cholesterol. This type of transition occurs when K_2 is small with respect to J , and is essentially the same as the transition described for the fixed- n model. In this transition, the total reactant fraction of complex changes while the growth parameter g remains close to zero (Fig. 9 *A* and the high-temperature transitions in Fig. 9, *D* and *F*). The enthalpy change associated with this transition is $(p + q)|\Delta\hat{H}_1|$ for a mixture at the stoichiometric composition.

The second type of transition ($\text{S} \leftrightarrow \text{L}$) is between small and large complexes. In this case, the total reactant fraction of complex remains high while the growth parameter g changes (Fig. 9 *E* and the low-temperature transitions in Fig. 9 *B* and *D*). This transition has an associated enthalpy change of $(p + q)|\Delta H_2 - \Delta\hat{H}_1|$ for a stoichiometric mixture.

The third type of transition ($\text{L} \leftrightarrow \text{NR}$) is between large complexes and free cholesterol and phospholipid. In this transition, which tends to be much sharper than the other two, both the total reactant fraction of complex and the growth parameter g change (Fig. 9 *C*, the high-temperature transition in Fig. 9 *B*, and the low-temperature transition in Fig. 9 *F*). The enthalpy change for this transition is $(p + q)|\Delta H_2|$ at the stoichiometric composition.

DISCUSSION

The model of extended complexes of cholesterol and phospholipids described here was inspired by earlier studies of the polymerization of sulfur. Tobolsky and Eisenberg developed a phenomenological model for the formation of

sulfur polymers, using separate equilibrium constants for ring opening and chain growth (Tobolsky and Eisenberg, 1959). Scott elaborated on this work by deriving a more general model to describe phase behavior in solutions of sulfur polymers (Scott, 1965). Further theoretical work on sulfur solutions using a lattice model was performed by Corrales and Wheeler (1989).

In our thermodynamic model, no assumption is made as to the structure of the condensed complex. In a two-dimensional membrane, possible structures are limited to two broad categories. In the first, the complex is constructed as a one-dimensional chain of phospholipid and cholesterol molecules, alternating according to the p/q stoichiometry, in analogy with polymeric sulfur (Tobolsky and Eisenberg, 1959; Corrales and Wheeler, 1989). In the second category, the complex has a two-dimensional structure with at least short-range compositional order dictated by stoichiometry. Note that a one-dimensional chain might fold on itself to form a two-dimensional structure, having characteristics of both.

The model implies stoichiometric intermediate-range ordering. It is likely that lattice models or other formulations can describe similar effects (Corrales and Wheeler, 1989). The chemical reaction formalism implies that the condensed complex is physically distinct from uncomplexed cholesterol and phospholipid. This is not the case, however, in the quasi-chemical solution method, a lattice model in which the energy of mixing is modeled such that interactions between like and unlike nearest neighbors are related in a way that resembles the mass-action law of chemical reactions (Rice, 1967; Hillert, 1998). Because the mixing of cholesterol, phospholipid, and complex is assumed to be ideal, phase separation using mean-field repulsions is not described by the model. Even so, complexes of very large size are a de facto separate phase for complexes that are two-dimensional. One-dimensional complexes are polymers and may or may not form a separate phase. In either case, in the limit of $n \rightarrow \infty$ the reactant fraction of complex (Fig. 2 *C*) and free energy (Fig. 4 *C*) inside the reaction zone are equivalent to those of a mixture of coexisting phases corresponding to pure complex and a mixture of free cholesterol and phospholipid with a composition at the boundary of the reaction zone (Fig. 10 *A*). The linearity of the free energy curve on either side of the stoichiometric composition for mixtures with large n (Fig. 4 *C*) indicates that these mixtures are susceptible to phase separation if mean-field repulsions are introduced. Furthermore, the phase diagram is not symmetric. Fig. 2 *C* shows that the distribution of complex, free cholesterol, and free phospholipid is different for the cases of excess phospholipid versus excess cholesterol. Varying degrees of repulsion among the coexisting phases would therefore be expected to have asymmetric effects vis-a-vis phase separation on either side of the stoichiometric composition. Experimental phase diagrams ob-

