The Influence of Cholesterol on Phospholipid Membrane Curvature and Bending Elasticity

Z. Chen and R. P. Rand
Department of Biological Sciences, Brock University, St. Catharines, Ontario L2S 3A1, Canada

ABSTRACT The behavior of dioleoylphosphatidylethanolamine (DOPE)/cholesterol/tetradecane and dioleoylphosphatidylcholine (DOPC)/cholesterol/tetradecane were examined using x-ray diffraction and the osmotic stress method. DOPE/tetradecane, with or without cholesterol, forms inverted hexagonal (H_{II}) phases in excess water. DOPC/tetradecane forms lamellar phases without cholesterol at lower temperatures. With tetradecane, as little as 5 mol% cholesterol in DOPC induced the formation of H_{II} phases of very large dimension. Increasing levels of cholesterol result in a systematic decrease in the H_{II} lattice dimension for both DOPE and DOPC in excess water. Using osmotic pressure to control hydration, we applied a recent prescription to estimate the intrinsic curvature and bending modulus of the H_{II} monolayers. The radii of the intrinsic curvature, $R_p°$, at a pivotal plane of constant area within the monolayer were determined to be 29.4 Å for DOPE/tetradecane at 22°C, decreasing to 27 Å at 30 mol% cholesterol. For DOPC/tetradecane at 32°C, $R_p°$ decreased from 62.5 Å to 40 Å as its cholesterol content increased from 30 to 50 mol%. These data yielded an estimate of the intrinsic radius of curvature for pure DOPC of 87.3 Å. The bending moduli k_c of DOPE/tetradecane and DOPC/tetradecane, each with 30 mol% cholesterol, are 15 and 9 kT, respectively. Tetradecane itself was shown to have little effect on the bending modulus in the cases of DOPE and cholesterol/DOPE. Surprisingly, cholesterol effected only a modest increase in the k_c of these monolayers, which is much smaller than estimated from its effect on the area compressibility modulus in bilayers. We discuss possible reasons for this difference.

INTRODUCTION

Cholesterol is found in almost all mammalian cells; the human erythrocyte plasma membrane contains as much as 45 mol% cholesterol (Cooper et al., 1978). Cholesterol's physiological significance is not clear, but many studies show that cholesterol alters a number of phospholipid membrane physicochemical properties.

^{2}H-NMR and x-ray studies have shown that cholesterol is oriented perpendicular to the membrane surface, with its 3β-hydroxyl group located in the vicinity of the phospholipid ester carbonyl groups, and the hydrophobic steroid ring oriented parallel to the acyl chains of phospholipids (Rand and Luzzati, 1968; Taylor et al., 1981; Dufourc et al., 1984; Franks, 1976; Worcester and Franks, 1976). The hydrophobic tail of cholesterol experiences relatively rapid motional rates among several conformations, in contrast to the steroid ring, which is confined to a single conformation (Opella et al., 1976; Kroon et al., 1975). Cholesterol can induce a liquid-crystal state in lipids that would otherwise form a gel state and induce a less fluid state in disordered hydrocarbon chains (Oldfield and Chapman, 1972). Cholesterol decreases the molecular area of liquid-crystalline phospholipid monolayers composed of saturated and monounsaturated chains (the condensing effect) (Demel and Van Deenen, 1977), but has no effects on 18:2, 18:2 phosphatidylcholine (PC) or on 18:3, 18:3PC (Bittman et al., 1984).

Cholesterol is found to destabilize PE and PE-PC bilayers and to induce the formation of the H_{II} phase in these systems (Tilcock et al., 1984; Cullis and De Kruijff, 1978; Noordam et al., 1980; Gallay and De Kruijff, 1982; Simon et al., 1982). The incorporation of cholesterol into polyunsaturated PC (C18:3, C20:4, C22:6) (Dekker et al., 1983) and into glucolipid mixtures extracted from *Acholeplasma laidlawii* (Khan et al., 1981; Rilfors et al., 1987) can promote the H_{II} transition. Cholesterol reduces the amount of diacylglycerol required to induce the lamellar-hexagonal transition (Coorssen and Rand, 1990).

The significance of H_{II}-prone lipids in membranes is not clear. The fact that the lipid composition of several biological membranes appears to be regulated so that the membrane is close to, but below, the bilayer-to-hexagonal phase transition temperature (T_H) (Lindblom et al., 1993; Rilfors et al., 1994; Rietveld et al., 1994) suggests that this physical property of the membrane plays an important role in the modulation of membrane function. This is supported by the finding that the activity of a number of enzymes is increased (Cornell, 1991; Senisterra and Epand, 1993; McCallum and Epand, 1995) and membrane channel gating changed (Keller et al., 1993) in the presence of non-lamellar-forming lipids. The activity of Ca-ATPase, reconstituted into liposomes composed of lipid mixtures, was found to increase upon increasing the amount of cholesterol (Cheng et al., 1986). Membrane fusion must involve changes in membrane curvature during the formation of structural intermediates (Siegel, 1993; Chernomordik et al., 1995).

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Address reprint requests to Dr. R. P. Rand, Department of Biological Sciences, Brock University, St. Catharines, ON L2S 3A1, Canada. Tel.: 905-688-5550, X3390; Fax: 905-688-1855; E-mail: rrand@spartan.ac.brocku.ca.

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Little is known about the effects of cholesterol on the structural dimensions and the energetics of H_{II} phases. We have investigated, by x-ray diffraction and osmotic stress, the effect of cholesterol on the DOPE and DOPC H_{II} phases. Small amounts of alkanes in phospholipids facilitate the formation of H_{II} phase in lipid-water mixtures (Kirk and Gruner, 1985; Gruner et al., 1985; Sjolund et al., 1987, 1989) by providing hydrocarbons to fill the intersticies between cylinders to relieve the hydrocarbon chain packing stress (Rand et al., 1990). In this study, a constant amount of tetradecane was used to relieve any hydrocarbon packing stress and allow the lipid to express its spontaneous curvature by forming the H_{II} phase. Tetradecane otherwise has only a small effect on the structural dimensions of the H_{II} phase and, as we show, on the bending modulus of hexagonal phase monolayers. It has been shown that cholesterol substantially increases the area compressibility and the derived bending modulus of bilayers (Evans and Needham, 1987; Evans and Rawicz, 1990; Needham and Nunn, 1990).

