Chemical Activity of Cholesterol in Membranes†

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Received March 6, 2000; Revised Manuscript Received May 3, 2000

ABSTRACT: Measurements are reported for the rate constants for the release of cholesterol (and dihydrocholesterol) to β-cyclodextrin from mixtures with phospholipids in homogeneous monolayers at constant pressure at the air−water interface. In each mixture, it is found that the release rate shows a sharp decrease as the cholesterol concentration in the monolayer decreases through a composition corresponding to the stoichiometry of a cholesterol−phospholipid complex. The stoichiometry of the complex was established previously by the position of a sharp cusp in the thermodynamic phase diagram of each mixture and also by a minimum in average molecular area versus composition measurements. A theoretical model used earlier to account for the phase diagrams predicts the chemical potential and chemical activity of cholesterol in these mixtures. The calculated chemical activity also shows a sharp change at the complex stoichiometry in homogeneous monolayers. The similarities in change of observed release rate and calculated chemical activity are expected from reaction rate theory where the release rate is proportional to the cholesterol chemical activity. The chemical activity of cholesterol as determined by complex formation between some phospholipids and cholesterol in the plasma membrane of cells may serve a regulatory function with respect to intracellular cholesterol transport and biosynthesis.

There are a number of theoretical calculations of the properties of liquid mixtures where the chemical components undergo reversible chemical reactions with one another. For example, liquid A reacts reversibly with liquid B to produce a third liquid, C. Under conditions where A, B, and C tend to be immiscible with one another, a reacting mixture of this type is predicted to have an unusual phase diagram with pairs of upper miscibility critical points (*1*−*4*). This model of reacting liquids has provided an explanation for a recent discovery of pairs of upper miscibility critical points in liquid monolayers of Chol[1] (or DChol) and phospholipids at the air−water interface (*5*, *6*). That is, this unusual thermodynamic phase behavior can be interpreted by a model in which cholesterol and certain phospholipids undergo a reversible reaction to form a liquid complex of cholesterol and phospholipid. In monolayers at low pressures, the liquid complex is immiscible with a phospholipid-rich liquid and is also immiscible with a cholesterol-rich liquid. A sharp cusp seen in the phase diagrams between the two regions of immiscibility occurs at a composition corresponding to the stoichiometry of the complex. This stoichiometry is often around 35 or 45 mol % cholesterol, depending on the phospholipids involved. As noted below, these physical−chemical properties may have significant implications with respect to cholesterol homeostasis in cell membranes. The monolayer systems, while being simplified models of membranes, provide an efficient way to dissect molecular interactions that are also relevant in bilayers.

At the lower pressures in cholesterol−phospholipid monolayers, two distinct liquid phases are often present. In the case of two upper miscibility critical points, three liquid phases can be formed. According to the "condensed complex" model (*6*), at the lower cholesterol concentrations, the phases present are mostly liquid phospholipid and the liquid cholesterol−phospholipid complex. At higher cholesterol concentrations, the phases present are mostly liquid complex and liquid cholesterol. As the concentration of cholesterol in the membrane is increased, there is a sharp increase in the concentration of free cholesterol. An important feature of the thermodynamic model is that this switch in free cholesterol concentration also takes place at higher pressures and molecular densities where the monolayer is homogeneous. This condition is appropriate to bilayers and biological membranes. We use the thermodynamic term *chemical activity*, rather than free cholesterol concentration. The two quantities are usually approximately equal to one another. Chemical activity is the same quantity as the classical term *fugacity*, meaning tendency to flee.

We have used β-CD to facilitate the release of Chol (and DChol) from phospholipid−sterol monolayers. β-CD is a cyclic polysaccharide with a polar surface and a hydrophobic cavity. Cyclodextrins have been shown to extract efficiently hydrophobic compounds into polar environments, particularly membrane cholesterol from monolayer membranes (*7*, *8*), red blood cells (*9*), and cultured cells (*10*−*12*). Since β-CD has no surface activity of its own (*7*), it is a useful, nonintrusive probe to study cholesterol−phospholipid inter-

† This work was funded by the NSF and by the NIH Biotechnology Program.

