Cholesterol Displacement from Membrane Phospholipids by Hexadecanol

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ABSTRACT Adding cholesterol to monolayers of certain phospholipids drives the separation of liquid-ordered from liquid-disordered domains. The ordered phases appear to contain stoichiometric complexes of cholesterol and phospholipid. Furthermore, it has been suggested that the cholesterol in these complexes has a low chemical activity compared to that of the free sterol; i.e., that in excess of the phospholipid binding capacity. We have now tested the hypothesis that the membrane intercalator 1-hexadecanol (HD) similarly associates with phospholipids and thereby displaces the complexed cholesterol. HD introduced into monolayers of pure dimyristoylphosphatidylcholine generated highly condensed (stable and solid) domains. In contrast, the phase behavior of mixed monolayers of the phospholipid, sterol, and alcohol suggested that HD could substitute for cholesterol mole for mole in promoting liquid-ordered domains. We also found that the transfer of cholesterol from mixed monolayers to aqueous cyclodextrin was greatly stimulated by the presence of HD, but only at levels sufficient to competitively displace the sterol from the phospholipid. This enhanced efflux was interpreted to reflect an increase in uncomplexed cholesterol. We conclude that HD forms complexes with dimyristoylphosphatidylcholine that are surprisingly similar to those of cholesterol. HD competitively displaces cholesterol from the phospholipid and thereby increases its chemical activity.

INTRODUCTION

Sterols are essential components of all eukaryotic plasma membranes, serving to reduce their permeability and increase their mechanical strength. Cholesterol (CH) also drives the formation of immiscible liquid-ordered bilayer phases (rafts). The partition of various membrane proteins into cholesterol-rich domains is important in many cell functions (1,2). Lipid monolayers of specified composition offer a powerful approach to the analysis of the properties of CH-phospholipid (PL) interactions under physically and chemically well-defined conditions, thereby providing precise information complementary to that obtained from synthetic bilayers and biological membranes.

Of relevance here is the observation that adding CH to certain PL membranes can, under specified conditions, drive the formation of coexisting liquid-ordered and liquid-disordered phases (i.e., immiscible liquid domains). This phase separation appears at both low and high CH concentrations but is minimal at intermediate mole fractions (3,4). The fact that the two immiscibility regions were separated by a sharp cusp was interpreted to signify that CH forms complexes with these PLs and that their stoichiometry is given by the membrane composition at the cusp point. The limited solubility of these complexes in the PL would favor the formation of sterol-rich domains; at low CH levels, these would be surrounded by a PL-rich continuum. The sterol-rich phase, although liquid, would tend to be more tightly packed (i.e., ordered) compared to the PL-rich phase, a disordered liquid (5). According to this model, when CH is in excess, there will be little or no uncomplexed PL (3,4). Being poorly miscible with the sterol-PL complexes, the free CH will accumulate in discrete domains dispersed in a complex-rich continuum (5,6). At CH levels between these two limits, monolayers will be dominated by a major phase rich in complexes with small amounts of free CH and free PL dissolved therein. The CH/PL mole ratio at which this intermediate region of maximal miscibility appears corresponds to the stoichiometry of the putative complexes (4).

It has also been proposed that the CH in complexes has a relatively low chemical activity by virtue of its association with the PL, whereas uncomplexed CH (i.e., that exceeding the capacity of the PL) has a high chemical activity (3,4). Certain membrane-intercalating amphipaths have been observed to increase the chemical activity of the CH in mixed membranes, and this has been taken to signify their ability to displace CH from PL complexes. Among these intercalators are octanol, ceramides, and diglycerides (7–10).

This study explores these concepts using 1-hexadecanol (HD). This long-chain primary alcohol was chosen because of its insolubility in water and its ability to interact with PL membranes. HD is also the smallest alcohol that can form a stable monolayer by itself. Grazing incidence x-ray diffraction shows that phosphatidylcholine forms complexes with both CH and HD (11,12). We reasoned that if octanol, with only eight carbon atoms, displaces CH from its association with PLs (7), then HD should be even more potent and, when

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studied in a monolayer system, provide precise information about the nature of the complexes between the PLs and these intercalators. We have therefore studied monolayers of ternary mixtures for evidence of CH displacement from PL by HD. To do so, we followed phase behavior and both the kinetics of the transfer of the sterol from monolayers to aqueous cyclodextrin and its equilibrium partition (7,13,14). Our results support the hypothesis stated above.

MATERIALS AND METHODS

Materials

Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids (Alabaster, AL), and dihydrocholesterol (DChol), HD, β-cyclodextrin (β-CD), and methyl-β-cyclodextrin (Mβ-CD) were obtained from Sigma (St. Louis, MO). Texas Red-dihexadecanoyl-phosphatidylethanolamine (TR-DHPE) was obtained from Molecular Probes (Eugene, OR). The lipids were dissolved in chloroform without further purification, stored in the dark at −20°C, and warmed to ambient temperature before use. DChol was used in place of CH because, in monolayers, the latter undergoes rapid air oxidation. The properties of DChol are very similar to those of CH (3,13).

Phase diagrams

Film balance experiments were conducted in a Langmuir trough atop water that was purified to a resistivity of 18.2 MΩ-cm with a Millipore UF Plus system (Millipore, Bedford, MA). We recorded the phase behavior of monolayers doped with 0.1 mol % TR-DHPE using a fluorescence microscope (15). All experiments were performed at 13 ± 0.5°C, well below the main transition temperature of DMPC (24°C); our conditions fostered liquid-liquid immiscibility at both low and high DChol mole fractions (3).

The lipids were deposited from chloroform onto the interface at a low surface density and negligible surface pressure. The solvent was allowed to evaporate for 10-15 min. Monolayers were then compressed to a single phase, and the pressure was held constant for 30 min to allow uniform lipid mixing. The film was then expanded slowly to determine the point where uniform fluorescence was replaced by fields of micrometer-scale dark and bright regions. (This reflected the demixing of liquid-ordered from -disordered phases, respectively.) The surface pressure at which two coexisting phases first appeared, called the transition pressure, was mapped for replicate monolayers containing varied DChol and/or HD. The error bars in the figures denote standard deviations; generally, these values were 5–10% of the mean.

Transfer to cyclodextrin

To obtain a relative measure of the chemical activity of the CH in the mixed monolayers, we analyzed both the time course and the extent of its transfer to 2 mM β-CD or Mβ-CD dissolved in the aqueous subphase. For these studies, we used a home-built Langmuir trough equipped with a Wilhelmy plate and movable barriers (15). The surface pressure of the monolayer was brought to 28 mN/m and kept constant thereafter by means of a feedback link between the pressure sensor and the movable barriers. This surface pressure was greater than the transition pressure of all of the mixtures, ensuring that all of the monolayers examined were in one homogeneous phase. This pressure is also close to the bilayer equivalent pressure (16).