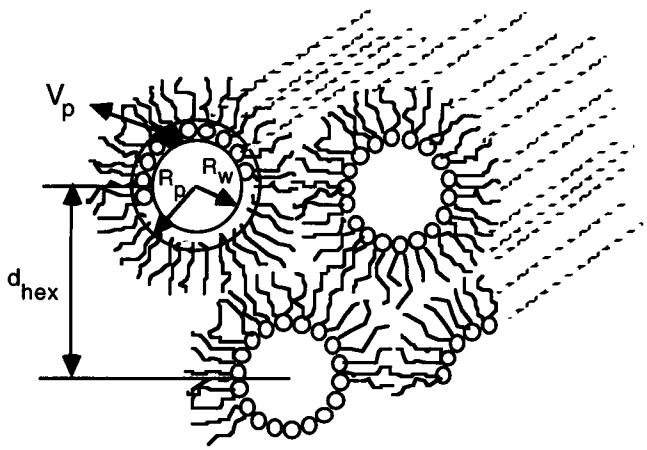


FIGURE 1 Schematic diagram of the structure of the hexagonal phase, showing the dimensions determined as described in the text and used for the structural and energetic analysis.

MATERIALS AND METHODS

Sample preparation

Synthetic L-α-dioleoylphosphatidylethanolamine (DOPE), L-α-dioleoylphosphatidylcholine (DOPC), and cholesterol were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. The lipid was checked for impurities by thin-layer chromatography and judged to be at least 98% pure. Tetradecane is a product of Sigma Chemical Co. (St. Louis, MO).

The lipids were stored under nitrogen at −18°C until used. Lipid mixtures were produced by combining the appropriate amounts of DOPE or DOPC with cholesterol, using stock solutions in chloroform, and then removing the solvent, first by rotary evaporation and then under vacuum. Tetradecane was added to these dry mixtures by weighing directly. The concentration of tetradecane was kept constant at 16% of the total dry weight of the sample. After 48 h of equilibration with tetradecane, dry lipid mixtures were hydrated by adding either known weights of double-distilled water or excess amounts of polyethylene glycol solutions of known osmotic pressure, sealing, and equilibrating them at room temperature for 48 h. Each sample was reweighed, combined with some powdered Teflon as an x-ray calibration standard, and then sealed between mica windows 1 mm apart.

X-ray diffraction

X-ray diffraction was used to characterize the structures formed by the hydrated lipid. The $CuK_{\alpha 1}$ line (λ = 1.540 Å), from a Rigaku rotating anode generator, was isolated by using a bent quartz crystal monochromator, and diffraction patterns were recorded photographically with Guinier x-ray cameras operating in vacuo. The sample temperature was controlled with thermoelectric elements to approximately ±0.5°C. All samples formed hexagonal phases characterized by at least three x-ray spacings bearing ratios to the dimension of the first order, d_{hex}, of 1, $1/\sqrt{3}$, $1/\sqrt{4}$, $1/\sqrt{7}$, $1/\sqrt{9}$, $1/\sqrt{12}$, etc. d_{hex} is measured to ±0.1 Å on any one sample: sample-to-sample variation, approximately ±2%, represents experimental error in sample composition.

Structure analysis

H_{II} phases are two-dimensional hexagonal lattices formed by the axes of indefinitely long, parallel, regular prisms (Fig. 1). Water cores, centered on the prism axes, are lined with the lipid polar groups, and the rest of the lattice is filled with the hydrocarbon chains. Here the cross-sectional shape of the water core prism within that lattice is assumed to be circular, although it has been shown that that the cross section can be distorted from circularity (Turner et al., 1992).

For a hexagonal phase of known composition, the measured lattice can be divided into compartments, as shown in Fig. 1, each containing defined volume fractions of the lipid and water. This volume average division follows the method originally introduced by Luzzati (e.g., see Luzzati and Husson, 1962) and depends only on a knowledge of the specific volumes of the molecular components and their relative amounts, and on the assumption of their linear addition. For the lipid components in this study, some physicochemical and structural parameters are listed below:

	DOPE	DOPC	Chol	td
MW	744	786	387	198
Density (g/cm^3)	1	1.01	0.94	0.76
Sp. vol. (cm^3/g)	1	0.99	1.06	1.31
Mol. vol. (Å^3)	1235	1292	605	431

The water and lipid compartments are divided by an idealized cylindrical interface of radius R_w that encloses a volume equal to the volume of water in the H_{II} phase. We refer to the surface of this cylinder as the Luzzati plane. The radius of the water cylinder, R_w, is related to the first-order Bragg spacing in the hexagonal phase, d_{hex}, and to the volume fraction of water in the sample, ϕ_w, as follows:

$$R_w = d_{hex}\sqrt{\frac{2\phi_w}{\pi\sqrt{3}}}. \quad (1)$$

The area per lipid molecule at the Luzzati plane is given by

$$A_w = \frac{2\phi_w V_l}{(1-\phi_w)R_w}, \quad (2)$$

where V_l is the volume of a lipid molecule, and where

$$\phi_w = \frac{V_{water}}{V_{water} + V_{PL} + V_{cholesterol} + V_{tetradecane}}.$$

We use the notion of an effective molecule that is one phospholipid, (DOPE or DOPC) + x-cholesterol + y-tetradecane, where x is the molar

ratio of cholesterol to phospholipid, and y is the molar ratio of tetradecane to phospholipid in the samples. The effective molecular volume, $V_L = V_{PL} + xV_{cholesterol} + yV_{tetradecane}$. For the mixtures used in this study, tetradecane was constant at 16% of the total dry weight and ratios of DOPE/chol were as follows:

DOPE/chol	10/0	9/1	8/2	7/3
x	0	0.11	0.25	0.428
y	0.714	0.757	0.809	0.875
V_l (Å^3)	1543	1628	1735	1870

DOPC/chol	4/0	4/1	4/2	4/3	4/4
x	0	0.25	0.5	0.75	1
y	0.756	0.849	0.94	1.035	1.128
V_l (Å^3)	1631	1818	2012	2205	2396

Elastic energy of the hexagonal phase

The H_{II} phase is usually described in terms of curvature and molecular area on either of two cylindrical surfaces lying inside the lipid monolayer (Fig. 1):

1. A neutral surface of bending where the bending and stretching (compression) deformations are energetically uncoupled (Kozlov and Winterhalter, 1991a,b);

2. A pivotal plane where the molecular area remains constant (Rand et al., 1990). Here we analyze the experimental data using the pivotal plane, because its position can be found from the data with much higher accuracy (Leikin et al., 1996)

Using the radius of curvature at the pivotal plane, R_p, the elastic free energy, F, of the hexagonal phase (normalized per lipid molecule) can be approximated by the energy of bending (Helfrich, 1973; Kirk et al., 1984):

$$F = \frac{1}{2} k_{cp} A_p \left(\frac{1}{R_p} - \frac{1}{R_{0p}} \right)^2 \tag{3}$$

where k_{cp} is the bending modulus, and A_p and R_{0p} are the molecular area and the spontaneous radius of curvature at the pivotal plane. The goal of the measurement is to find the position of the pivotal plane, the spontaneous curvature, molecular area, and bending moduli for different phospholipid/cholesterol mixtures.