[1] Abbreviations: Chol, cholesterol; DChol, dihydrocholesterol; CD, cyclodextrin; ER, endoplasmic reticulum; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DMPS, dimyristoylphosphatidylserine; SM, sphingomyelin; ACAT, acyl-coenzyme A:cholesterol acyltransferase.

10.1021/bi0005097 CCC: $19.00
Published on Web 06/17/2000

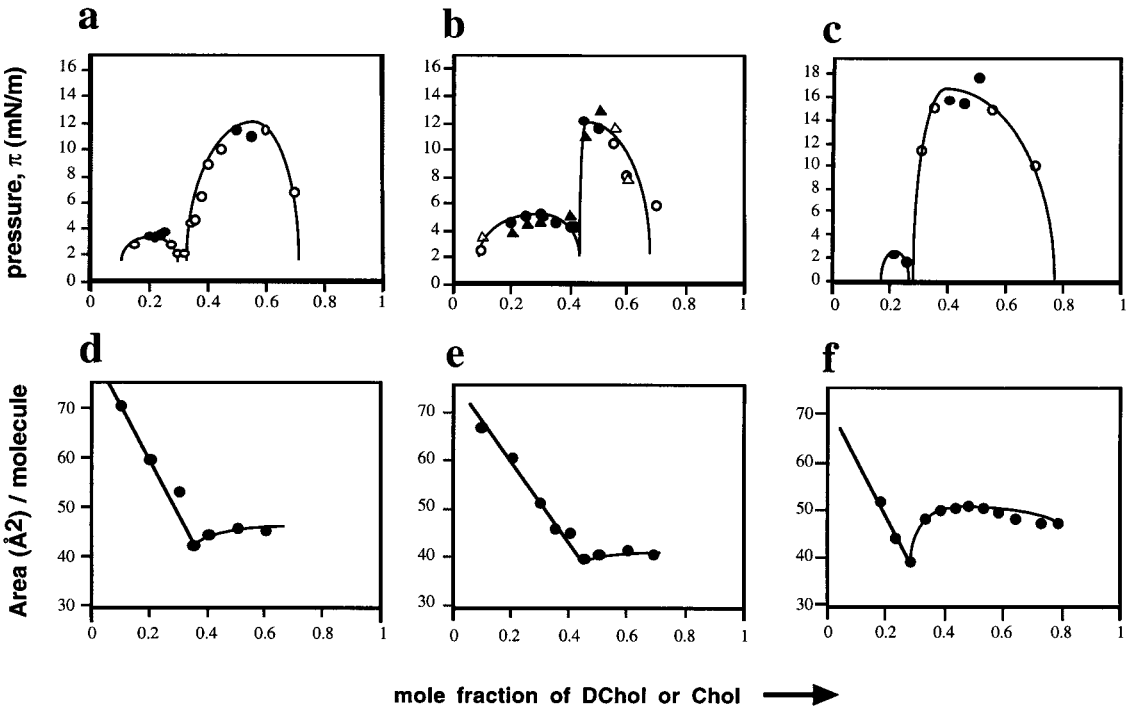

FIGURE 1: Phase diagrams showing liquid−liquid miscibility critical points and average molecular areas for mixtures of DChol or Chol (mole fraction x_0) and phospholipids ($1 - x_0$). Typical errors for these measurements are about 3−8%. (a) The phospholipid is a 2:1 molar mixture of DMPS and DMPC. (b) The phospholipid is a 1:1 molar mixture of DMPC and DPPC. In this case phase diagrams are shown both for Chol (triangles) and for DChol (circles). (c) The phospholipid is egg SM. The plotted data points in the phase diagrams represent the transition pressures that mark the disappearance of two-phase coexistence during monolayer compression and thus define a phase boundary between a two-phase liquid−liquid coexistence region at low pressures and a one-phase liquid region at higher pressures. Stripe superstructures characteristic of a proximity to a critical point (*33*) were observed at the transitions marked by filled circles and filled triangles and not at those marked by open circles and open triangles. (d) Average molecular area for the lipid mixture of (a) at a pressure of 15 mN/m. (e) Average molecular area for the lipid mixture of (b) at a pressure of 20 mN/m. (f) Average molecular area for the lipid mixture of (c) at a pressure of 22 mN/m. The cusp positions in the phase diagrams are the same as those in the molecular area measurements to within the experimental errors for a given phospholipid composition.