The time courses could not be initiated by the injection of cyclodextrin into the subphase because of its slow diffusion in the unstirred bath. Instead, a predetermined amount of lipid was spread on subphases of water containing 2 mM cyclodextrin at 13°C. The barrier was immediately freed, and the pressure maintained constant at 28 mN/m as lipid was lost to the subphase. The rate of area loss was recorded over time. The rate processes were often biphasic; we took the fast and slow components to signify, respectively, the transfer of uncomplexed and complexed DChol to the cyclodextrin. It was straightforward to estimate the initial fractional rate of transfer as the linearized loss of monolayer surface area with time in the steepest part of the curve. That is, $-((A_t - A_o)/A_o)/t$, where A_t is the total area after a short time, t, and A_o is the initial area. All experiments were repeated at least three times, and representative results are presented. Standard deviations were ≤10%.

Two forms of cyclodextrin were tested: β-CD and Mβ-CD. The former had no surface activity and was therefore a good acceptor in CH transfer experiments. The latter, though its surface activity added complexity to the system, allowed us to relate the results from this model system to those obtained in biological experiments (14).

RESULTS

Pressure-area isotherms of the lipids under study

With increasing pressure, monolayers of pure DMPC condensed from an expanded, liquid-disordered phase to increasingly compact forms (Fig. 1, *squares*). In contrast, monolayers of DChol (*solid triangles*) and HD (*stars*) showed no liquid-expanded (LE) phase; rather, they condensed completely at very low pressures. The addition of increasing amounts of HD shifted the isotherms of DMPC films to the left, suggesting condensation effects (*open inverted triangles* and *open circles*). These results are in accord with earlier observations on HD (12). Incorporation of increasing DChol into the DMPC monolayer also served to shift the isotherm to the left, revealing the similar condensation effect of DChol on DMPC (*solid inverted triangles* and *solid circles*).

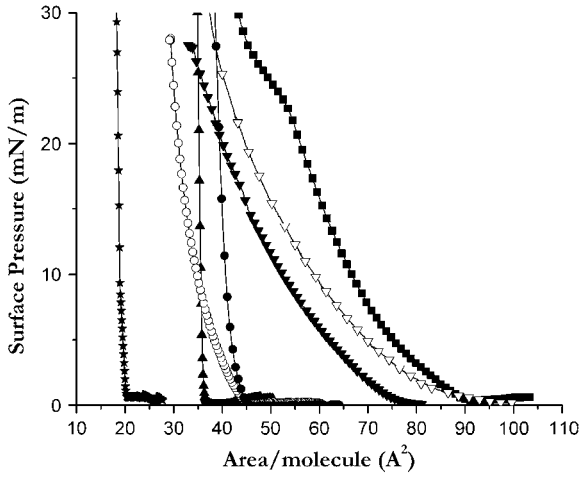

FIGURE 1 Isotherms of pure and mixed lipid monolayers. Monolayers of varied composition were spread on pure water at 13°C and compressed to the recorded areas and surface pressures as described in Materials and Methods. (■) Pure DMPC; (▲) pure DChol; (★) pure HD; (▽) 20 mol % HD plus 80 mol % DMPC; (○) 50 mol % HD plus 50 mol % DMPC; (▼) 20 mol % DChol plus 80 mol % DMPC; and (●) 50 mol % DChol plus 50 mol % DMPC.

Phase behavior of binary DMPC/DChol, DMPC/HD, and HD/DChol monolayers

Domains of relatively low and high molecular order were visualized in monolayers by the partition of a fluorescent reporter lipid, TR-DHPE. Pure films of DMPC at low pressures had a uniform brightness characteristic of a single phase (Fig. 2 *A*). The introduction of small amounts of DChol resulted in the appearance of dark circular domains against a bright background (Fig. 2 *B*). The area fraction occupied by the dark domains grew in rough proportion to the DChol. The known partition characteristics of the fluorescent probe suggest that the bright regions are the more fluid (presumably liquid-disordered, PL-rich) phases whereas the dark domains are the less fluid (presumably liquid-ordered, sterol-rich) phases (3,5). That both the emergent domains and the continuous phases in Fig. 2 *B* are liquids can be inferred from the circular shapes of the circumscribed regions (17). The observed immiscibility of all DMPC/DChol mixtures could be abolished by raising the surface pressure to 25 mN/m or above (see Fig. 5).

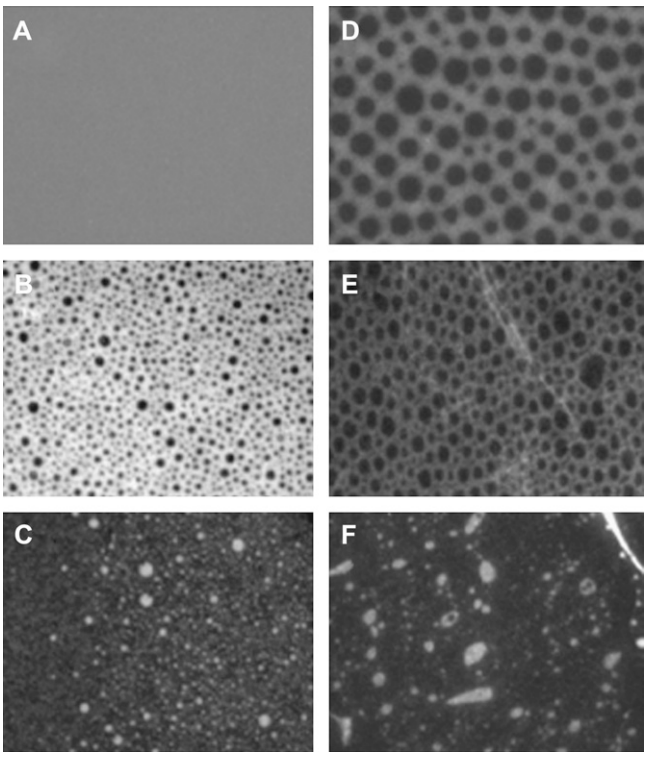

FIGURE 2 Phase behavior of lipid monolayers. Pure and mixed monolayers containing 0.1 mol % TR-DHPE were spread on pure water at 13°C at low surface density, then compressed to the desired surface pressure, and fluorescence micrographs recorded. (*A*) Pure DMPC. (*B*) 20 mol % DChol plus 80 mol % DMPC. (*C*) 40 mol % DChol plus 60 mol % DMPC. (*D*) 30 mol % HD plus 70 mol % DMPC at 30 mN/m. (*E*) 40 mol % HD plus 60 mol % DMPC at collapse. (*F*) 90 mol % DChol plus 10 mol % HD at 6 mN/m. The width of each panel is 200 μm.

