Thermomechanical and Transition Properties of Dimyristoylphosphatidylcholine/Cholesterol Bilayers†

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ABSTRACT: Mixtures of dimyristoylphosphatidylcholine (DMPC) and cholesterol (Chol) have been used to examine the effects of cholesterol on the chain crystallization transitions and thermomechanical properties in phospholipid bilayer membranes. The mechanical properties—elastic moduli and level of tension at membrane rupture—were derived from micropipet pressurization of giant single-walled vesicles. Also, the micropipet method allowed temperature-dependent area transitions to be measured at constant membrane tension. X-ray diffraction measurements were made on selected lipid/cholesterol mixtures. Wide-angle patterns and electron density profiles were used to measure bilayer thickness as an indication of chain tilt and fluidity. Vesicle area versus temperature plots showed that the main acyl chain crystallization transition of DMPC broadened and shifted to higher temperatures. Both above and below the broad transition, the elastic area compressibility modulus, K, was greatly increased with cholesterol addition. The value for the 1:1 DMPC/Chol complex was found to be ~700 dyn/cm, comparable to that for DMPC in the $L_{\beta'}$ phase. However, for all concentrations above 12.5 mol % (which was weakly solid), vesicle bilayers behaved as surface liquids with no surface shear rigidity even at temperatures well below the DMPC phase transition. Area changes over the broadened transitions were reduced by cholesterol and disappeared with the addition of 50 mol % to leave the thermal area expansivity at 1.3×10^{-3}/°C. These area changes are consistent with separate formation of a 1:1 DMPC/Chol complex that does not condense plus residual free lipid and lipid loosely associated with the 1:1 complex that freezes normally. X-ray diffraction measurements indicated that at low cholesterol concentrations (12.5 mol %) lipid chain tilt still existed and that for 50 mol % Chol the bilayer remained in a liquid phase to temperatures as low as 10 °C.

The interaction between cholesterol (Chol)[1] and phospholipids in lipid bilayer structures has been the subject of many investigations in recent years (Presti et al., 1982; Yeagle, 1985; Demel & Dekruijff, 1976). Attempts have been made to rationalize the wealth of calorimetric, spectroscopic, and microscopic data. Presti et al. (1982) proposed row formation by a tightly bound 1:1 phospholipid/cholesterol complex with intercalated, free, and loosely associated phospholipid up to 50 mol % cholesterol. High-sensitivity DSC measurements show that from 0 to 25 mol % cholesterol a sharp transition, attributable to pure phospholipid, gradually disappears while from 0 to 50 mol % a broad higher temperature transition appears, whose enthalpy reaches a maximum and finally disappears (Estep et al., 1978; Blume, 1980; Mabrey et al., 1978; Vist, 1984). The total transition enthalpy for phospholipid/Chol mixtures decreases monotonically with increasing cholesterol content (Estep et al., 1978; Blume, 1980). The broad higher temperature transition has been attributed to perturbed, "boundary" phospholipid which is loosely associated with the rows of 1:1 phospholipid/cholesterol complex resulting in a reduced lipid cooperativity (Presti et al., 1982; Estep et al., 1978). ^{2}H NMR and X-ray diffraction studies largely agree with this interpretation (Vist, 1984; Hui & He, 1983). Eutectic behavior and a thermodynamically distinct lipid phase are proposed to exist above 20 mol % Chol (Vist, 1984), although compound formation is favored by others (Mabrey et al., 1978; Jacobs & Oldfield, 1979). More recently, small-angle neutron scattering indicates complete miscibility in the liquid (L_α) phase up to 14 mol % cholesterol while evidence for three (and possibly four) phase boundaries has been obtained for the frozen ($L_{\beta'}$) phase (Knoll et al., 1985). A cholesterol solubility limit is also implicated at 42 mol % cholesterol.

Broadening of the "second" transition to higher temperatures with increasing cholesterol has also been observed by dilatometric measurements (Melchior et al., 1980) and from wide-angle X-ray peak positions (Hui & He, 1983).