actions in monolayer membranes. As noted later, the cholesterol release rate should be a good measure of the chemical activity of cholesterol in the membrane. The experimental method builds on earlier work by Slotte and others (*7*). We find that there is a sharp change in the release rate as a function of monolayer composition, and this change occurs at the condensed complex stoichiometry.

There is evidence that changing the concentration of cholesterol, or that of phospholipids such as sphingomyelin, in the outer plasma membranes of cells affects the rate of cholesterol biosynthesis in the membranes of the ER (*13*−*18*). There is also evidence that the (total) cholesterol concentration in the ER membrane depends sharply on the cholesterol concentration in the plasma membrane (*12*). Thus a switch in the chemical activity of cholesterol in the plasma membranes of cells may serve a regulatory function. A sharp switch in the chemical activity of cholesterol can be related to the model of a reacting mixture of two liquids, A and B, discussed above. Note that, for any mixture, there is either an excess of A or an excess of B, except at the stoichiometric composition. If we start with pure liquid B and add A, much of the free A will be consumed to form complex if the binding constant is large enough and the other thermodynamic parameters are appropriate (*6*). However, as the concentration of A is increased past the stoichiometric concentration, then the concentration of free A, and its chemical potential, can rise rapidly. In our discussion, the roles of A and B are played by liquid cholesterol and liquid phospholipid, both of which are two-dimensional liquids under the conditions of our experiments.

EXPERIMENTAL PROCEDURES

DMPC, DMPS, DPPC, and egg SM were obtained from Avanti Polar Lipids, Alabaster, AL. Chol and DChol were obtained from Sigma, St. Louis, MO. These lipids were used without further purification. All experiments were carried out at room temperature, 23 ± 0.5 °C. Monolayers containing various mixtures of phospholipids and Chol (or DChol) were prepared at the air−water interface of a 9 cm × 3.5 cm Teflon trough with a movable barrier to modulate the surface pressure. These mixtures have been studied before, and their phase behavior has been determined (*6*, *19*). In the desorption measurements, the subphase contained distilled water at pH 5.3 (substitution with phosphate-buffered saline made no significant difference) with 2 mM β-CD (cycloheptaamylose, Sigma, St. Louis, MO). Some experiments were also carried out with lower β-CD concentrations. The desorption of Chol (or DChol) to the subphase was monitored by the decrease in monolayer area required to maintain a constant surface pressure. This surface pressure is chosen so as to be well above the critical pressures in the phase diagrams of the respective mixtures (Figure 1). We have previously determined the mean molecular area of these mixtures as a function of Chol or DChol mole fraction (*6*, *19*). Using this information, one can calculate the amount of cholesterol

removed as a function of time. Cholesterol-containing monolayers undergo detectable air oxidation in 10−20 min. This oxidation results, for instance, in increases in phase transition pressures (*20*). The experiments with cholesterol were carried out with Ar-saturated water in a chamber flooded with Ar gas in order to minimize cholesterol oxidation.

RESULTS

The phase diagrams and average molecular areas for the mixtures used in this work are given in Figure 1. Figure 1a shows the phase diagram of a 2:1 DMPS:DMPC/DChol mixture, Figure 1b shows the phase diagram of a 1:1 DMPC:DPPC/DChol (and Chol) mixture, and Figure 1c shows the phase diagram of an egg SM/DChol mixture. In most of the experiments dihydrocholesterol rather than cholesterol is used so as to minimize possible artifacts due to cholesterol oxidation. Controls using cholesterol were always carried out. For example, in Figure 1b data points are given for both Chol and DChol. We have never detected any significant difference between cholesterol and dihydrocholesterol in the phase diagrams (to within ±1 mN/m) or in average molecular areas. We find the desorption rate of cholesterol is systematically 10−15% higher than dihydrocholesterol, probably due to the more hydrophilic environment in the activated state for steroid release and the slightly more hydrophobic character of dihydrocholesterol.