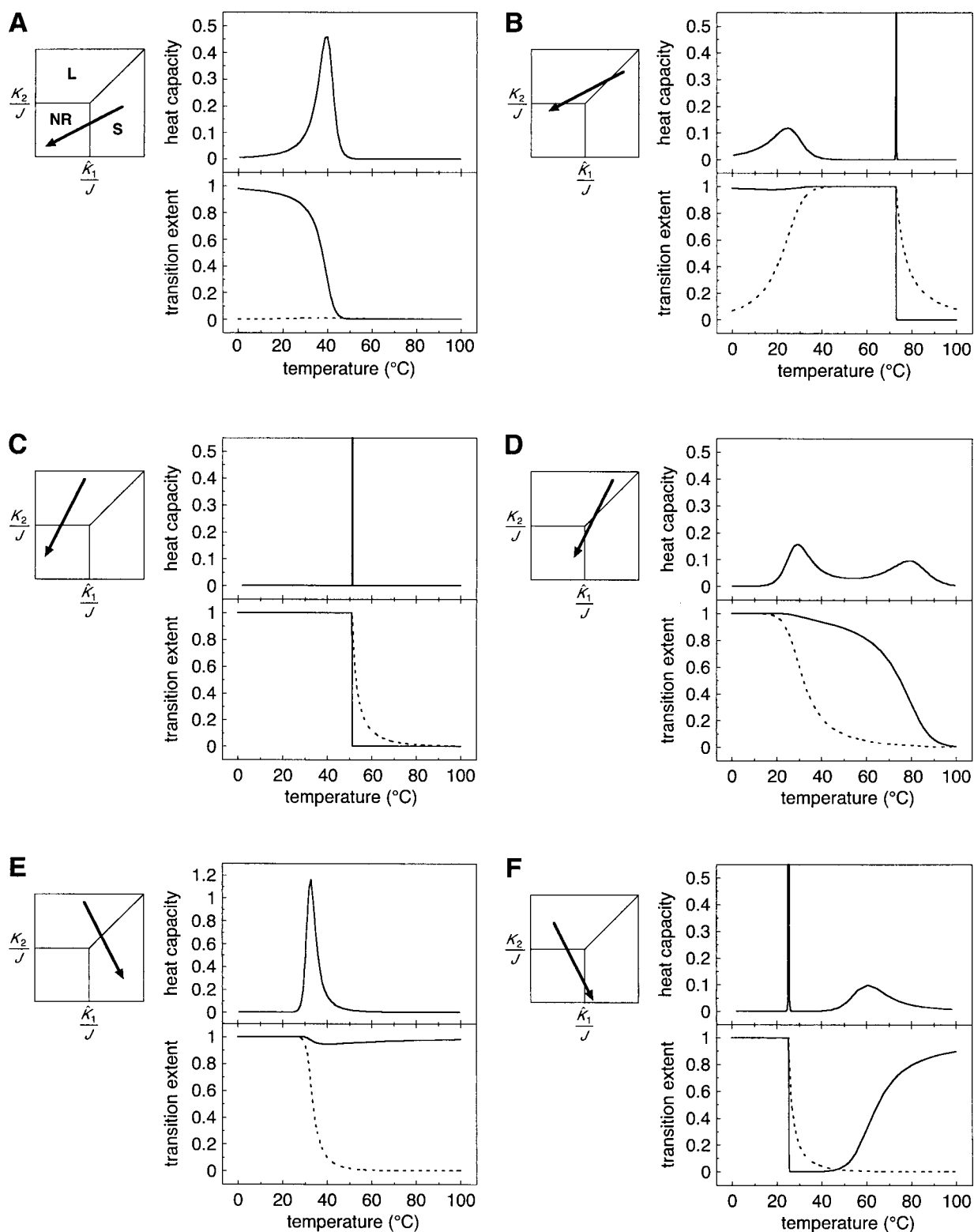


FIGURE 9 Thermal transitions in the variable size model for representative sets of reaction parameters. For each case, the equilibrium constants are plotted parametrically at left in the manner of Fig. 8, with an arrow indicating the values taken by K_1 and K_2 as the temperature is increased from 0 to 100°C. At right are plots of (top) heat absorption and (bottom) the extent of the transition in terms of total reactant fraction of complex $r_{X(tot)}$ (solid line) and the growth parameter g (dotted line). In units of kcal/mol, the heats of reaction used are: (A and B) $\Delta\hat{H}_1 = -20$, $\Delta\hat{H}_2 = -10$; (C and D) $\Delta\hat{H}_1 = -10$, $\Delta\hat{H}_2 = -20$; (E and F) $\Delta\hat{H}_1 = +10$, $\Delta\hat{H}_2 = -20$. For each set of heats of reaction, the values of the standard equilibrium constants K_1° and K_2° were chosen so as to produce one (A, C, E) or two (B, D, F) thermal transitions. All calculations are for a 3:1 stoichiometric mixture with a minimum complex size of $n_0 = 6$.