We have very recently described a recipe for determining these structural parameters and elastic moduli (Leikin et al., 1996).

1. The molecular area, A, and radius of curvature, R, at any cylindrical dividing surface, separated by a volume V per lipid molecule from the Luzzati plane (Fig. 1), are given by

$$A^2 = A_w^2 + 2V\frac{A_w}{R_w} \quad \text{or} \quad A = A_w\sqrt{1 + \frac{1-\phi_w}{\phi_w}\frac{V}{V_l}} \tag{4}$$

$$R = R_w\sqrt{1 + \frac{1-\phi_w}{\phi_w}\frac{V}{V_l}}. \tag{5}$$

2. We verify whether the system has a well-defined pivotal plane. From Eq. 4, in a form that uses normalized areas (Leikin et al., 1996),

$$\frac{A_w^2}{V_l^2} = \frac{A_p^2}{V_l^2} - 2\frac{V_p}{V_l}\left(\frac{A_w}{V_l R_w}\right) \tag{6}$$

we establish whether the plot $(A_w/V_L)^2$ versus $(A_w/V_L)R_w$ is a straight line. If so, the system has a dividing surface of constant area that is the pivotal plane. We determine its location from V_p, the volume separating this plane and the Luzzati plane, and the molecular area (A_p), from the slope and the intercept of the plot.

3. From the value of V_p we calculate the radii of curvature (R_p) at the pivotal plane by using Eq. 5. We use these radii and follow the previously suggested procedure (Gruner et al., 1986; Rand et al., 1990) for determining the elastic parameters of the lipid mixture from osmotic stress experiments. Specifically, comparing the elastic energy given by Eq. 3 with the osmotic work done by the osmotic stress (Π), we find the following relationship:

$$\Pi R_p^2 = 2k_{cp}\left(\frac{1}{R_p} - \frac{1}{R_{0p}}\right). \tag{7}$$

The plot of (ΠR^{p2}) versus ($1/R_p$) gives, simultaneously from the slope and the intercept, the monolayer bending modulus (k_{cp}) and the spontaneous curvature ($1/R_{0p}$) (Gruner et al., 1986; Rand et al., 1990). We use the subscript p to be consistent with our previous notation and particularly to emphasize that these measurements are at the pivotal plane and not at the neutral plane of the monolayer.

RESULTS

We have used 16 wt% tetradecane throughout this study to be consistent with our earlier studies. The tetradecane is required to allow the hexagonal phase to form by reducing chain stress, particularly for small-curvature lipids, where it fills larger interstitial spaces otherwise inaccessible by the phospholipid chains. However, we have noted that even for pure DOPE itself, a lipid of large curvature and near its intrinsic curvature even without tetradecane, its lattice dimension increases continuously with added tetradecane. In excess water d_{hex} increases from 64 to 67 Å with 16 wt% added tetradecane.

For cholesterol contents up to cholesterol/DOPE 1/1, hexagonal phases are found with and without 16% tetradecane at all water contents. However, for cholesterol/DOPE ratios above 3/7, that hexagonal phase coexists with dry crystalline cholesterol. We conclude that the DOPE hexagonal phase can accommodate up to about one cholesterol molecule for every two DOPE molecules.

Without tetradecane, DOPC with up to equimolar cholesterol and up to 50°C forms only lamellar structures in excess water. However, with 16% tetradecane, hexagonal phases of large dimension form. Fig. 2 shows the phase diagram of cholesterol/DOPC mixtures, all containing 16% tetradecane and in excess water. The lamellar-hexagonal transition depends on temperature and cholesterol content. At higher temperatures, DOPC alone forms coexisting hexagonal and lamellar phases. For cholesterol/DOPC ratios up to 1/4, and at lower temperatures, the hexagonal phases coexist with lamellar phases. Pure hexagonal phases exist at higher cholesterol contents. An unexpected region of disorder occurs at the cholesterol/DOPC ratio of 1/3, where the x-ray diffraction lines disappear. On each side of that region the x-ray diffraction lines are sharp and the hexagonal lattice dimension appears to change continuously through it (Fig. 3). For cholesterol/DOPC ratios greater than 1/1, crystalline cholesterol separates from the hexagonal phase. Therefore DOPC hexagonal phases appear to accommodate more cholesterol than DOPE. We conclude that cholesterol endows DOPC monolayers with intrinsic curvature sufficient to result in the formation of the hexagonal phase when

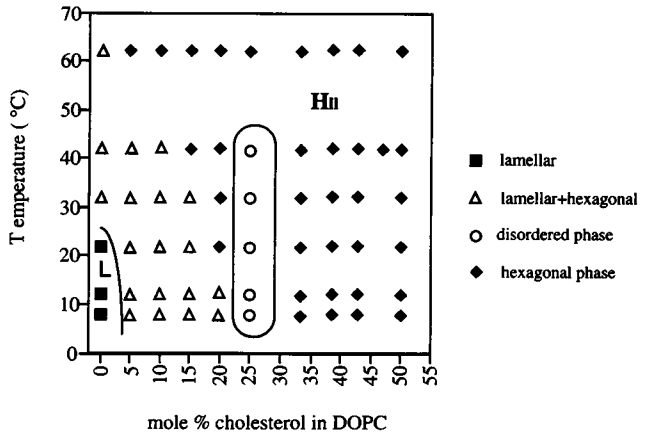


FIGURE 2 Phases formed by dioleoylphosphatidylcholine containing increasing amounts of cholesterol. Sixteen percent of the dry weight of every sample was tetradecane. All samples were suspended in excess water. Higher cholesterol contents resulted in separation of crystalline cholesterol coexisting with the hexagonal phase.

tetradecane is available to fill the interstitial spaces. The dimensions of these hexagonal phases are shown in Fig. 3

Fig. 3 shows that in excess water and with tetradecane, increasing amounts of cholesterol produce a systematic decrease in the lattice dimension of the hexagonal phase formed with either DOPE or DOPC. In the case of DOPE, the dimension change is systematic but relatively small. In the case of DOPC, hexagonal lattices of very large dimension form at low cholesterol contents. That dimension decreases continuously with increasing cholesterol content, even with the "intervention" of the unusual region of disorder wherein it cannot be measured.