In the liquid phase, lateral diffusion rates of phospholipid (egg yolk lecithin) as measured by ^{31}P NMR decrease with increasing cholesterol content (Cullis, 1976). The diffusion rate approaches a constant value at ~25 mol % Chol, which coincides with the disappearance of the "free" phospholipid component. This appears to imply that the phospholipid molecule in the 1:1 tight complex and the phospholipid loosely associated with this complex have similar lateral diffusion characteristics.

With regard to mechanical properties, the only measurements which have been made on lipid bilayer systems containing cholesterol were by Lis et al. (1982). In this study (Lis et al., 1982), it was found that the presence of cholesterol at certain concentrations greatly enhanced the compressibility of bilayers in multilayer arrays. This result, however, is op-

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[1] Abbreviations: Chol, cholesterol; DMPC, dimyristoylphosphatidylcholine; DSC, differential scanning calorimetry; SANS, small-angle neutron scattering; SOPC, stearoyloleoylphosphatidylcholine; RBC, red blood cell(s).

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posite to what we measure here using micropipet pressurization of individual single-walled lipid vesicles. It is generally accepted that the addition of cholesterol condenses and restricts lipid motion above T_c and expands and fluidizes lipid motion below T_c (Demel & Dekruijff, 1976; Vist, 1984). For a series of PC's of varying chain length, the location of cholesterol and ordering (restricted motion) of the hydrocarbon chains near the lipid head group have been observed by X-ray (McIntosh, 1978) and NMR (Godici & Landsberger, 1975). However, excess acyl chain overlap for the longer lipids tends to fluidize the bilayer center.

Our approach in this paper has been to use micropipet aspiration of single giant (2×10^{-3} cm) unilamellar vesicles in order to control and determine the bilayer isotropic tension and the changes in vesicle area (at constant volume) under conditions of varying membrane tension and temperature (Kwok & Evans, 1981; D. Needham and E. Evans, to be published). Hence, we report how cholesterol modifies the vesicle bilayer compressibility, cohesion, and phase transition properties for a series of lipid (DMPC)/cholesterol compositions ranging from 0 to 50 mol % cholesterol. A temperature range of 7–45 °C was covered, thereby including both the pretransition and main acyl chain melting transition of DMPC. X-ray diffraction measurements (wide-angle patterns and electron density profiles) are also included on multilayer specimens to corroborate inferences regarding the effect of cholesterol on area per molecule, chain tilt of gel phase DMPC, and bilayer fluidity.

Experimental Procedures

Two experimental procedures were employed: the thermomechanical behavior of phospholipid/cholesterol bilayers was investigated by micropipet aspiration of giant, unilamellar vesicles, and X-ray diffraction was carried out on hydrated multilayer suspensions.

Vesicle Measurements. Giant lipid vesicles were made from mixtures of dimyristoylphosphatidylcholine (Avanti Polar Lipids Inc., Birmingham, AL) and cholesterol (Sigma) dissolved in a 2:1 chloroform/methanol solution, where the Chol:DMPC ratio ranged from 0 to 50 mol %. The preparation followed a procedure introduced by Reeves and Dowben (1969) which was recently modified to provide a higher yield of unilamellar structures (D. Needham and E. Evans, to be published). Briefly, a clean, roughened Teflon disk was used as a substrate on which to dry the lipid film from the volatile solvent. Fifty microliters of a 10 mg/mL solution of lipid was added to the warm disk with a syringe needle and was quickly spread over the entire surface. Most of the solvent evaporated immediately, leaving the small amount of lipid as a thin film over the whole surface (~ 10 cm^2). The film of lipid was evacuated overnight to remove last traces of solvent. The lipid was prehydrated with water-saturated argon at 35 °C for 30 min (in a loosely parafilm-sealed beaker) and subsequently fully hydrated by adding 10 mL of distilled water at 35 °C; the beaker was left covered, in an oven at the same temperature (i.e., above the gel–liquid-crystalline bilayer phase transition of DMPC), to allow the lipid to swell undisturbed. Vesicles were harvested from the "cloud" which forms in suspension by gentle pasteur pipet aspiration into a 1-mL Eppendorf tube. The suspension was diluted to provide a suitable working concentration of vesicles (i.e., dilute enough so as not to degrate the microscopic image). The dilute vesicle suspension was injected into a temperature-controlled (±0.1 °C) microchamber mounted on the microscope stage. The temperature was monitored by a micro-thermocouple, and heating/cooling rates were 5 °C/min.