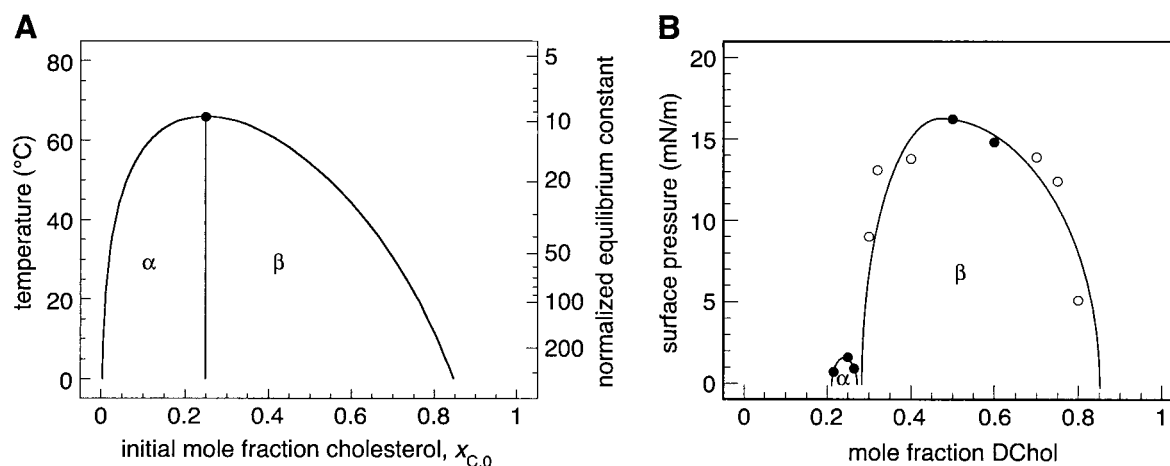


FIGURE 10 Comparison of model and experimental phase diagrams. (A) Phase diagram representation of the reaction mixture shown in Fig. 3. Below the critical point (black dot), pure complex coexists with a phospholipid-rich liquid phase (α) or a cholesterol-rich liquid phase (β). (B) Experimental phase diagram for a monolayer mixture of dihydrocholesterol and dimyristoylphosphatidylserine, showing two distinct regions of two-phase coexistence; black dots are in proximity to critical points (adapted from Radhakrishnan and McConnell, 1999b).

tained from lipid monolayers exhibit strong asymmetry (Fig. 10 B).

An analogy may be made between condensed complex formation in the limit of $n \rightarrow \infty$ and the freezing of a liquid solution. At the stoichiometric composition, when the equilibrium constant (\hat{K}_1 in the single- n model; K_2 in the variable- n model with $\hat{K}_1 = 0$) is increased above J , all of the cholesterol and phospholipid molecules are consumed abruptly to form complex in a first-order transition (black dot in Fig. 3 B). In the presence of excess phospholipid or cholesterol, as the reaction transition line is crossed (dotted line in Fig. 3 B), small amounts of cholesterol and phospholipid react in the stoichiometric ratio to form complex initially, leaving a solution slightly enriched in the excess reactant. As the equilibrium constant is raised further by lowering the temperature, additional complex is formed (that is, r_X increases), further enriching the surrounding solution in the excess reactant. This continues until all of the limiting reactant is consumed. Similar behavior is observed when a liquid containing a dissolved solute is cooled below its (depressed) freezing point.

In the calculations, the formation of large complexes is strongly exothermic, with a heat of reaction in the range of -10 to -20 kcal/mol per stoichiometric unit. This is comparable to the reaction enthalpy inferred from DSC studies of bilayer mixtures of cholesterol and phospholipids (Anderson and McConnell, 2001) and measurements of monolayers over a range of temperatures (Radhakrishnan and McConnell, submitted for publication). This similarity indicates that the structures of the two are similar. That is, there may be comparable numbers of similar hydrocarbon chain-hydrocarbon chain interactions in the solid phospholipid phase and the condensed complex. Furthermore, the average molecular area at the stoichiometric composition in

monolayers is often close to the area of cholesterol itself, $\sim 40 \text{ \AA}^2$, indicating that the hydrocarbon chains of the phospholipid are extended and relatively closely packed (Radhakrishnan and McConnell, 2000a). This average area is typically found at both high and low monolayer pressures, including pressures judged to correspond to bilayers (Seelig, 1987).

In the case of the nucleation/growth model of complexes, we have considered nucleation steps that are exothermic (Fig. 9, A–D) and those that are endothermic (Fig. 9, E and F). An endothermic nucleation step could arise from the need to create a boundary between the nascent complex and the surrounding mixture. For coexisting liquid domains in monolayers, line tensions on the order of 2×10^{-7} dyne have been reported (Benvegnu and McConnell, 1993); this translates to a boundary energy of ~ 3 kcal/mol for a 3:1 complex with $n_0 = 6$. In large complexes, the boundary energy is small relative to the total energy of the complex.