Elevating the DChol content of the DMPC monolayer above 35 mol % reversed the pattern of fluorescence, making the continuum dark and the discrete domains bright (Fig. 2 *C*). Again, the round contours of the phases indicate that both the continuous and discontinuous phases were fluid. These results are consistent with earlier studies (3).

Adding increasing HD to DMPC monolayers induced the proportionate appearance of dark domains in a brighter continuum at low surface pressures (Fig. 2 *D*). Addition of HD to DMPC has the effect of lowering the pressure for the liquid-expanded/condensed phase coexistence plateau (see Fig. 1), as if the incorporation of HD acts to condense the film. The smallest amount of HD addition to the DMPC film we have examined was 10% HD, and at this low HD concentration, dark domains with irregular contours were observed starting at 8–9 mN/m. At higher HD content, the appearance of these dark domains occurred at even lower surface pressures. Unlike DChol, phase separation persisted at pressures $\geq$ 60 mN/m (Fig. 2 *E*), even for the DMPC film with only 10% HD. These are signs of strong condensation into solid phases. Above equimolar HD/DMPC, we observed a single condensed phase (not shown, but see Fig. 1). Similar condensation effects of HD have previously been reported for mixed HD and dipalmitoylphosphatidylcholine (DPPC) films (18).

We also examined the phase behavior of mixtures of HD with DChol by fluorescence microscopy. Adding 10 mol % HD to monolayers of DChol created two immiscible phases (Fig. 2 *F*). These domains persisted at high surface pressures, and their shape is irregular. As with mixtures of DMPC and HD, HD/DChol monolayers remained biphasic up to the collapse pressure, ~40 mN/m. The persistence of phase separation at high pressures in binary mixtures of HD with either DMPC or DChol contrasts with the greater miscibility seen in mixtures of DChol with DMPC.

Phase transitions of DMPC/DChol monolayers

Adding small amounts of DChol to DMPC monolayers induced liquid-liquid phase separation, seen in Fig. 2 *B* as dark circular domains dispersed in a bright continuum. This immiscibility disappeared as films were compressed to a characteristic pressure, above which only a single homogeneous phase was seen. This characteristic mixing-demixing pressure gives a measure of the tendency toward phase separation of the components. A plot of this transition pressure as a function of monolayer composition is shown for DMPC/DChol monolayers by the open squares in Fig. 3. The transition pressure rose to a maximum at 25–30 mol % DChol and then fell to a minimum near 35 mol % DChol, at which point the monolayer exhibited a homogeneous bright field at very low pressures (the cusp in Fig. 3). As the proportion of DChol rose above 35 mol %, the monolayer showed increasing phase separation of coexisting fluid and ordered phases. At these high DChol levels, however, the circumscribed domains were bright and the continuum dark, as

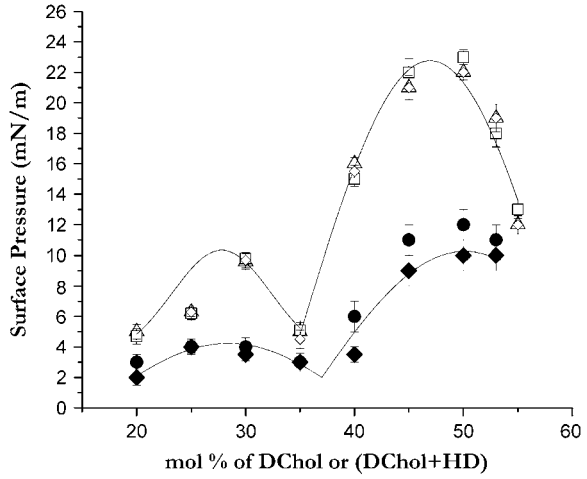

FIGURE 3 Effect of composition on the transition pressure of binary and ternary monolayers. Monolayers containing DMPC, DChol, and/or HD plus 0.1 mol % TR-DHPE were spread on pure water at 13°C, and the surface pressure varied to determine transition pressures as described in Materials and Methods. The *x* axis denotes the combined mole percent of DChol plus HD. (□) DMPC plus varied DChol. (●) DMPC plus 10 mol % DChol and varied HD. (♦) DMPC plus 15% DChol and varied HD. (△) DMPC plus 20 mol % DChol and varied HD. (◇) DMPC plus 25 mol % DChol and varied HD. Solid lines were added arbitrarily to guide the eye. The error bars are standard deviations.

shown in Fig. 2. This is the inverse of that seen at low DChol content. At very high proportions of DChol, the transition pressures dropped, defining a second critical point in the phase diagram at ~48 mol % DChol (Fig. 3).

Phase transitions of ternary mixtures of DMPC, DChol, and HD

Also shown in Fig. 3 are plots of the transition pressures of DMPC monolayers containing different fixed mole fractions of DChol supplemented with varied HD. The fixed mole fractions of DChol were chosen so that the DChol content was below that given by the cusp point of the binary DMPC/DChol system, where, according to the complex-formation model, virtually all of the DChol will be complexed with DMPC. The phase diagrams of monolayers containing 20 mol % DChol (*open triangles*) or 25 mol % DChol (*open diamonds*) and varied HD were virtually superimposable on those of binary DMPC/DChol films (*open squares*) when plotted as the sum of the two intercalators (DChol and HD). These three curves all had two regions of immiscibility, one at low and one at high intercalator abundance. In addition, the ternary systems had precisely the same maximal transition pressures as the binary DMPC/DChol mixture and were separated by the same well-defined cusp near 35 mol % intercalator. In mixtures of DMPC, DChol, and low levels of HD, the discrete domains were dark and round (i.e., liquid-ordered). Thus, we did not see the solid phases persistent to high surface pressures that were characteristic of HD-rich domains in DMPC alone. Rather, the area fraction of the dark (presumably DChol-rich) domains increased with low mole fractions of HD, suggesting that HD and/or DMPC/HD complexes accumulated in the liquid-ordered domains created by DChol.

At a combined abundance of DChol plus HD exceeding 35 mol %, the ternary mixtures formed biphasic systems composed of a dark continuum with dispersed bright circular domains. This pattern was similar to that seen in binary systems of DMPC plus high levels of DChol (Fig. 2 *C*). Adding HD in excess of 35 mol %, so that HD plus DChol exceeded 55 and 60 mol %, respectively, in the two ternary systems created irregular domains, even at pressures exceeding 40 mN/m. These images were reminiscent of the solid domains seen in binary mixtures of HD and DMPC, discussed above. Other than this evidence for solid phases at high HD fractions, the ternary systems containing 20 and 25 mol % DChol closely matched the binary system of DMPC plus DChol in all respects examined if the intercalator concentration was expressed as the sum of DChol plus HD.