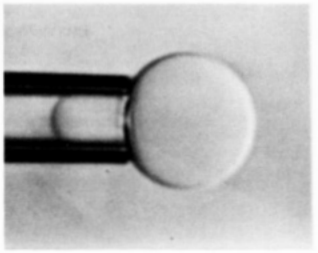

FIGURE 1: Video micrograph of giant (DMPC/Chol, 1:1) bilayer vesicle aspiration. (Vesicle diameter $\sim 30 \times 10^{-4}$ cm; pipet diameter $\sim 10 \times 10^{-4}$ cm.) Vesicle contains 0.18 M sucrose inside and has equiosmolar NaCl outside.

In order to facilitate measurements on single bilayers, only vesicles which appeared most optically transparent (by interference contrast microscopy) were chosen for tests. Then, by subsequent evaluation of membrane elastic modulus, it was possible to discriminate between one, two, or more layers, since the elastic modulus groups around discrete values; the lowest value is characteristic of a single bilayer (Kwok & Evans, 1981).

Vesicle size was typically $(20–30) \times 10^{-4}$ cm diameter. For vesicles which had sufficient excess area (over a sphere of the same volume), aspiration by a 10^{-3} cm diameter micropipet produced a projection inside the pipet as shown in Figure 1. Micropipets were produced from a 1-mm glass tube pulled to a fine point and broken by a glass knife to obtain flat tips. Pipet suction pressure was controlled hydrostatically by micrometer-driven displacement of a water reservoir relative to the pipet tip, giving resolution at microatmospheres. The following two experimental tests were performed: (i) at constant temperature, suction pressure was increased, and the increase in projection length inside the pipet was measured; (ii) at fixed suction pressure, ambient temperature was increased, and again the increase in vesicle membrane projection inside the pipet was measured.

In the first experiment, we obtained a measure of the elastic area compressibility modulus for the various DMPC/Chol bilayers over a range of temperatures both below and above the DMPC gel to liquid-crystalline phase transition and the membrane tension required to cause lysis. In the second experiment, we observed directly membrane area changes due to lipid phase transitions and thermal expansivities in the different phases.

Multilayer Suspensions. DMPC and cholesterol were used as obtained from Sigma. Appropriate lipid mixtures were codissolved in chloroform and rotary evaporated to remove all solvent. Multilamellar suspensions were prepared by adding excess (70% by weight) doubly distilled water, vortexing, and allowing the lipid/water suspensions to incubate above the lipid's main phase transition temperature for several hours. The specimens were sealed in quartz X-ray capillary tubes and mounted in a temperature-regulated specimen holder in a pinhole collimation X-ray camera. Diffraction patterns were recorded on Kodak DEF X-ray film. Specimen to film distance was 10 cm, and exposure times were on the order of 5 h. The films were densitometered with a Joyce–Loebl microdensitometer, Model MKIIIC, and integrated intensities, $I(h)$, where h is the diffraction order, were obtained as described previously (McIntosh, 1980). For these unoriented specimens, the structure amplitude for each order h was set equal to $[h^2 I(h)]^{1/2}$. Electron density profiles were calculated by using the phase angles determined by Janiak et al. (1979). Similar measurements were also made on 1:1 DMPC/dodecane in excess water, in order to obtain structural parameters for DMPC bilayers with no hydrocarbon chain tilt.