Gravimetric phase diagrams

Fig. 4 shows the hexagonal dimension (d_{hex}) as a function of water volume fraction (ϕ_w) of gravimetrically prepared samples of DOPE or DOPC containing four different concentrations of cholesterol. For all of these mixtures, 16% of the dry weight was tetradecane. All traces of a coexisting lamellar phase, seen in the case of DOPC at room temperature, were eliminated at 32°C, and d_{hex} changed insignificantly with that temperature change.

Remarkably, for both DOPE and DOPC, regardless of cholesterol content, the lattice dimension of the hexagonal phase was the same at any fixed ϕ_w that was less than excess water. Consequently, for each lipid we pooled the data for all four cholesterol contents to get a common relation between lattice dimension and water content. We use this relation to determine the water contents, from lattice dimension, of samples of unknown aqueous content, such as those prepared osmotically.

The maximum hexagonal dimension reached reflects the highest degree of swelling, or hydration, for the hexagonal phase of each composition. As shown in Fig. 4, we determine the water content at maximum hydration ϕ_w° from the intersection of the common best-fit line to the pooled data with the averaged maximum dimension in excess water. In Fig. 5 we plot ϕ_w° as it varies with cholesterol content for both DOPE and DOPC.

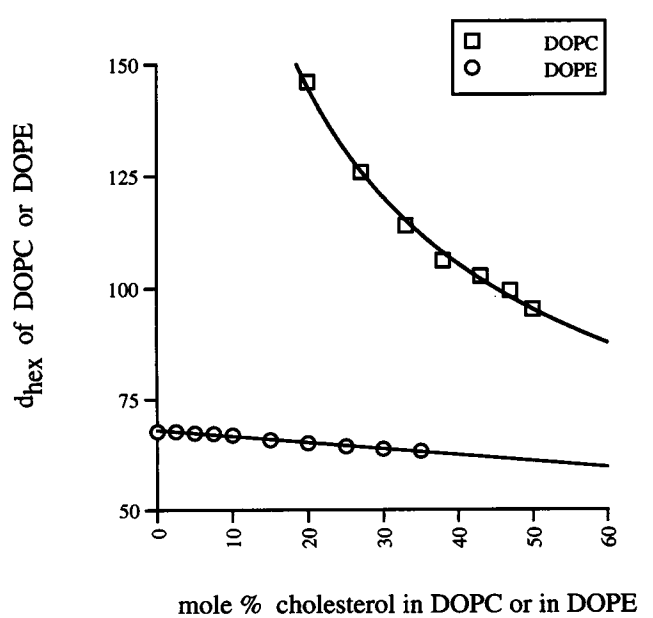


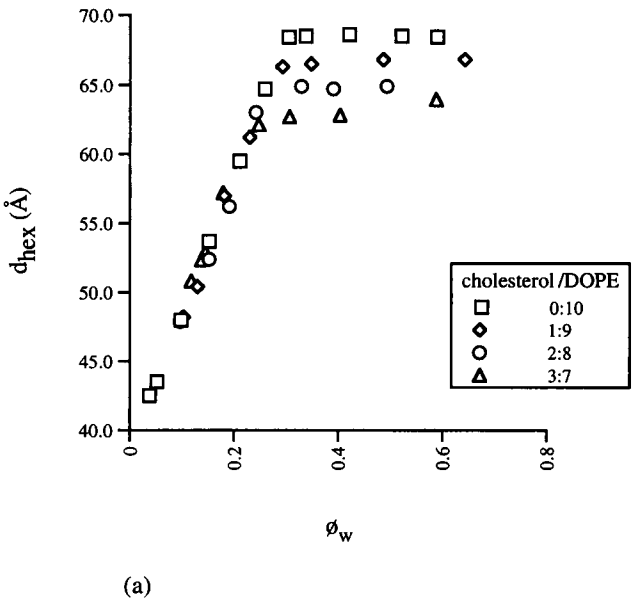
FIGURE 3 Lattice dimension d_{hex} of the single hexagonal phases formed by DOPE and DOPC with added cholesterol (16% of the dry weight is tetradecane) and in excess water. DOPC/cholesterol forms hexagonal phases of extremely large dimension.

Osmotically stressed hexagonal phases

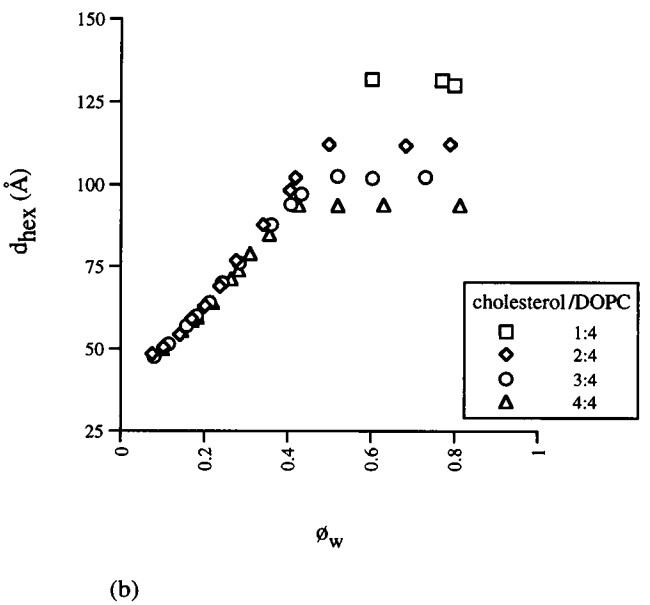
The lattice dimensions of osmotically stressed hexagonal phases of different mixtures of DOPE/cholesterol and of DOPC/cholesterol are shown in Fig. 6. The dimensions appear to converge at high pressures, or low water content. Under the same osmotic pressure, as the cholesterol content increases, the lattice dimensions decrease. The water content of each of these phases (ϕ_w) is determined from the ϕ_w versus d_{hex} dependence (Fig. 4).