Figure 2 gives illustrative monolayer area versus time plots for the release of Chol or DChol from the monolayer into β-CD in the subphase. In these experiments the monolayer pressure is kept constant, well above the critical pressures, while the change in monolayer area is monitored. This method is similar to that used previously by Slotte and others (*7*, *21*). The rate of cholesterol release increases with higher β-CD concentrations in the subphase, as reported previously (*7*). Each of these experiments was repeated at least three times with the subphase containing both distilled water and phosphate-buffered saline. It is clear that there is a single pronounced break in slope for each of these curves. As discussed below, these breaks correspond to large changes in the rate constant for cholesterol release. The composition of the membranes at the break in slope was established in two ways. In one method, the initial composition of the monolayer was varied, to find which initial compositions did or did not yield area versus time curves with breaks in slope. This method enabled us to bracket the composition at the break points to within ±5% (data not shown). While this method is not very accurate, the accuracy is good enough to be confident that the breaks in slope are close to the complex stoichiometries implied by the data in Figure 1. A more accurate method is described below. The earlier work by Ohvo and Slotte (*7*) did not examine in detail the lower cholesterol concentration used in the present work, where we observe the sharp break in release rate.

Data Analysis. The experimental data on the release of cholesterol were analyzed as follows. Let A represent the total area of the homogeneous monolayer, N the total number of molecules in the monolayer (cholesterol plus phospholipid), and a the average area per molecule. Data on average areas are given in Figure 1 and in previous work (*6*, *19*). The mole fraction of cholesterol in the sample is x_0. The