We performed similar analyses on ternary mixtures containing 10 or 15 mol % DChol plus varied HD (Fig. 3, *solid symbols*). As noted earlier, introducing 10 or 15 mol % DChol to DMPC monolayers created dark circular domains. Adding HD to these monolayers produced additional small, irregular, and even darker domains that persisted at high pressures, unlike those dependent on DChol. Furthermore, the dark domains grew in number with HD content. It appears that these were solid HD-rich phases, similar to those seen in Fig. 2, *D* and *E*, except that they coexisted with two immiscible fluid phases, a bright one rich in DMPC and a dark one rich in DChol.

The transition pressures that eliminated the coexistence of the two fluid phases (but not the solid domains) for mixtures of DMPC with 10 or 15 mol % DChol plus varied HD were plotted as solid symbols in Fig. 3. These profiles resembled those for the binary DMPC/DChol system (*open symbols* in Fig. 3). That is, they too had two immiscibility regions separated by a cusp of maximal miscibility. They also had lower transition pressures to the left of the cusp than to the right. Near the cusp point, there was an inversion of the pattern of light and dark fluorescence between the continuous and discontinuous phases (not shown), just as seen in Fig. 2 for the binary system. Despite these similarities, there were two notable differences between the profiles at 10 and 15 mol % DChol and those at 20 and 25 mol % DChol. One was the formation of solid HD-rich phases, even at low HD content. The other was the lower transition pressures for the profiles of monolayers containing 10 and 15 mol % DChol plus HD. The second phenomenon may be linked to the first; i.e., a substantial fraction of the HD was sequestered in solid phases. The immiscibility of HD and DChol, such as seen in Fig. 2 *F*, may also have affected the phase behavior of the ternary systems.

Kinetics of transfer of lipids from monolayers to β-cyclodextrin

One measure of the chemical activity of sterols in monolayers is the kinetics of their transfer to an aqueous acceptor such as cyclodextrin (13,14). We gauged this process by the rate of change of the surface area of monolayers held at constant surface pressure over a subphase containing the acceptor. We first performed control experiments on monolayers of the individual lipid components (Fig. 4). No area change was detected in any system in the absence of cyclodextrin (not shown) or in the case of pure DMPC monolayers (*squares*). In contrast, the area change for pure DChol (*triangles*) was so rapid that the movable barrier could not move quickly enough to maintain the pressure at 28 mN/m. Consequently, the slope of the plot of fractional residual area, A_t/A_o, versus time for DChol monolayers reflects the velocity of the unrestrained barrier and underestimates the actual transfer rate. In any case, it is clear that the transfer of pure HD to β-CD (*stars*) is less than one-fourth as rapid as that of DChol (*triangles*). The rates of area change for monolayers composed of 1:1 DMPC/DChol (*diamonds*) and 1:1 DMPC/HD (*circles*) were intermediate between those of the corresponding pure constituents. The area loss of the DMPC/DChol mixture exhibited biphasic kinetics. We argue below that this pattern may represent the rapid dissociation rate processes of the free form of the DChol in the film and the slow dissociation of the complexed form.

The initial fractional rates of area loss to β-CD from monolayers of DMPC containing varied DChol and/or HD are plotted in Fig. 5. Again, the kinetics for pure DMPC were immeasurably slow (*solid square* in the lower left corner of Fig. 5). The (underestimated) fractional rate for DChol alone was 0.06 min^{-1} (*open star* in the upper right corner of Fig. 5). The fractional rate for pure HD was 0.01 min^{-1} (*open circle* in the far right of Fig. 5). The area loss from DMPC monolayers containing HD was slow, but it increased gradually and without inflection with increasing HD content (*solid triangles* in Fig. 5). The behavior of monolayers of DMPC/DChol was distinctive and informative (Fig. 5, *open squares*). Here, the fractional rate of area loss rose minimally with DChol below 35 mol %. Beyond this point, however, the transfer rate increased sharply, rising ~5.5-fold between 35 and 40 mol % DChol before reaching a plateau.

We next examined monolayers containing DMPC plus DChol fixed at 10, 15, 20, and 25 mol % of the total plus varied HD (Fig. 5). In each case, the rate of fractional area loss was relatively slow at low intercalator content. But transfer rates increased abruptly when the combined fraction of DChol plus HD exceeded ~30 mol %. The transfer rates all reached a plateau at a combined fraction of DChol plus HD of ~40 mol %. We note that the behavior of these ternary systems differed from that of binary DMPC/DChol mixtures in a few minor ways. First, the area loss curves reached plateaus (maximal values) at lower transfer rates than for binary DMPC/DChol. Given that the plateaus increased in rough proportion to DChol content, we suggest that the maximal rate of transfer simply varied with the abundance of the major transportable component, DChol.

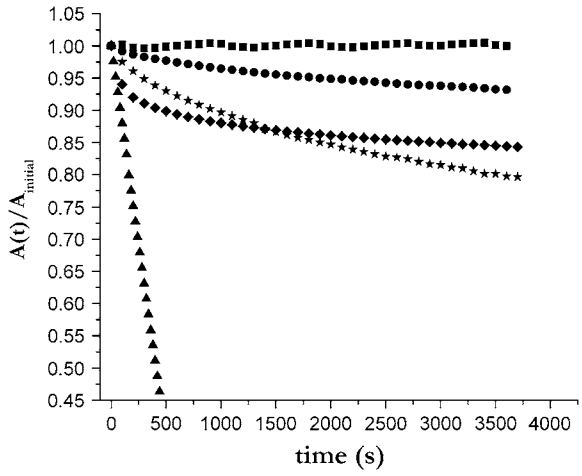

FIGURE 4 Time course of transfer of lipids from monolayers to β-cyclodextrin. Lipid mixtures were spread as monolayers over 2 mM solutions of β-CD at 13°C. The fractional change in total area was immediately recorded over time at a constant surface pressure of 28 mN/m as described in Materials and Methods. Monolayer compositions: (▲) pure DChol; (■) pure DMPC; (★) pure HD; (●) 50 mol % HD plus 50 mol % DMPC; and (♦) 50 mol % DChol plus 50 mol % DMPC.