There is an important distinction between the osmotic and gravimetric experiments that results in the different behavior of the lipids. In the osmotic experiments, the large reservoir of polymer solution sets the chemical potential of water in the system, and the lipid imbibes water to equilibrium and forms one phase. In the gravimetric samples, the composition is fixed, and the hexagonal and lamellar phases compete for the available water to minimize the free energy of the system. Under these latter conditions, lamellar and hexagonal phases can coexist, as we observe for DOPC at 22°C.

The structural dimensions of the single hexagonal phases formed at each osmotic pressure are calculated from Eqs. 1 and 2.



(a)



(b)

FIGURE 4 Lattice dimension, d_{hex}, of the phases formed by the indicated mole ratios of (a) DOPE/cholesterol and (b) DOPC/cholesterol, as a function of volume fraction water. Sixteen percent of the dry weight of all samples was tetradecane. In less than excess water, the lattice dimensions at different cholesterol contents were not detectably different. However, with increasing cholesterol content, both the maximum lattice dimension, indicated by the horizontal lines, and the volume fraction water of the fully hydrated phase decreased.

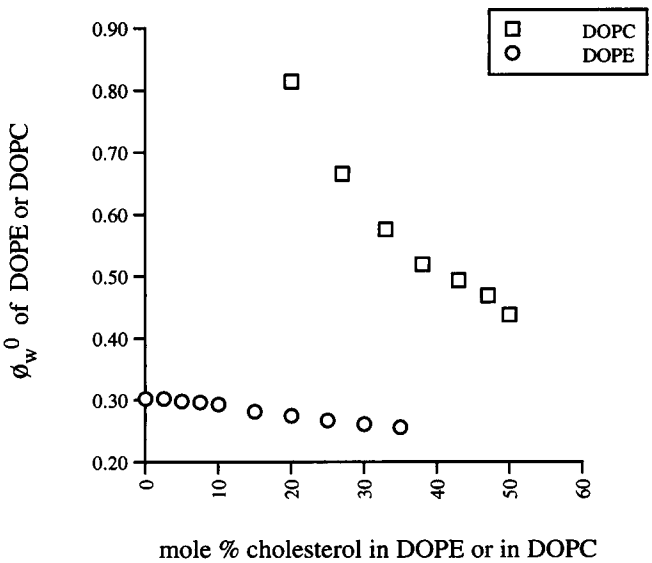


FIGURE 5 Maximum hydration, volume fraction water, of the single hexagonal phases shown in Fig. 4.

Pivotal plane

The diagnostic plots (Leikin et al., 1996) of $(A_w/V_l)^2$ versus $(A_w/V_l)R_w$ for all cholesterol/DOPE mixtures and for cholesterol/DOPC are shown in Fig. 7, *a* and *b*. The linearities in Fig. 7 indicate that there is a well-defined pivotal plane, the position of which, V_p/V_l, is independent of cholesterol content. We use these linear fits to determine 1) the normalized molecular area at the pivotal plane A_p/V_l for DOPE (= 0.0491 $Å^{-1}$) and for DOPC (= 0.0408 $Å^{-1}$), and 2) the relative volume between the Luzzati and pivotal planes, V_p/V_l, for DOPE (= 0.348) and for DOPC (= 0.241).

Because A_p/V_l and V_p/V_l are constant in each case, the pivotal plane area, A_p, and the volume separating the Luzzati and pivotal planes, V_p, increase with the cholesterol content. We have attempted to get a measure of the individual contributions of phospholipid and cholesterol to A_p and V_p. For this we define the phospholipid molecule as one phospholipid plus the tetradecane. Taking the phospholipid and cholesterol contributions as additive, so that

$$A_p = A_p^{dope/td} + xA_p^{chol} = 0.049\ (V_l)$$

$$V_p = V_p^{dope/td} + xV_p^{chol} = 0.348\ (V_l)$$

and

$$A_p = A_p^{dopc/td} + xA_p^{chol} = 0.041\ (V_l)$$

$$V_p = V_p^{dopc/td} + xV_p^{chol} = 0.241\ (V_l)$$

we estimate the following individual contributions of each lipid component to A_p and V_p,

for DOPE	$A_p^{dope/td} = 75$ Å^2;	$V_p^{dope/td} = 538$ Å^3;
	$A_p^{chol} = 37$ Å^2;	$V_p^{chol} = 266$ Å^3;
and for DOPC	$A_p^{dopc/td} = 67$ Å^2;	$V_p^{dopc/td} = 393$ Å^3;
	$A_p^{chol} = 31$ Å^2;	$V_p^{chol} = 184$ Å^3.

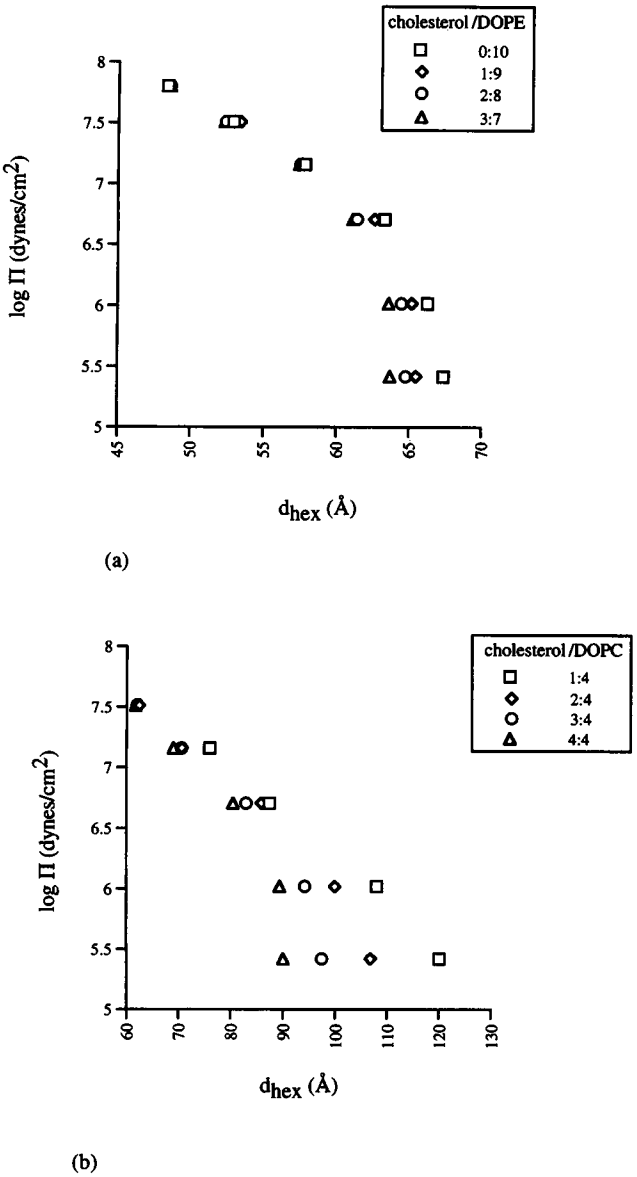


FIGURE 6 Experimental data relating the osmotic pressure, Π, of the equilibrating solutions and the hexagonal lattice dimension d_{hex} formed by (a) DOPE and (b) DOPC with different cholesterol contents. With increasing cholesterol contents, the lattice dimension starts from ever smaller lattice size at low pressures, but converges at higher pressures.