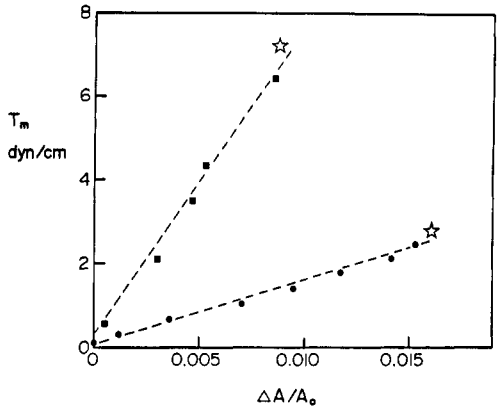

FIGURE 2: Vesicle bilayer tension induced by pipet suction pressure versus relative area change, comparing pure L_α phase DMPC with a 1:1 DMPC/Chol mixture. The slope gives the elastic area compressibility modulus, K. Stars indicate tension levels at vesicle lysis (rupture). (■) DMPC/Chol, 1:1 (15 °C), K = 685 dyn/cm; (●) DMPS (29 °C), K = 145 dyn/dm; (☆) lysis.

Methods of Analysis and Results

Vesicles. In each experiment, area changes were derived from aspiration lengths in the micropipet either as a function of membrane tension *at* constant temperature or as a function of temperature *at* constant tension.

Membrane tension (T_m) is uniform over the entire vesicle surface and is given by the pipet suction pressure (P) and the pipet/vesicle geometry:

$$T_m = PR_p/(2 - 2R_p/R_o)$$

where R_p is the pipet radius and R_o is the radius of the outer spherical segment of the vesicle.

Changes in vesicle membrane projection length (ΔL) inside the pipet are a direct measure of the fractional change in total vesicle membrane area (ΔA):

$$\Delta A = 2\pi R_p(1 - R_p/R_o)\Delta L$$

This relationship is valid only if the volume of the vesicle is constant. Changes in volume (due to filtration of water by pipet suction) were found to be negligible when a vesicle was held under maximum suction for periods well in excess of the duration of the experiment. This was expected because of the limited permeability of the membrane and the relatively low suction pressures involved in these experiments. Since the number of molecules in the membrane is fixed (due to extremely low lipid solubility in aqueous media), changes in vesicle area represent changes in surface area per lipid molecule. Consequently, we are able to determine the change in vesicle membrane area in response to a change in membrane tension at constant temperature, as shown in Figure 2. The slope of each plot yields the elastic area compressibility modulus (K), and the maximum tension at vesicle rupture gives the lysis tension (T_{lysis}). Compared with L_α phase DMPC, the 50 mol % Chol mixture was much less compressible and exhibited lysis tensions approximately 3–5 times higher. Table I shows that compared to the L_α phase the compressibility modulus was found to be elevated both above and below the main transition by the addition of cholesterol and approached values comparable to the solid, crystalline L_β' phase of the pure lipid when 50 mol % Chol was present. However, for all concentrations above 12.5 mol % (which was only weakly solid), the vesicle bilayers behaved as liquids with *no* surface shear rigidity even at temperatures well below the DMPS phase transition.

The change in vesicle membrane area with change in ambient temperature at constant suction pressure was used to map out the lipid phase transitions and the thermal area expansivity both above and below the transition for pure DMPC. Figure 3 is a plot of vesicle area vs temperature which shows the effect of increasing cholesterol content on the main acyl chain crystallization transition of DMPC. Vesicle areas in Figure 3 are arbitrarily expressed relative to the area measured at 20 °C where the pure DMPC vesicle surface was forced into a planar L^*_β' crystalline state (i.e., the P_β' ripples were "pulled flat"); the acyl chains are therefore tilted with respect to the bilayer normal to an angle of 25° (D. Needham and E. Evans, to be published).

Table I: Thermoelastic Properties of DMPC/Chol Mixtures

composition	area compressibility modulus, K (dyn/cm)	thermal area expansivity, α ($\times 10^3$/°C)	temp, T (°C)
DMPC (L_α)	144 ± 10.5	6.81 ± 1.0	29
DMPC(L_α)		4.17 ± 0.2	35
DMPC (L_β')	855.3 ± 140.1	1.0	8
Chol/(DMPC + Chol)			
0.125	396.9	2.83	15.5
0.33	646.9	1.97	15
0.33	559.0	3.1	25
0.40	600	2.3	35
0.50	685	1.33	22
0.50	633		25