As indicated in Fig. 6, the average size of complexes in the variable- n model is strongly dependent on the composition of the mixture. For the representative mixture described by Fig. 6, the average size of the complexes in the mixture, $\langle n \rangle$, is $>10^4$ at the stoichiometric composition, but decreases by more than an order of magnitude within a few mole percent of this composition. This effect is reminiscent of electric-field experiments conducted with cholesterol/phospholipid monolayers, in which the response to the electric field was found to be restricted to a narrow range around the stoichiometric composition (Radhakrishnan and McConnell, 2000b). A complex having 1:3 stoichiometry and $n = 10^4$ would have a length of $\sim 30 \text{ }\mu\text{m}$ in the case of a linear complex, or a diameter of $\sim 0.2 \text{ }\mu\text{m}$ in the case of a round two-dimensional complex. The concept of condensed complexes of large size is potentially related to the

phenomenon of “rafts” reported in cell membranes (Simons and Ikonen, 1997; Brown and London, 2000). If one were to identify complexes of variable size with the lipid component of membrane rafts, the size of the rafts might be exquisitely sensitive to the cholesterol concentration (Fig. 6).

A key piece of evidence for condensed complexes is distinctive jumps in the chemical activity of cholesterol in the vicinity of the stoichiometric composition (Radhakrishnan and McConnell, 2000a). Under the ideal-mixing assumption, the chemical activity of cholesterol is equal to its mole fraction in solution. As in earlier work (Radhakrishnan et al., 2000; Radhakrishnan and McConnell, 2000a,b), the calculated cholesterol mole fraction—and hence activity—exhibits a pronounced jump in the vicinity of the stoichiometric composition (Fig. 1, *B* and *C*, and Fig. 5 *A*).

In the calculations, temperature is used as the independent variable that affects equilibrium constants. Because formation of complexes in monolayers is associated with area condensation (Radhakrishnan and McConnell, 1999a, b), surface pressure also affects equilibrium constants and could be used as the variable. In this case, elevated surface pressures correspond to larger equilibrium constants. In the case of complexes of variable size, it is likely that both the nucleation and higher complexes are stabilized by higher pressures in monolayers. This effect might be tested by measurements of the electric field effect at different pressures.

In previous work (Anderson and McConnell, 2001), an effort was made to fit the single-complex model to experimental DSC results. A persistent problem was the appearance of a spurious “foot” in the calculated temperature-composition phase diagram, corresponding to the decomposition of complex into a mixture of solid phospholipid and cholesterol-rich liquid at temperatures $\sim 20^\circ\text{C}$ below the observed thermal transition. The relative instability of the complex at low temperatures arose because the free energy of the solid phospholipid phase decreased to a greater degree than the free energy of the complex-rich liquid phase as the temperature was lowered.

This effect may be avoided in the variable- n model of condensed complexes. Consider for example the $S \leftrightarrow L$ transition shown in Fig. 9 *E*. In this case, small complexes are present at high temperatures and grow into large complexes when the temperature is lowered below a transition temperature T^\ddagger . At low temperatures, K_2 is large and the free energy of a stoichiometric reaction mixture will be approximately equal to the free energy of the complex (compare with the fixed- n free energy plots in Fig. 4). With the standard chemical potentials of phospholipid and cholesterol set equal to zero, it can be shown that the temperature derivative of the free energy at this composition is

$$\partial G_{\text{stoich}}/\partial T = \Delta S_2 = -(k_B \ln K_2^\ddagger + \Delta H_2/T^\ddagger)/(p + q) \quad (36)$$

where ΔS_2 is the entropy change associated with the complex growth step and K_2^\ddagger is the value of the equilibrium constant K_2 at the transition temperature. By comparison, the rate of change in the free energy of the solid phospholipid with temperature is related to the enthalpy, entropy, and temperature of fusion by

$$\partial G_{P(s)}/\partial T = \Delta S_{\text{fus}} = \Delta H_{\text{fus}}/T_{\text{fus}} \quad (37)$$

Since the enthalpy of the $S \leftrightarrow L$ transition is proportional to $|\Delta H_2 - \Delta \hat{H}_1|$, the value of ΔH_2 may be adjusted freely so long as the difference between the ΔH values is preserved to fit the measured transition enthalpy H^\ddagger . The transition equilibrium constant K_2^\ddagger is similarly flexible. By setting these parameters appropriately, the problem of complex destabilization relative to a mixture of solid phospholipid and cholesterol-rich liquid at low temperatures may be avoided.

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