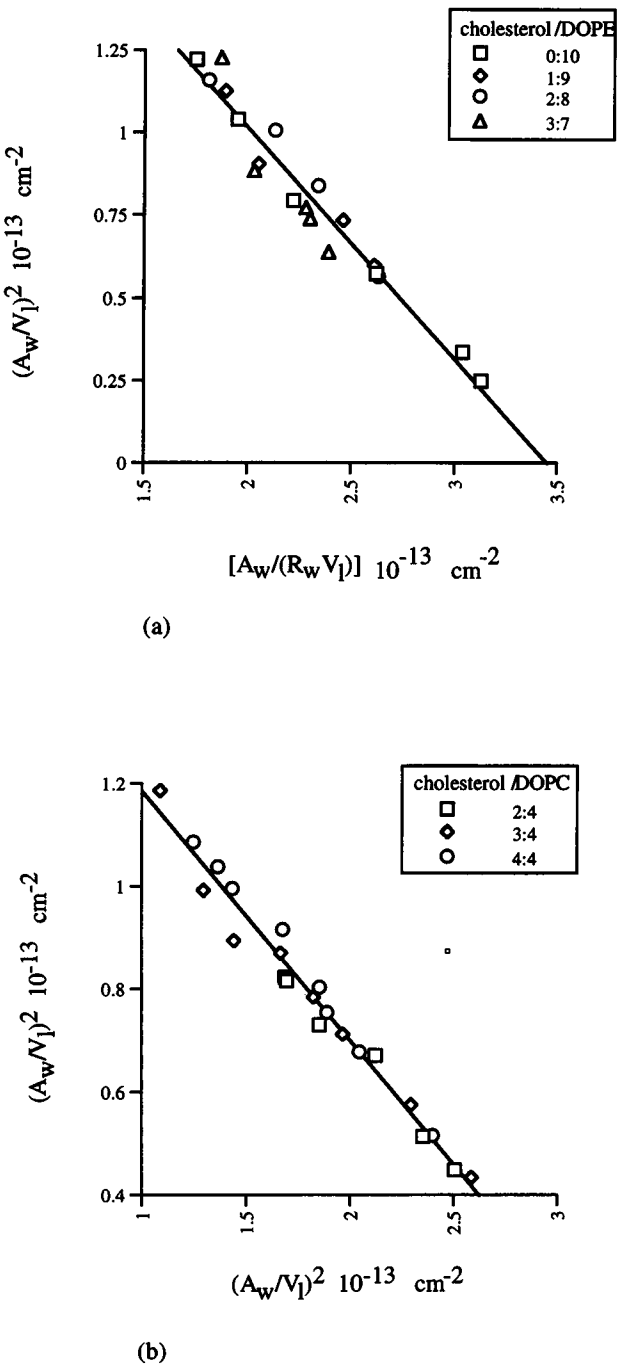
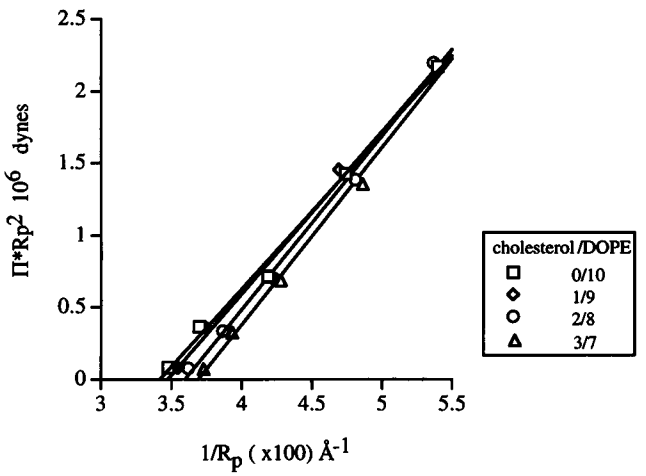


FIGURE 7 Diagnostic plots, as described in Eq. 6, relating molecular area at the Luzzati plane, A_w, with the radius of the water cylinder, R_w. When the area is normalized by the volume of an effective molecule (V_l) as described in the text, no systematic variation can be seen with mole ratio cholesterol in either DOPE (a) or DOPC (b). The results were pooled to yield the plotted linear least-squares fit. The slope of this plot ($2V_p/V_l$) defines the fraction of molecular lipid volume included inside the pivotal surface.

Intrinsic curvature R_{op} and bending modulus k_{cp}

We now calculate the radius of curvature of the pivotal plane, R_p, and the monolayer bending modulus, k_{cp}, for the osmotically stressed hexagonal phases, using Eq. 5 with R_w and V_p, and using the ϕ_w determined from Fig. 4. Fig. 8, *a* and *b*, shows the plots of $\Pi * R_p^2$ vs $1/R_p$ for the different mixtures of cholesterol/DOPE/td and cholesterol/DOPC/td, where Π is the osmotic pressure of the equilibrating solution. From the intercepts at $\Pi = 0$ and the slopes, we find the radii of spontaneous curvature (R_{op}) and bending moduli k_{cp}.