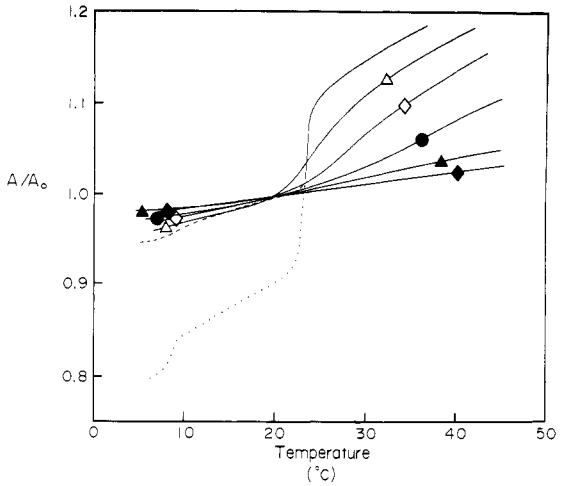

FIGURE 3: Relative areas derived from single-vesicle heating/cooling experiments for mixed DMPC/Chol bilayers. Also shown is the area–temperature plot for pure DMPC vesicles (D. Needham and E. Evans, unpublished results). Chol/(Chol + DMPC) ratio is (△) 0.125, (◇) 0.25, (●) 0.33, (▲) 0.40, and (◆) 0.50, (---) DMPC L^*_β' phase; (···) DMPC P_β' phase; (—) DMPC L_α phase.

As cholesterol concentration was increased, the transition broadened (the liquidus line shifted to higher temperatures), and the apparent transition area change decreased. Also, a small shift of the onset of the transition (solidus line) to lower temperatures was observed. This is consistent with other experimental phase diagrams determined by calorimetry and NMR. No transition area change was detectable at 50 mol % Chol, where the thermal area expansivity was 1.33 × 10^{-3}/°C.

The tension level at vesicle lysis for 50 mol % Chol was increased to >8 dyn/cm, from ~2 dyn/cm for the pure L_α-state lipid. Also, the bilayer permeability (as evidenced by the extremely slow response of vesicles to osmotic dehydration) was significantly reduced.

Multilayer Suspensions. The diffraction pattern of fully hydrated DMPC at 10 ± 2 °C consists of 5 lamellar orders of a periodicity of 61.6 Å and a sharp wide-angle reflection at 4.21 Å with a broad band centered at about 4.1 Å. This

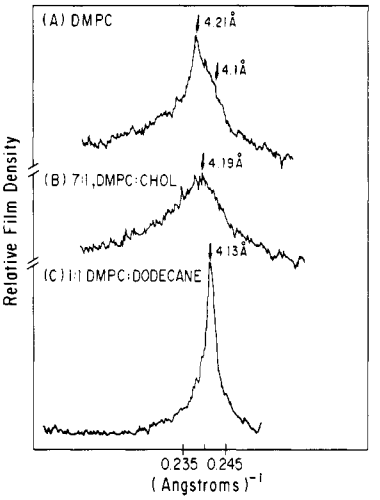

FIGURE 4: Densitometer traces of wide-angle diffraction patterns from (A) DMPC, (B) DMPC containing 12.5 mol % cholesterol, and (C) DMPC containing 50 mol % dodecane. All patterns were recorded at 10 °C.

pattern is very similar to that reported by Janiak et al. (1979). The double wide-angle reflection is characteristic of the $L_{\beta'}$ phase of saturated phosphatidylcholines, where the hydrocarbon chains are in an ordered "quasi-hexagonal" array and tilted relative to the bilayer normal (Tardieu et al., 1973). The introduction of cholesterol changes both the lamellar and wide-angle diffraction patterns. For 12.5 mol % Chol at 10 ± 2 °C, the X-ray pattern consists of 5 lamellar orders of a periodicity of 69.3 Å and a single wide-angle band centered at 4.19 Å. Densitometer traces of the wide-angle regions of the diffraction patterns for both DMPC and DMPC with 12.5 mol % Chol are shown in Figure 4A,B. For 50 mol % Chol at 10 °C, the lamellar repeat period is 61.7 Å, and the wide-angle pattern contains a very broad band centered at about 4.5 Å, characteristic of lipids with liquid acyl chains (data now shown).