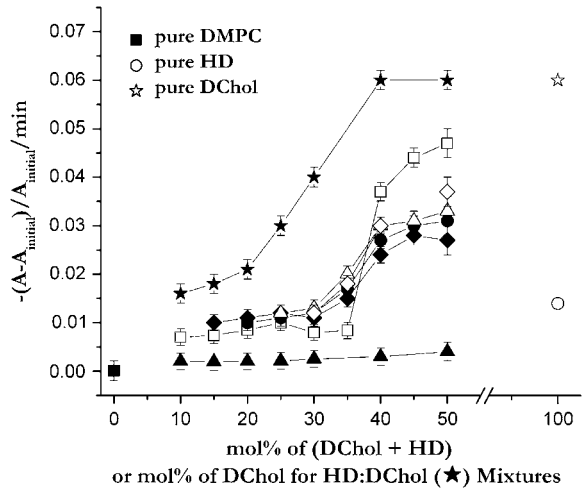

FIGURE 5 Rates of transfer of lipids from monolayers to β-cyclodextrin. Lipid mixtures were spread as monolayers over 2 mM solutions of β-CD at 13°C. The fractional change in total area was followed over time at a surface pressure of 28 mN/m as described in Materials and Methods. The rates of lipid loss from monolayers (expressed as area fraction per minute) are plotted against the mole fraction of DChol plus HD (or the mole fraction of DChol in the binary HD/DChol (★) mixtures). Pure components are identified by single points as noted: DMPC, (■); DChol, (☆); and HD, (○). Binary mixtures of DMPC and DChol are shown by open squares; binary mixtures of DMPC and HD by solid triangles; and binary mixtures of HD and DChol are shown by solid stars. Ternary mixtures of DMPC were prepared with varied HD plus DChol at these fixed mole fractions: (♦) 10 mol % DChol; (●) 15 mol % DChol; (△), 20 mol % DChol; and (◇) 25 mol % DChol.

Less explicable was the observation that the rate of area loss began to accelerate at a lower concentration of intercalator (DChol plus HD) and rose less sharply than that seen with DChol alone.

For completeness, we also tested binary mixtures of HD and DChol (Fig. 5, *solid stars*). At low DChol content, transfer rates were not much greater than that of pure HD and rose slowly with increasing sterol. Then, as DChol rose from 20 and 40 mol %, the rate of area loss increased linearly by ~3-fold. Beyond 40 mol % DChol, the desorption rate was comparable to the movable barrier itself and therefore indeterminate.

Partition of monolayer DChol to cyclodextrin

A better measure of chemical activity than rate of transfer is fugacity. Here, this thermodynamic parameter is reflected in the equilibrium partition of DChol between the monolayer and the cyclodextrin. We estimated the final extent of transfer between DMPC/DChol films and β-CD (i.e., the sum of the fast and slow processes) from a fit of time courses to a two-exponential equation. A plot of these estimates as a function of monolayer composition is shown as the squares in Fig. 6. The fraction of the monolayer area transferred increased very slightly and rather linearly with DChol content up to ~30 mol %. Thereafter, the fraction of the area lost increased dramatically with the DChol content.

We sought to infer from Fig. 6 how much of the DChol was transferred to cyclodextrin in the early and late parts of the curve. We therefore determined the molecular cross sections of pure DMPC and DChol in monolayers at 28 mN/m, the surface pressure used in those experiments. The values

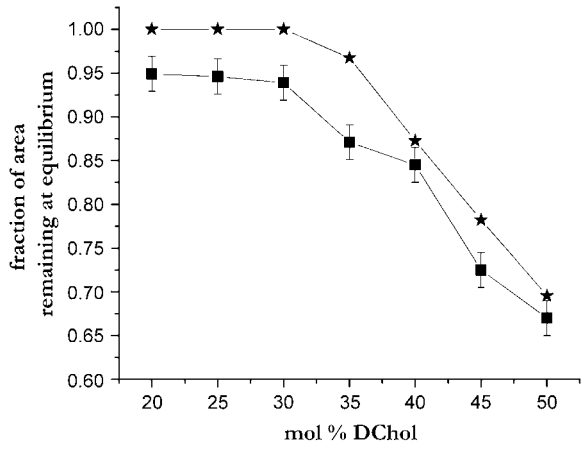

FIGURE 6 Extent of transfer of DChol from mixed DMPC monolayers to β-cyclodextrin at equilibrium. Mixtures of DChol plus DMPC were spread as monolayers over 2 mM solutions of β-CD at 13°C. The fractional change in total area was followed at a surface pressure of 28 mN/m to obtain time courses such as those seen in Fig. 4. These were fit to a two-exponent equation, and the partition values at infinite time obtained (■). Also shown (★) is a theoretical curve of area loss calculated by assuming no transfer from 2:1 DMPC/DChol complexes and complete transfer of DChol in excess of the equivalence point with DMPC (see text).

obtained were 53 and 35 Å^2/molecule, respectively. Because the cusp at 35% DChol in Fig. 3 suggests the formation of DMPC/DChol complexes with a stoichiometry of ~2:1 (see Discussion), we also estimated the average molecular cross section of 2:1 DMPC/DChol complexes in monolayers at 28 mN/m to be ~35–40 Å^2 per molecule. From these values, we generated the hypothetical curve marked by stars in Fig. 6 by assuming the following: 1), All of the area loss was caused by DChol transfer to cyclodextrin. 2), All of the monolayer sterol below 33% was held tightly in 2:1 DMPC/DChol complexes and therefore not transferable to cyclodextrin. (Fig. 6 shows that ~5% of the mass of the film was lost at DChol levels below 33% DChol; this suggests that DChol actually partitions weakly from the complexes to the cyclodextrin.) And 3), None of the DChol above this stoichiometric equivalence point was complexed but rather was free in the monolayer and available to partition into the cyclodextrin compartment. The calculated curve (*stars*) in Fig. 6 paralleled the experimental curve (*squares*) quite well. Most instructive was the fact that the fractional area loss in the steep part of the experimental curve was just that predicted from the calculation if every one of the DChol molecules above the putative stoichiometric equivalence point was lost to cyclodextrin and, hence, uncomplexed in the monolayer.

Kinetics of transfer of lipids from monolayers to methyl-β-cyclodextrin

Cholesterol transfer experiments have been carried out on biological membranes using Mβ-CD rather than β-CD (7). We therefore tested Mβ-CD in this constructed system. As expected, the results, shown in Fig. 7, closely resembled and therefore confirmed those for β-CD shown in Fig. 5. Most importantly, DMPC/DChol exhibited an abrupt and major increase in the rate of area loss at DChol >35 mol % (Fig. 7, *squares*). Mixtures of DMPC with DChol fixed at 20% plus varied HD also showed a maximal fractional rate of area loss above ~35 mol % of intercalators. As seen with β-CD (Fig. 5), the acceleration of transfer to Mβ-CD began at lower intercalator levels and was less sharp in the ternary than the binary mixtures (Fig. 7). Furthermore, as with β-CD, area loss reached a maximum rate at 40% total intercalator at slightly lower values (~0.05 min^{-1}) than did DMPC/DChol monolayers (~0.06 min^{-1}). DMPC/HD mixtures (*triangles*) again showed little change in monolayer area. In addition, the area lost from single-component films was negligible for DMPC and far greater for DChol than for HD (not shown). Thus, the results obtained using the two cyclodextrins were equivalent.