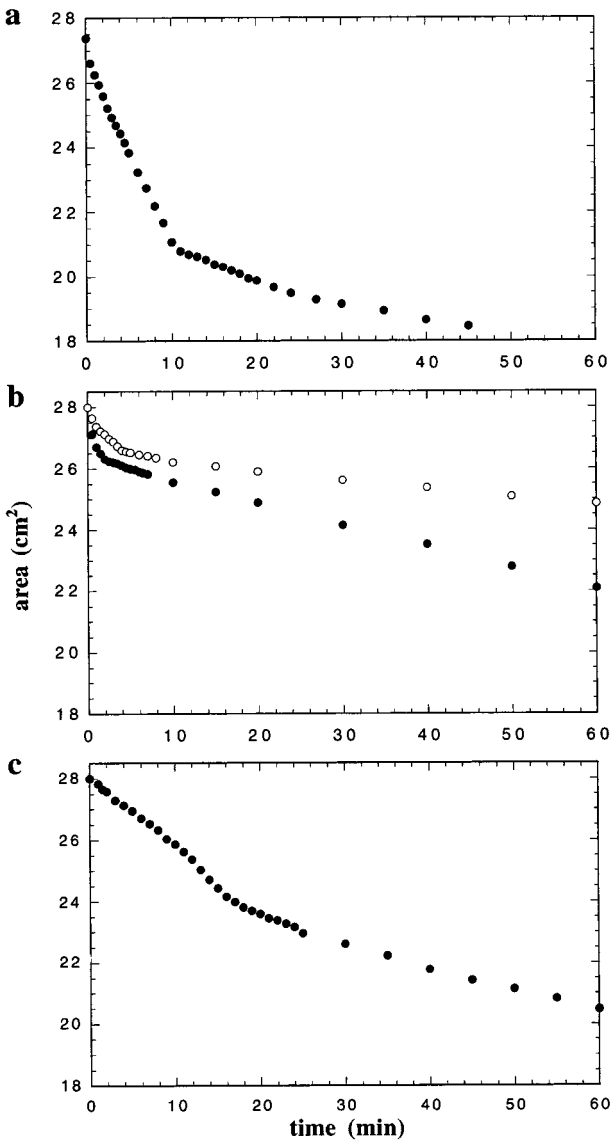

FIGURE 2: Release of cholesterol (or dihydrocholesterol) from single phase, homogeneous monolayer membranes with various phospholipid mixtures. These figures show the area decrease necessary to maintain a constant surface pressure during the desorption of Chol or DChol to the subphase of β-CD. (a) Desorption from a 2:1 DMPS:DMPC/DChol mixture, where the initial concentration of DChol was 50 mol %. The surface pressure was kept constant at 15 mN/m, and the subphase contained 2 mM β-CD. (b) Desorption from a 1:1 DMPC:DPPC/Chol mixture, where the initial concentration of Chol was 50 mol %. The surface pressure was kept constant at 20 mN/m. The solid circles are for desorption to a 2 mM β-CD subphase, while the open circles are for desorption to the subphase containing 1 mM β-CD. (c) Desorption from an egg SM/DChol mixture, where the initial concentration of DChol was 50 mol %. The surface pressure was kept constant at 22 mN/m, and the subphase contained 2 mM β-CD.

following set of equations can be easily derived using the above notation:

$$A = Na \tag{1}$$

$$dA = a\,dN + N\,da \tag{2}$$

$$dA/A = [(1 - x_0)^{-1} + a^{-1}\partial a/\partial x_0]\,dx_0 \tag{3}$$

From these equations one then arrives at the results

$$\frac{1}{n_x}\frac{dn_x}{dt} = x_0^{-1}(1 - x_0)^{-1}\, dx_0/dt \quad (4)$$

$$= x_0^{-1}[1 + (1 - x_0)a^{-1}\partial a/\partial x_0]^{-1} A^{-1}\, dA/dt \quad (5)$$

Starting with a sample of given area A and mole fraction x_0, one first measures a small change in monolayer area dA over a small time interval dt from which one derives the small change in mole fraction dx_0 using eq 3 along with appropriate experimental values of a and $\partial a/\partial x_0$. This procedure is then repeated by iteration in small time steps. At each step of this iteration the cholesterol release rate constants are determined using eqs 4 and 5. In eq 4, n_x is the number of Chol or DChol molecules in the membrane. Cholesterol release rates obtained by this method are given in Figure 3. It will be seen that there are marked changes in release rate constants at the complex stoichiometries. Note that the release rate $(1/n_x)(dn_x/dt)$ is discontinuous when the slope $(1/A)(dA/dt)$ is discontinuous.

Calculated Chemical Activities. The chemical activity of cholesterol ζ is defined by the chemical potential of cholesterol μ

$$\zeta = e^{\mu/kT} \quad (6)$$

The calculation of the chemical potentials for cholesterol has been described previously (*6*). A calculated chemical activity of cholesterol as a function of membrane composition is illustrated in Figure 4 for a binary mixture where a 2:1 phospholipid:cholesterol complex is formed. The chemical potentials used in this calculation are the same as those used earlier to account for the effects of electrical fields on cholesterol−phospholipid complexes (*19*). Similar results were obtained in earlier work where chemical potentials were modeled to account for the phase diagrams showing pairs of upper miscibility points (*6*, *22*). Three important parameters go into these model calculations. They are the equilibrium constant for complex formation, K_0, the oligomerization parameter, n, and immiscibility parameters, especially the parameter governing the immiscibility of the phospholipid and cholesterol. The reaction of complex formation is written

$$nq\mathrm{C} + np\mathrm{P} \leftrightarrow \langle \mathrm{C}_{nq}\mathrm{P}_{np}\rangle \quad (7)$$

In this reaction C and P refer to cholesterol and phospholipid molecules, p and q are relatively prime numbers, and n is the oligomerization parameter. The brackets are a reminder that solution nonideality leading to immiscibility can facilitate complex formation. That is, the complex formation can involve a many-body effect, and the complex should not be viewed as an isolated group of $nq + np$ molecules. The term condensed complex was introduced previously to indicate the cooperative nature of the complex formation and its high molecular density. In reaction *7* the molecules C, P, and $\mathrm{C}_{nq}\mathrm{P}_{np}$ interact with one another as in a nonideal solution, irrespective of whether the membrane is homogeneous or not (*6*, *19*).