Electron density profiles for DMPC and DMPC with 12.5 and 50 mol % Chol are shown in Figure 5A–C. In each profile, the high-density peaks correspond to the lipid polar head groups. The lowest density dip in the center of each profile corresponds to the localization of terminal methyl groups in the geometric center of the bilayer, and the medium density regions between the head-group peaks and the terminal methyl trough correspond to the methylene chain regions of the bilayer. The electron density of the methylene chain region is increased by the incorporation of cholesterol, consistent with the localization of cholesterol in this region of the bilayer (McIntosh, 1978; Franks, 1976). The medium density regions at the edges of the profile correspond to the fluid spaces between adjacent bilayers. In these profiles, the head-group peak separation across the bilayer is 39.5 Å for DMPC, 40.8 Å for 12.5 mol % Chol, and 42.0 Å for 50 mol % Chol. This can be compared to the 40-Å head-group separation determined for DMPC by Janiak et al. (1979). The fluid space between bilayers is largest for the multilayers containing 12.5 mol % Chol.

In order to assess the effect of cholesterol on bilayer width, it was useful to have electron density profiles for a DMPC bilayer with no chain tilt (McIntosh, 1978). Incorporation of long-chain alkanes such as dodecane into the bilayer removes chain tilt and causes only a small change in the main transition temperature (McIntosh et al., 1980). For 1:1 DMPC/dodecane in excess water, a repeat period of 64.9 Å and a sharp wide-angle reflection at 4.13 Å were obtained (Figure 4C).

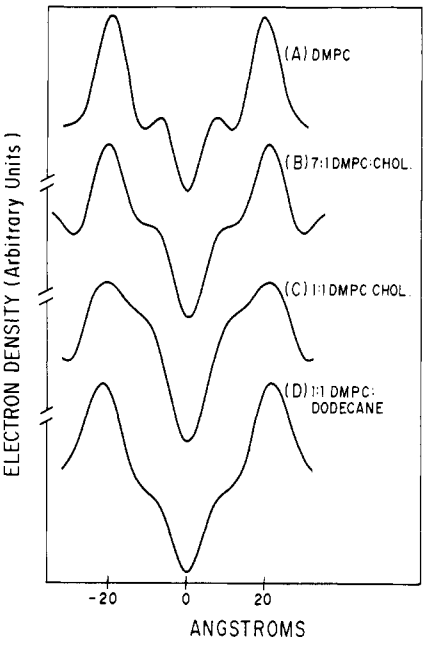

FIGURE 5: Electron density profiles for (A) DMPC, (B) DMPC containing 12.5 mol % cholesterol, (C) DMPC containing 50 mol % cholesterol, and (D) DMPC containing 50 mol % dodecane. The origin is at the geometric center of each bilayer.

The electron density profile shown in Figure 5D has a head-group peak separation of 43.2 Å.

Discussion

A major observation from the vesicle experiments is that for all concentrations above 12.5 mol % Chol (which was only weakly solid), the bilayers showed no surface shear rigidity even at temperatures well below the DMPC phase transition. This implies that for high cholesterol concentrations (>25 mol %), the bilayers remain fluid; i.e., they behave as surface liquids. Below 25 mol %, patches of frozen DMPC may also exist in this liquid phase.

Both above and below the observable transition, the elastic area compressibility modulus (K) is greatly increased compared to the L_α phase of DMPC as the cholesterol content is increased (Table I and Figure 2). The value for the 1:1 complex (i.e., 50 mol % Chol bilayer) was found to be comparable to that for DMPC in the $L_{\beta'}$ crystalline phase, and the bilayer was still fluid. Not only was the area compressibility reduced by the addition of cholesterol, but also the bilayer cohesion was greatly increased on the basis of tension levels required for vesicle lysis. This general feature has been seen in all of our vesicle studies; i.e., the tension (mechanical stress) levels at lysis increase as the elastic area compressibility is reduced. The results are consistent with a simple critical surface density fluctuation model for the origin of membrane failure (Evans & Needham, 1987). A similar increase in cohesion over that shown by the pure phospholipid has been measured in SOPC/Chol bilayers (Evans & Needham, 1987). The compressibility of the whole RBC membrane (whose lipid component is ~1:1 phospholipid/cholesterol) is greater than that of the purely 1:1 phospholipid/cholesterol bilayers measured here (Waugh & Evans, 1979). Thus, in the RBC membrane, some compressibility must be introduced by defects, i.e., the integral membrane proteins and their surrounding lipid.