DISCUSSION

Our evidence supports a five-tiered hypothesis regarding the interactions of sterols and certain intercalators with

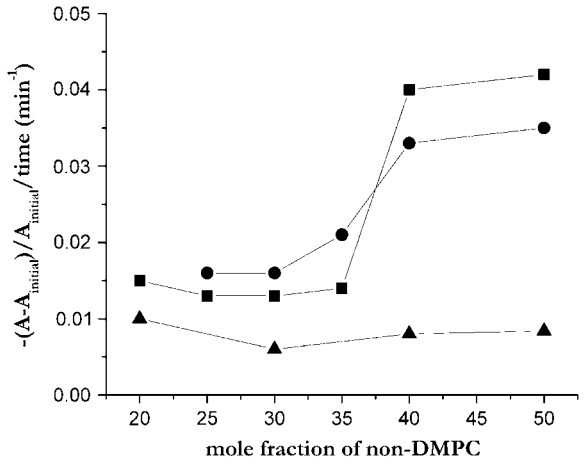

FIGURE 7 Rate of transfer of lipids from monolayers to methyl-β-cyclodextrin. Different lipid mixtures were spread as monolayers over 2 mM solutions of β-CD at 13°C. The fractional change in total area was followed over time at a surface pressure of 28 mN/m as described in Materials and Methods. These values are plotted against the mole fraction of DChol plus HD. (■) Binary mixtures of DMPC and DChol; (▲) binary mixtures of DMPC and HD; and (●) ternary mixtures of DMPC plus 20 mol % DChol plus varied HD.

membrane PLs. The five premises are: 1), membrane sterols form stoichiometric complexes with PLs; 2), sterols have a low chemical activity when bound to the PLs, whereas free sterols (namely, those exceeding the capacity of the PLs) have a relatively high chemical activity and, consequently, distinctive properties; 3), certain intercalating amphipaths form complexes with the PLs similar to those of the sterols and can therefore substitute for them; 4), it follows that these amphipaths compete with the sterols for association with the PLs and can displace them from the PLs; and 5), the displaced sterol molecules are free and therefore have a high chemical activity relative to the complexed forms.

We tested this compound hypothesis in a well-defined system by examining the effects of the intercalating amphipath, HD, on monolayers of DMPC plus DChol. Our approach was to assess the influence of HD on the formation of immiscible liquid phases in DMPC/DChol mixtures. In addition, we examined the effect of HD on the chemical activity of DChol in the monolayer, as gauged by the rate and extent of its transfer to the aqueous acceptor, cyclodextrin.

By itself, DChol forms condensed (solid) films at surface pressures ~1 mN/m (Fig. 1), a reflection of the well-known propensity of the ring system of CH toward intermolecular alignment and close packing at the air-water interface. DChol also promoted lateral phase separation of DMPC films into liquid-ordered and liquid-disordered regions (Figs. 2, *A–C,* and 3). The bright versus dark staining pattern of the discontinuous domains and the continuum switched at ~35 mol % DChol (Fig. 2, *B* and *C*). This kind of behavior has suggested a novel molecular mechanism (3). Briefly, sterols such as DChol can form stoichiometric complexes with PLs such as DMPC that are poorly miscible in the bulk PL. These complexes drive phase separation even at low DChol levels (see Fig. 2 *B*, the left-hand region of Fig. 3, and Radhakrishnan and McConnell (4)). However, at a characteristic composition (~35 mol % DChol in Fig. 3), phase separation becomes minimal because the preponderant complexes serve as a solvent for the small amounts of uncomplexed PC and DChol remaining (19). At DChol proportions higher than this point of maximal miscibility, phase separation reappears because the DMPC/DChol complexes have limited miscibility with free, uncomplexed DChol. It follows that the cusp point separating the two peaks in Fig. 3 corresponds to the stoichiometry of the two components in the complexes (3,4). The composition of the monolayer at this point, ~35 mol % sterol (Fig. 3), suggests that the proportions of the complexes are ~2:1 DMPC/DChol. It is possible that the stoichiometry of the complexes is actually a multiple of this ratio; i.e., $(2:1)_n$. There could even be a mixture of varied complex forms with a weighted average stoichiometry of 2:1.

The premise that the sterol was complexed with DMPC up to a 2:1 equivalence point and free beyond that stoichiometry was confirmed by the interaction of monolayer DChol with cyclodextrin in the subphase. As predicted by the model, there was little loss of monolayer area at equilibrium and a low rate of desorption when the DChol content was <35 mol %. The rate and extent of area loss rose dramatically thereafter (Figs. 5–7). Furthermore, the increment in area loss to the cyclodextrin at DChol content exceeding 35 mol % corresponded precisely to the entirety of the DChol added beyond that point. It therefore appears that only a little DChol passed to the acceptor below the apparent DMPC saturation point found in Fig. 3, whereas above this point, essentially all of the excess sterol was transferred. The experiments shown in Figs. 4–7 were carried out on monolayers held above the transition pressure so that they were monophasic; thus, their composition-dependent threshold behavior was presumably not related to any phase transition. Rather, the results affirm two of our main premises: that DMPC and DChol form ~2:1 molecular complexes and that the excess DChol above the stoichiometric equivalence point is free and therefore has a relatively high fugacity. These findings are all consistent with those reported earlier (20).

A third premise was that intercalators such as HD can bind to DMPC, thereby competing with sterols, displacing them, and activating them. Like CH, HD by itself forms condensed (solid) films at 13°C and surface pressures of ~1 mN/m (Fig. 1). Similar condensation effects of HD on other phospholipid films have been previously reported (12,18). This behavior presumably reflects the ability of this long-chain primary alcohol to align and pack closely at the interface. Also like sterols, HD condensed DMPC monolayers (Fig. 1) and induced phase separation in them (Fig. 2, *D–E*). That the HD-dependent domains were apparently solid, whereas those produced by DChol were liquid, could signify that

DMPC/HD complexes pack more tightly than DMPC/DChol; structural evidence for this has been reported (11). Mixtures of HD and DChol show immiscibility up to high surface pressures (Fig. 2 *F*). Consistent with this behavior, adding HD to DMPC monolayers containing 10 or 15 mol % DChol created coexisting solid (DMPC/HD rich) and liquid-ordered (DMPC/DChol-rich) phases in the liquid-disordered DMPC-rich continuum. In contrast, monolayers containing 20 or 25 mol % DChol and HD up to 35 mol % total intercalator showed no evidence of solid domains. It would seem that, in Fig. 3, the liquid-ordered phases rich in DMPC/DChol complexes took up the HD or, more likely, its complexes with DMPC. That is, DMPC/HD complexes were somewhat miscible with DMPC/DChol complexes.