DISCUSSION

It is known from much previous work that the rate of cholesterol loss from mixtures with phospholipids depends on the lipid composition (*7*, *23*). It is plausible to assume

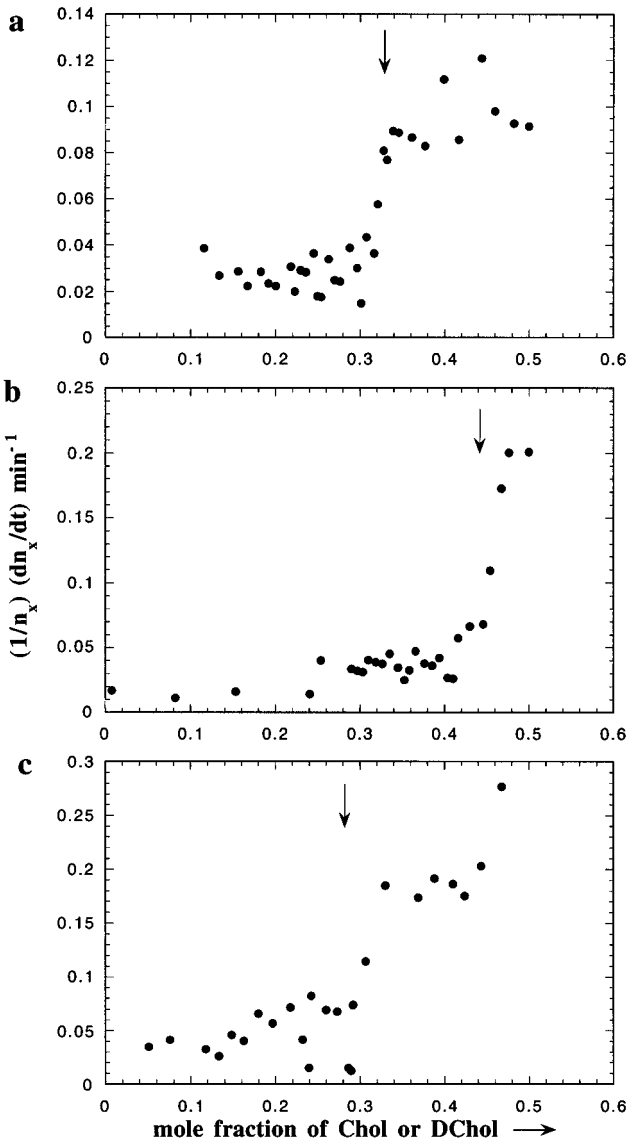

FIGURE 3: Rate constants (min^{-1}) for Chol (or DChol) release from single phase, homogeneous membranes to β-CD. These rate constants are calculated from the area decrease data such as those in Figure 2 by using eqs 4 and 5 and the molecular area data from Figure 1d−f. Arrows show complex stoichiometries determined from phase diagrams such as those in Figure 1.

that the rate constant for loss of cholesterol from the monolayer membrane is proportional to the chemical activity of cholesterol. That is, the rate constant for cholesterol loss is proportional to $\exp[-(\mu^+ - \mu)/kT]$, where μ^+ is the chemical potential of cholesterol in the activated state and μ is the chemical potential of cholesterol in the membrane. If we assume that this activated state has a free energy that is substantially independent of membrane composition, then one sees that the rate constant for cholesterol loss is simply proportional to the chemical activity of cholesterol in the membrane.