Fig. 9 shows that the addition of cholesterol both to DOPE and to DOPC results in a linear increase in monolayer spontaneous curvature ($1/R_o$) with molar fraction cholesterol [$m_{chol} = x/(1 + x)$]. Based on these linear relationships, the intrinsic curvatures of the individual lipids are



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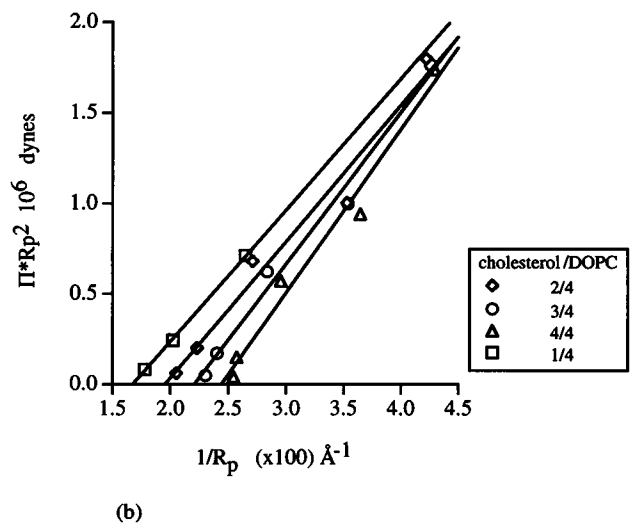

(b)

FIGURE 8 Plot, following Eq. 7, to determine the intrinsic radius of curvature R_o and the bending modulus k_{cp} for the cholesterol-containing lipid monolayers (a) DOPE, (b) DOPC. Standard errors in intercept and slope are ±5%

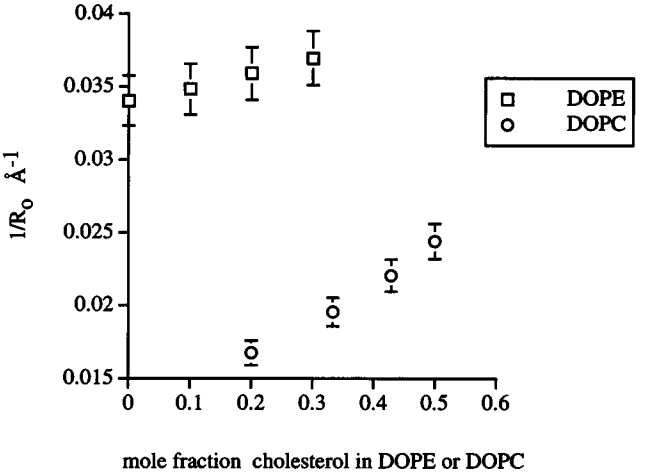

FIGURE 9 Plot of the intrinsic curvature, $1/R_o$, measured for DOPE and DOPC monolayers as their cholesterol content increases. Standard error bars are 5%, determined from the fits to the data of Fig. 8.

calculated by

$$\frac{1}{R_{0p}} = (1 - m_{chol})\frac{1}{R_{0p}^{DOPE/PC}} + m_{chol}\left(\frac{1}{R_{0p}^{chol}}\right)$$

At 22°C, R_{op} is found to be 22.8 (±2) Å for cholesterol in DOPE and 29.4 (±2) Å for DOPE/td. At 32°C, R_{op} is found to be 27.2 (±2) Å for cholesterol in DOPC and 87.3 (±4) Å for DOPC/td. These specific values are incorporated into Table 1. Interestingly, cholesterol appears to behave as a molecule with a slightly larger radius of curvature in the presence of DOPC than in DOPE, perhaps reflecting different interactions with those different polar groups.

Fig. 10 shows the bending moduli of DOPE and DOPC monolayers and how it they are affected by cholesterol. Values for the specific lipids are shown in Table 1. Except for the small effect of cholesterol seen here, the range of values ($0.72 - 1.2 \times 10^{-12}$ dyn cm for a bilayer) are remarkably similar to those derived from area dilation for bilayer vesicles ($0.44 - 0.90 \times 10^{-12}$ dyn cm) (Evans and Rawicz, 1990). In the present system, however, cholesterol appears to only modestly increase the monolayer bending modulus, measured at the pivotal plane, of both DOPE and DOPC monolayers.

Although tetradecane is required to relieve chain stress to measure R_o, it may affect the monolayer bending modulus. Fig. 11 shows that tetradecane increases k_{cp} slightly in both DOPE and cholesterol/DOPE 3/7 monolayers. The tetradecane-free values are plotted in Fig. 10. Cholesterol has the same small effect on the bending modulus of DOPE monolayers whether or not they contain tetradecane. Similar experiments could not be done on a tetradecane-free DOPC system because tetradecane is required for hexagonal phase formation.

DISCUSSION

Whereas studies of cholesterol in bilayers have usually focused on its position and effects in restricting molecular motions, the present studies indicate that cholesterol also induces curvature in DOPE and DOPC monolayers. For DOPC, which is normally considered to form particularly stable bilayers with cholesterol, that curvature is expressed in the transition from the lamellar to the hexagonal phase when hydrocarbon is present to reduce chain packing stress. This is consistent with a few previous observations that cholesterol destabilizes PC bilayers (Dekker et al., 1983; Coorssen and Rand, 1990).

The phase diagrams of DOPE/cholesterol and DOPC/cholesterol provide two particularly interesting observations:

TABLE 1 Molecular and elastic parameters for individual lipids as determined through studies of the pure lipids or mixtures

	MW	v (ml/g)	V_l (Å^3)	V_{pol} (Å^3)	V_{hc} (Å^3)	V_p (Å^3)	A_p (Å^2)	R_0 (Å)	K_c/kT
DOPE	744	1.00	1235	312	923	374	65	28.5 (22°C)	11
DOPE/td	886	1.05	1543	312	1231	538	75	29.4 (22°C)	13
CHOL in DOPE/td	386	0.94	604	0	604	266	37	22.8 (22°C)	
DOG in DOPE	621	1.08	1116	193	923	338	59	11.5 (22°C)	
DOPC	786	0.99	1292	377	915				
DOPC/td	936	1.03	1631	377	1254	393	67	87.3 (32°C)	9
CHOL in DOPC	386	0.94	604	0	604	184	31	27.2 (32°C)	

1. The maximum amount of cholesterol that can be accommodated in the hexagonal phase monolayers before it forms a separate phase is 30 mol% in DOPE and 50 mol% in DOPC. How this difference is related to the specific polar group, hydrocarbon chain characteristics, and maximum monolayer curvature or other molecular packing considerations remains to be explored.

2. The unusual region of disorder at 25 mol% cholesterol in DOPC indicates conditions where the hexagonal phase destabilizes; on either side of that composition, the hexagonal lattice is well ordered. Studies of this phenomenon could inform our understanding of the stability of the hexagonal lattice.

Tetradecane is required for the hexagonal phase to be expressed, at least for DOPC, and it changes the dimension of the hexagonal phase of DOPE slightly. Comparison of the elastic parameters of DOPE and of cholesterol/DOPE with and without 16 wt% tetradecane (Figs. 10 and 11) shows that tetradecane has only a small effect on bending modulus. Although we cannot completely rule out the unlikely possibility that tetradecane has a different effect on DOPC monolayers, we take it that our measurements of bending modulus reflect values close to the tetradecane-free system.