Remarkably, adding HD to monolayers of DMPC containing 20 or 25 mol % DChol gave transition pressure profiles that were essentially superimposable on those for mixtures of DMPC/DChol alone, when plotted against total intercalator mole fractions (three upper curves with *open symbols* in Fig. 3). That is, these ternary monolayers had the same cusp point as did binary mixtures. Furthermore, as the total intercalator content was increased from below to above the cusp point, HD evoked the same reversal of dark and bright phases as did adding DChol. That HD and DChol contributed equivalently to the transition pressure profiles suggests that each molecule of HD had a similar effect on the propensity of the monolayer to demix as did DChol itself. Furthermore, the cusp point at ~35 mol % suggests that HD may itself form DMPC complexes with the same net stoichiometry as DChol. We presume that, above the cusp point, domains rich in complexes separate from a continuous phase rich in uncomplexed DChol and/or HD. It should be pointed out that the phase diagram for binary mixtures of DMPC and HD does not exhibit the type of liquid immiscibility regions as shown in Fig. 3 for binary mixtures of DMPC and DChol and ternary mixtures of DMPC, DChol, and HD. Instead, the addition of HD to DMPC makes the monolayer pack more tightly (Fig. 1, *open inverted triangles* and *open circles*) and gives rise to a dark, condensed DMPC/HD phase in coexistence with a bright, disordered DMPC phase (Fig. 2, *D–E*) at HD content <50 mol %. Our earlier x-ray work has further shown that the addition of HD to PC monolayers induces the formation of ordered phases, giving rise to Bragg peaks (12). It is intriguing that the binary and ternary transition pressure curves are congruent for DChol ≥ 20 mol % (*open symbols* in Fig. 3) despite the miscibility differences between DChol and HD. This complex behavior is worth further study.

The phase behavior of ternary mixtures of DMPC, HD, and DChol at a DChol content of 10 and 15 mol % qualitatively resembled that at 20 and 25 mol % DChol (compare *solid* and *open symbols* in Fig. 3). That is, the transition pressure profile described two immiscibility regions with a cusp of maximal miscibility near 35% total intercalator. These data therefore support the premise stated above that HD substitutes for DChol mole for mole to form complexes with DMPC. It follows that HD will compete with DChol in forming complexes with DMPC. We tested for such competition by examining the ability of HD to displace DChol to the aqueous acceptor, cyclodextrin. Figs. 4–6 show that whereas HD has only a weak propensity to leave DMPC monolayers for cyclodextrin, it potentiated the rate and extent of transfer of DChol. The fact that the rates of area loss at the plateaus for all four ternary systems examined in Fig. 5 (and that for the ternary system with Mβ-CD in Fig. 7) were higher than that of HD itself strongly indicates that DChol and not HD was transferred to the subphase. The DChol content of all of the ternary systems was so low that, in the absence of HD, all the DChol should have been complexed with DMPC according to Fig. 3. Complexed DChol exits only slowly and to a minor degree (Fig. 5). The rapid transfer results in Figs. 5 and 7 therefore signify that the sterol in the ternary systems was displaced from DMPC by the HD. That the transfer rates and extents for the various ternary mixtures all increased abruptly near 35 mol % total intercalator confirms our overall hypothesis that HD forms complexes with DMPC and, in doing so, displaces and activates the sterol.

We recognize that the system under study is not ideal. That is, the ternary mixtures can contain uncomplexed molecules of DMPC, DChol, and HD as well as binary and ternary complexes of each. Furthermore, each of these molecular species can partition among solid, liquid-ordered, and liquid-disordered phases. It is therefore striking that the data obtained conform to a simple hypothesis. But why should HD so effectively mimic DChol in its interaction with DMPC? Both are highly hydrophobic alcohols of comparable length. Grazing-incidence x-ray diffraction suggests that HD can complex with and condense monolayers of phosphatidylcholine (12) and that DMPC/DChol and DMPC/HD complexes can coexist in ternary mixtures (21). HD and DChol both associate with DMPC through van der Waals forces and through hydrogen bonding of their hydroxyl groups (22–24). Furthermore, the headgroup of the disaturated DMPC molecule has a larger cross section than its tail, whereas the converse area differential holds for HD. By intercalating under the bulky DMPC headgroups, HD can offset this area mismatch and thereby eliminate the tilt of the DMPC tails so as to increase favorable short range contacts (12). This is how the slender HD molecules condense PC monolayers (12). In contrast, bulkier sterol molecules disorder disaturated PC membranes (11).

Because the favorable association of membrane CH and PL has evolved in eukaryotes over eons (25), it is intriguing that various intercalators such as HD, octanol, ceramides, and diglycerides can displace the sterol so effectively (7–10). One possibility is that the association of sterols with PLs has evolved to be weak so as to optimize certain physical properties of plasma membranes. For example, weakening close packing in membrane bilayers could optimize their intermediate fluidity and mechanical flexibility (26).

It was our working hypothesis that DMPC forms stoichiometric chemical complexes with both CH and HD (6). Presumably, this association is driven by a favorable enthalpy change arising from van der Waals interactions and hydrogen bonding. However, the data are also consistent with an alternative model that does not postulate such chemical interactions (27). Instead, the bulky headgroup of DMPC shields the sterol from hydrophobic interactions with water. Any CH not under these ''umbrellas'' would be destabilized by exposure to water and have an elevated chemical activity. In both this and the complexation model, there would be competition for the PL; free intercalator molecules would appear when the HD plus CH content exceeded an equivalence point with the PL. In this respect, both models account for the observed threshold and the relatively high chemical activity of the excess sterol. Because the chemical activity of the sterol in the umbrella model is basically entropically driven, it is consistent with the lack of steric similarity between HD and sterols as PL ligands. On the other hand, that model does not address the dramatic immiscibility behavior observed; that is, it does not predict the phase separation of these intercalators at low abundance (6). Our findings are therefore more in keeping with the former model and its stipulation of stoichiometric chemical complexes.