In previous work we showed theoretically that the concentration of free cholesterol undergoes a marked increase as membrane cholesterol concentration is increased beyond the stoichiometric composition (*6*). (From the phase diagrams, this is obviously true at low pressures where the liquids are largely phospholipid and complex or complex and cholesterol. It is also true at higher pressures in the homogeneous phase.) This is also true for the chemical

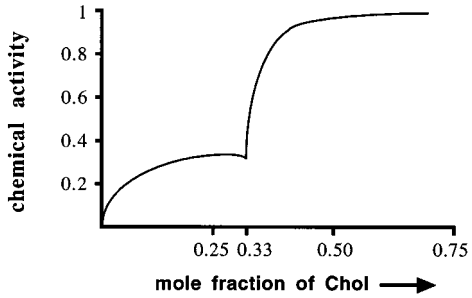

FIGURE 4: Theoretical cholesterol chemical activity as a function of cholesterol concentration in a homogeneous membrane. The activity is calculated using eq 6 and the parameter values given in Figure 4 of ref *19* (equilibrium constant $K_0 = 2$, $n = 5$, and 33 mol % cholesterol in the complex). The calculated sharp jump in chemical activity increases with increasing binding constant, K_0, for the reaction of eq 7. The sharpness of the change also increases with increasing n, but the magnitude of the change (between low and high cholesterol) decreases with increasing n.

activity of cholesterol, as illustrated in Figure 4. The results in Figure 4 are based on the theoretical model together with values of the parameters used previously (*19*). It will be seen in this calculation that the chemical activity of cholesterol increases by a factor of about 3 when the cholesterol mole fraction in the membrane is changed by a small amount in the vicinity of the stoichiometric composition. The experimental data in Figure 3 show that the rate of cholesterol release from the membrane also increases by a factor of 3−4 when the cholesterol mole fraction in the membrane is increased by a small amount in the vicinity of the stoichiometric compositions. The similarity of these results gives us confidence in our interpretation of these rates.

The quantitative details of the chemical potential calculations depend of course on the thermodynamic parameters chosen. However, the qualitative features of the increase in chemical activity of cholesterol are retained for any set of parameters that also simulate data such as those given in Figure 1. The stoichiometry of the complex in terms of $q/(p + q)$ is set by the position of the cusps in Figure 1. The sharpness of the cusps is sensitive to the value of the cooperativity n, and this also affects the size of the jump in the chemical activity. We have used values of n in the range $n = 3-5$ in our simulations (*6*, *19*). It will be noted that the experimental curves on release rate in Figure 2 may have more than a single break or inflection. This might be related to quantitative aspects of the chemical potential and area per molecule versus cholesterol concentration curves or possibly to complexes with more than one stoichiometry (*22*, *24*). In connection with the latter possibility, in recent work it has been reported that the hydrolytic activity of a phospholipase on certain cholesterol−phospholipid bilayers shows 8−10 minima at specific cholesterol concentrations (*24*, *25*). These minima may also correspond to minima in chemical potentials. Some of these minima have compositions similar to those often found in our work on monolayers, namely, about 35 and 45 mol %. At present we do not have a molecular model for the relation between this complex stoichiometry $[q/(p + q)]$ and phospholipid structure. There is a rough empirical correlation between $q/(p + q)$ inferred from phase diagrams and phospholipid chain melting temperatures (*26*).

The monolayer phase diagrams observed to date show no evidence for cholesterol complex formation with unsaturated

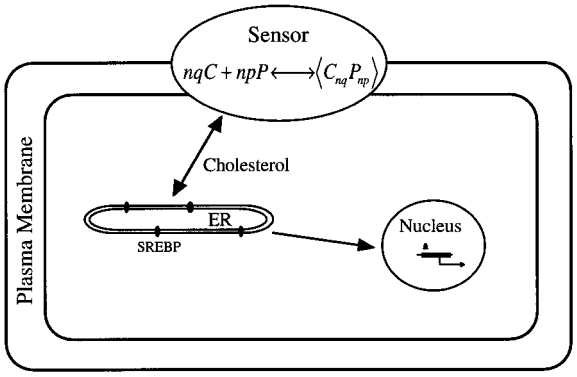

FIGURE 5: Schematic representation of cholesterol homeostasis in a cell [adapted from Lange and Steck (*31*)]. The chemical activity of cholesterol as determined by complex formation (eq 7) acts as a plasma membrane cholesterol sensor. The sensor regulates the transport of cholesterol to and from the ER and, hence, indirectly regulates the proteolytic release of sterol regulatory element binding protein (SREBP) and the subsequent expression of genes of the cholesterol biosynthetic and uptake pathways (*13*).