Vesicle area versus temperature plots (Figure 3) show that the transition broadens and the liquidus line shifts to higher temperatures, while the solidus line falls to ~20 °C with

increasing cholesterol concentration, in agreement with regular solution theory. For a more detailed discussion, see Hjort Ipsen et al. (1987). These results are in general agreement with scanning calorimetry (Mabrey et al., 1978; Vist, 1984), dilatometry (Melchior et al., 1980), and NMR (Vist, 1984) studies where greater resolution reveals a free lipid component up to cholesterol concentrations of ~20–25 mol % and a higher temperature phase (Vist, 1984), of undefined structure, which probably represents lipid loosely associated with a 1:1 DMPC/Chol complex (Hui & He, 1983). For pure DMPC (D. Needham and E. Evans, to be published), the magnitude of the area change at the liquid-crystalline to gel phase transition represents both acyl chain condensation and the introduction of chain tilt with respect to the bilayer plane. In the P_{β}' phase, the chains line up parallel to the normal to the projected plane because of a surface ripple or superlattice. When these ripples are removed (either by mechanical stress or at low temperatures), the chains become tilted with respect to the projected plane, and the projected area is increased. Upon the addition of even small amounts of cholesterol, the pretransition and P_{β}' phase ripple disappear which indicates a tilted geometry for any frozen lipid (Estep et al., 1978; Knoll et al., 1985; Copeland & McConnell, 1980). The total area change over the main transition is reduced by cholesterol and disappears at 50 mol %, where the thermal expansivity appears constant at about $1.33 \times 10^{-3}/°C$.

The magnitude of the projected area change as measured in these micropipet experiments can be rationalized by a simple geometric model in which the area contributions due to three bilayer components are summed (D. Needham and E. Evans, to be published; McIntosh, 1980; Tardieu et al., 1973; Demel et al., 1972). As outlined in the introduction, certain evidence implies the existence of three species:

(i) An untilted DMPC/Chol complex of 1:1 stoichiometry of as yet undefined nature is one line of evidence. The "tight" or time-averaged specific association may involve βOH hydrogen bonding at the head group (Huang, 1976), and/or enhanced van der Waals interaction between the planar rings of cholesterol and the all-trans upper chain segments of the phospholipid, and/or a steric association in which the larger phospholipid chains overlap and "hook" under the shorter cholesterol (A. Georgallas and M. J. Zuckermann, personal communication).

At 50 mol % Chol, that is, when all the bilayer consists of this 1:1 tight complex, no area transition or enthalpy change is observed in the temperature range studied (Mabrey et al., 1978). X-ray diffraction shows that the bilayer remains in a liquid phase to temperatures as low as 10 °C. The complex exhibits a small thermal expansivity of $1.33 \times 10^{-3}/°C$.

(ii) A free DMPC species up to 20–25 mol % Chol is additional evidence. This "free" phospholipid is evidenced, for example, by the disappearance of a sharp calorimetric peak centered at 24 °C (Estep et al., 1978; Mabrey et al., 1978) and the attainment of a constant lateral lipid diffusion rate at the same concentration (Cullis, 1976). This phospholipid is expected to undergo freezing to form crystallite domains of tilted, ordered gel phase.

(iii) A third line of evidence is DMPC which is "loosely" associated (and thereby perturbed) with the 1:1 DMPC/Chol complex. It is assumed to give rise to the broad calorimetric peak (Estep et al., 1978; Mabrey et al., 1978) and would be the B phase according to Vist (1984). In the present geometric model, this phospholipid undergoes the same total area change with temperature as free phospholipid but forms a tilted disordered gel phase.