Just as in the case of the monolayers studied here, excess CH in plasma membranes also appears to be chemically active (14). In particular, the level of plasma membrane CH seems to be maintained physiologically at an equivalence point that just matches the abundance of the PLs so that its chemical activity is minimal. Small increments of sterols beyond this balance point would create a sterol pool of high fugacity. Thus, when CH fluctuates above its physiological level, it would tend to exit the plasma membrane, just as seen in Figs. 5–7. In nucleated cells, a fraction of this high-fugacity CH appears to go to the endoplasmic reticulum, where it constitutes a feedback signal for the cell to reduce its sterol level through multiple regulatory pathways (14). Thus, the sterol-PL complexation mechanism has homeostatic implications. The impact of intercalators on the chemical activity of CH in natural membranes might therefore have biological significance worth exploring.

CONCLUSIONS

Our results support a mechanistic model for the modulation of CH chemical activity by membrane intercalators. In particular, HD was shown to displace CH mole-for-mole from stoichiometric complexes with PLs. Furthermore, under a broad range of conditions, the combination of the sterol and the alcohol evoked phase behavior in PL monolayers indistinguishable from the sterol itself. Thus, HD was a surprisingly good surrogate for CH in these experiments. The sterol molecules displaced from the PL by the alcohol were found to have high chemical activity. This system provides a new physicochemical platform for elucidating the role of sterols and lipid rafts in biological membranes.

SUPPLEMENTARY MATERIAL

To view all of the supplemental files associated with this article, visit www.biophysj.org.

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REFERENCES

1. Simons, K., and E. Ikonen. 1997. Functional rafts in cell membranes. *Nature*. 387:569–572.
2. Edidin, M. 2003. The state of lipid rafts: from model membranes to cells. *Annu. Rev. Biophys. Biomol. Struct.* 32:257–283.
3. Radhakrishnan, A., and H. McConnell. 2002. Thermal dissociation of condensed complexes of cholesterol and phospholipid. *J. Phys. Chem. B*. 106:4755–4762.
4. Radhakrishnan, A., and H. M. McConnell. 1999. Condensed complexes of cholesterol and phospholipids. *Biophys. J*. 77:1507–1517.
5. McConnell, H. M., and A. Radhakrishnan. 2003. Condensed complexes of cholesterol and phospholipids. *Biochim. Biophys. Acta*. 1610: 159–173.
6. Radhakrishnan, A., T. G. Anderson, and H. M. McConnell. 2000. Condensed complexes, rafts, and the chemical activity of cholesterol in membranes. *Proc. Natl. Acad. Sci. USA*. 97:12422–12427.
7. Lange, Y., J. Ye, and T. L. Steck. 2005. Activation of membrane cholesterol by displacement from phospholipids. *J. Biol. Chem.* 280:36126–36131.
8. Megha, and E. London. 2004. Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. *J. Biol. Chem*. 279:9997–10004.
9. Zitzer, A., R. Bittman, C. A. Verbicky, R. K. Erukulla, S. Bhakdi, S. Weis, A. Valeva, and M. Palmer. 2001. Coupling of cholesterol and cone-shaped lipids in bilayers augments membrane permeabilization by the cholesterol-specific toxins streptolysin O and *Vibrio cholerae* cytolysin. *J. Biol. Chem.* 278:14628–14633.
10. Alanko, S. M., K. K. Halling, S. Maunula, J. P. Slotte, and B. Ramstedt. 2005. Displacement of sterols from sterol/sphingomyelin domains in fluid bilayer membranes by competing molecules. *Biochim. Biophys. Acta*. 1715:111–121.
11. Ege, C., M. K. Ratajczak, J. Majewski, K. Kjaer, and K. Y. Lee. 2006. Evidence for lipid/cholesterol ordering in model lipid membranes. *Biophys. J*. 91:L01–03.
12. Lee, K. Y. C., A. Gopal, A. von Nahmen, J. A. Zasadzinski, J. Majewski, G. S. Smith, P. B. Howes, and K. Kjaer. 2002. Influence of palmitic acid and hexadecanol on the phase transition temperature and molecular packing of dipalmitoylphosphatidyl-choline monolayers at the air-water interface. *J. Chem. Phys*. 116:774–783.
13. Radhakrishnan, A., and H. M. McConnell. 2000. Chemical activity of cholesterol in membranes. *Biochemistry*. 39:8119–8124.

14. Lange, Y., J. Ye, and T. L. Steck. 2004. How cholesterol homeostasis is regulated by plasma membrane cholesterol in excess of phospholipids. *Proc. Natl. Acad. Sci. USA.* 101:11664–11667.

15. Gopal, A., and K. Y. C. Lee. 2001. Morphology and collapse transitions in binary phospholipid monolayers. *J. Phys. Chem. B.* 105: 10348–10354.

16. Seelig, A. 1987. Local anesthetics and pressure: a comparison of dibucaine binding to lipid monolayers and bilayers. *Biochim. Biophys. Acta.* 899:196–204.

17. Lee, K. Y. C., and H. McConnell. 1993. Quantized shape transitions in lipid monolayer domains: theory and experiment. *J. Phys. Chem.* 97: 9532–9539.

18. Gopal, A., and K. Y. C. Lee. 2006. Headgroup percolation and the collapse of condensed Langmuir monolayers. *J. Phys. Chem. B.* 110:22079–22087.

19. Radhakrishnan, A., and H. McConnell. 2002. Thermal dissociation of condensed complexes of cholesterol and phospholipid. *J. Phys. Chem. B.* 106:4755–4762.

20. Ohvo, H., and J. P. Slotte. 1996. Cyclodextrin-mediated removal of sterols from monolayers: effects of sterol structure and phospholipids on desorption rate. *Biochemistry.* 35:8018–8024.

21. Ratajczak, M. 2007. PhD thesis. The University of Chicago, Chicago, IL.

22. Xu, X., R. Bittman, G. Duportail, D. Heissler, C. Vilcheze, and E. London. 2001. Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. *J. Biol. Chem.* 276:33540–33546.

23. Porn, M. I., and J. P. Slotte. 1995. Localization of cholesterol in sphingomyelinase-treated fibroblasts. *Biochem. J.* 308:269–274.

24. Eliasz, A. W., D. Chapman, and D. F. Ewing. 1976. Phospholipid phase transitions. Effects of *n*-alcohols, *n*-monocarboxylic acids, phenylalkyl alcohols and quaternary ammonium compounds. *Biochim. Biophys. Acta.* 448:220–230.

25. Bloch, K. 1991. Cholesterol: evolution of structure and function. Biochemistry of Lipids, Lipoproteins and Membranes: 363–381.

26. Bloom, M. 1991. Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective. *Q. Rev. Biophys.* 24: 293–397.

27. Huang, J., J. T. Buboltz, and G. W. Feigenson. 1999. Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers. *Biochim. Biophys. Acta.* 1417:89–100.