phosphatidylcholines (*27*) or phosphatidylcholines with short saturated fatty acid chains (*26*). It is anticipated that the inclusion of these lipids in membranes will tend to increase the chemical activity of cholesterol. In other words, one membrane having a high cholesterol concentration and long-chain phosphatidylcholines (or sphingomyelin) may be in equilibrium with a second membrane with a low cholesterol concentration and unsaturated phosphatidylcholines, with respect to cholesterol interchange. In this connection it is interesting to note that human fibroblasts have a mole fraction of cholesterol of about 0.4 in the plasma membrane and a much lower concentration of cholesterol in the ER and other intracellular membranes (*12*). Nonetheless, they may be close to equilibrium with respect to cholesterol exchange in a quiescent cell. It has also been suggested that an uneven distribution of cholesterol may provide a mechanism for the sorting of membrane proteins of the Golgi apparatus (*28*).

The composition-dependent switch in the chemical activity of cholesterol may provide a regulatory factor for cholesterol transport and biosynthesis in cells. For example, the outer monolayer leaflet of the plasma membrane of eukaryotic cells contains both sphingomyelin and cholesterol. It is known that sphingomyelinase treatment of fibroblasts results in the downregulation of cholesterol biosynthesis in the ER (*16*). If we assume that under physiologic conditions the sphingomyelin−cholesterol ratio is close to stoichiometric, it will be seen that loss of sphingomyelin would lead to an increase in cholesterol chemical activity in the outer monolayer, and this increase in chemical activity could be transferred to the inner monolayer by cholesterol flip-flop and subsequent cholesterol transport to the ER, leading to downregulation of cholesterol biosynthesis. [A protein that acts as a cholesterol transporter between mitochondrial membranes is known (*29*).] A number of studies of the distribution of cholesterol between plasma membrane and intracellular membranes are consistent with the idea that while the concentrations of cholesterol in these membranes may be quite different, the chemical activities may be similar—the activity (concentration of free cholesterol) in the plasma membrane is low because complex formation consumes most of the cholesterol, whereas the activity of cholesterol in the ER is low because the concentration of cholesterol is low

and there is little complex formation. Xu and Tabas have found that activation of macrophage ACAT by lipoproteins proceeds only after the net expansion of cellular cholesterol pools to a critical threshold level (*30*). More recently, Lange and Steck have reported a threshold condition in the relation between plasma membrane cholesterol and intracellular membrane cholesterol (*12*). That is, when the cellular cholesterol is increased past its physiological value, there is a large increase in ER cholesterol. The threshold transition found by these investigators was proposed to arise from "a structural or organizational transition in the bilayer". We suggest that this transition is the composition-dependent switch in cholesterol chemical activity that is a consequence of condensed complex formation. This view can be summarized by the schematic drawing given in Figure 5, adapted from Lange and Steck (*31*). Here, a cholesterol sensor regulates the flux of cholesterol into and out of the plasma membrane. Our proposal is that this sensor is the chemical activity of cholesterol as governed by reaction *7*, shown in Figure 5 as functioning as the cholesterol sensor. The stoichiometry of this reaction depends on the phospholipid type, implying a coordinated regulation of phospholipid composition and cholesterol concentration (*32*).

NOTE ADDED IN PROOF

Recent evidence indicates that the cleavage of SREBP's (shown in Figure 5) may occur in a post-ER compartment such as the Golgi apparatus (*34*).

ACKNOWLEDGMENT

We thank Yvonne Lange and Ted Steck for helpful correspondence concerning cholesterol distributions and regulation in cells. We are also indebted to Robert Simoni, Sarah Keller, Tom Anderson, and Jane McConnell for helpful discussions.

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BI0005097