Thus, with increasing cholesterol concentration, the free phospholipid component forms smaller and smaller ordered crystalline domains while the loosely associated phospholipid and tightly associated phospholipid form a disordered gel phase. Such behavior is consistent with the observation that at 12.5 mol % Chol vesicles were only weakly solid and at higher concentrations the bilayers behaved as surface liquids with zero shear rigidity, indicating breakdown of long-range two-dimensional crystalline order. The multiple phases observed below T_c by SANS (Knoll et al., 1985) are not seen by our measurements of membrane area, presumably because upon crossing these purported gel phase boundaries there is no change in area per molecule or molecular tilt.

It appears that the free and loosely associated phospholipid species freeze into a tilted geometry. Support for this conclusion comes from the X-ray measurements which also indicate that at 10 °C the 12.5 mol % Chol bilayer is in a gel state with tilted hydrocarbon chains. Previously, it has been shown by analysis of the double wide-angle diffraction electron density profiles and by the partial lipid thickness as obtained from measurements of water content and repeat period (Janiak et al., 1976) that the hydrocarbon chains of DMPC in the L_{β}' phase have about a 35° chain tilt [also confirmed by stress history experiments (D. Needham and E. Evans, to be published)]. Since the bilayer width, as measured by peak to peak separation in the electron density profiles (Figure 5), is similar for both DMPC and 12.5 mol % Chol, and is smaller than the peak to peak separation in 1:1 DMPC/dodecane which has untilted chains (McIntosh, 1980), the cholesterol-containing bilayer must also have tilted hydrocarbon chains. Consistent with this is the width of the single wide-angle reflection observed for DMPC/Chol (Figure 4). This reflection is considerably broader than the wide-angle reflections observed from gel-state lipids which have untilted hydrocarbon chains, such as dipalmitoylphosphatidylethanolamine or 1:1 DMPC/dodecane (McIntosh, 1980) (Figure 4). It has been shown that tilting of the hydrocarbon chain relative to the normal to the plane of the bilayer causes a broadening of the wide-angle reflection (Tardieu et al., 1973). Therefore, the change in wide-angle pattern observed in Figure 4 indicates that cholesterol modifies the hydrocarbon chain packing, converting the double wide-angle reflection characteristic of a "quasi-hexagonal" arrangement of tilted hydrocarbon chains to a single broad reflection, consistent with a hexagonal packing arrangement of tilted chains (Tardieu et al., 1973).

A partial disordering of the hydrocarbon chains in the center of the bilayer would also tend to decrease the thickness of DMPC bilayers containing cholesterol. This explains why liquid-crystalline bilayers with 50 mol % Chol are not as wide as untilted gel-state bilayers (Figure 5). However, chain tilt must be the major factor in decreasing the width of DMPC bilayers containing 12.5 mol % Chol since these bilayers are not as wide as the liquid-crystalline bilayers containing 50 mol % Chol. That is, although both chain tilt and partial disordering of the bilayer interior would decrease the width of DMPC bilayers containing 12.5 mol % Chol, the relative widths of this bilayer (Figure 5B) and fully tilted gel phase bilayers (Figure 5A), untilted gel bilayers (Figure 5C), and liquid-crystalline bilayers (Figure 5D) indicate that a significant degree of chain tilt must be present in frozen bilayers containing free and loosely associated DMPC.

Conclusions

Cholesterol forms a tight complex with DMPC, which greatly reduces bilayer compressibility and permeability compared to liquid-crystalline-state bilayers, but maintains

a liquidlike (fluid) state, in that no surface shear rigidity is observed, even at temperatures well below the acyl chain crystallization temperature of the phospholipid. The outer regions of the acyl chains therefore appear to be condensed, thereby tightening up the surface, but the central bilayer region seems to remain fluid.

A simple geometric model explains the area data and liquid character of the DMPC/Chol bilayers. Each cholesterol appears to interact strongly with one phospholipid molecule, forming a "tight" 1:1 complex, while the remaining "uncomplexed" phospholipid is either free (up to ~25 mol % Chol) or loosely associated with the 1:1 complex. In the low-temperature region, the free phospholipid freezes to form an ordered gel phase while loosely associated phospholipid together with the 1:1 complex forms a disordered gel phase with no shear rigidity.

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