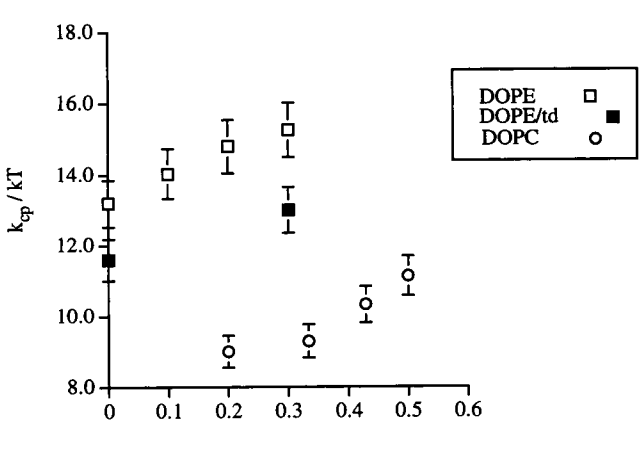

FIGURE 10 Plot of the derived bending modulus k_{cp}, in units of kT, measured at the pivotal plane, of DOPE and DOPC monolayers as their cholesterol content increases. The two solid symbols represent tetradecane-free DOPE samples. Standard error bars are 5%, determined from the fits to the data of Figs. 8 and 11.

The increase in bending modulus produced by the incorporation of cholesterol in these hexagonal phase monolayers is much smaller than might be expected from cholesterol's increase in area compressibility modulus in bilayers (Need-

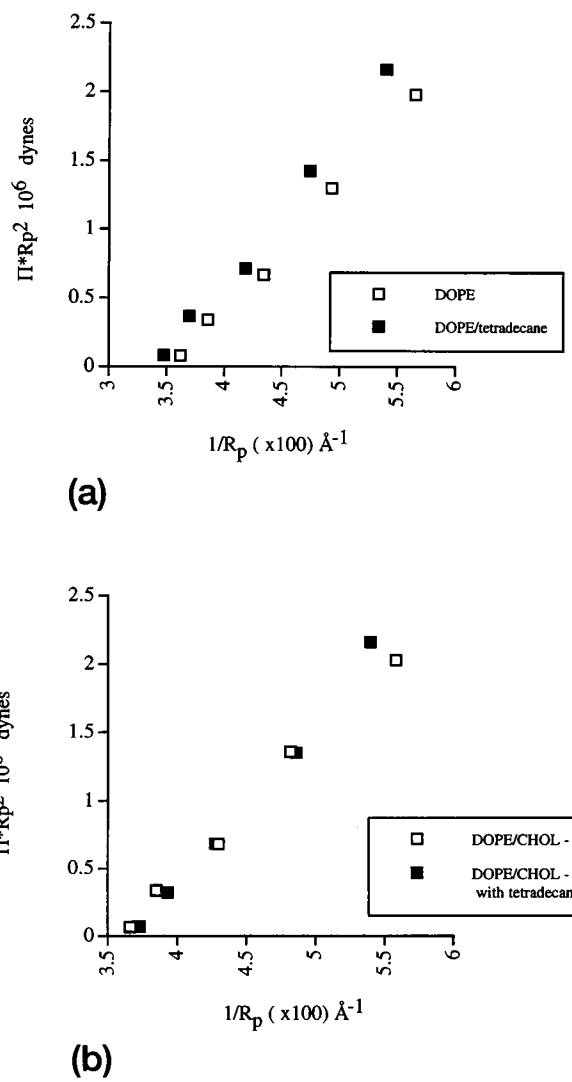

FIGURE 11 Plot, following Eq. 7, to determine the bending modulus k_{cp} for (a) DOPE and (b) DOPE/cholesterol (3/7), with and without tetradecane. For DOPE with added tetradecane, k_{cp} increases from 11.6 to 13.4 (±0.5 kT). For DOPE/cholesterol with added tetradecane, k_{cp} increases from 12.8 to 15.4 (±0.5 kT).

ham and Nunn, 1990). Needham and Evans showed that 50 mol% cholesterol produced a three- to fourfold increase in area compressibility modulus of stearoyloleoylphosphatidylcholine (SOPC) bilayers, and that most of that increase occurred in the 30–50 mol% range. Evans and Rawicz (1990) measured, on the basis of such area compressibility change, a threefold increase in bending modulus for bilayers of SOPC/cholesterol 1/1. Our measured bending modulus with DOPC shows an increase beginning at ~30 mol%, but an increase of only 30% at DOPC/cholesterol 1/1.

Except for the cholesterol-containing layers, the similarity of the bending moduli, measured in bilayer vesicles through vesicle aspiration (Evans and Rawicz, 1990), and measured in hexagonal phases by this osmotic stress method, is remarkable, considering the radically different lipid configurations and methods. How then to account for the different measured effects of cholesterol? Several possibilities are being considered. Briefly:

1. Our measurements are for a bending modulus measured at the pivotal plane. Estimating this parameter at the neutral plane where the comparison must be made (Leikin et al., 1996) cannot make up the difference. The separation of neutral and pivotal planes is determined by a parameter γ (Leikin et al., 1996), which is inversely proportional to both the area compressibility modulus and the square of R_o. Certainly for DOPC/cholesterol, γ makes the neutral and pivotal planes coincide.

2. It is possible that cholesterol and/or tetradecane change position during bending and soften the hexagonal monolayer. However, because we see a well-defined pivotal plane, this means that each lipid contributes a constant proportion of its volume between the Luzzati plane and the pivotal plane, and occupies a constant area on the pivotal plane. Therefore these components do not appear to be moving.

3. It is possible that cholesterol does behave differently in the highly curved monolayers of the hexagonal phase, because within such monolayers the molecular area of the hydrocarbon chains at its terminals is double that near the pivotal plane at the polar group end (Rand and Fuller, 1994). But similarly, such pivotal plane acts as one of remarkably constant molecular area compared to molecular positions some distance from it. This suggests that the treatment of the phospholipid monolayer as one of homogeneous mechanical properties through its thickness, with concomitant coupling of area and bending elastic moduli, may be unrealistic.

Similar to the effects of diacylglycerol added to DOPE (Leikin et al., 1996), a pivotal plane also exists for added cholesterol, and its position stays remarkably constant as the amount of cholesterol increases. We consider it significant that, given no a priori reason for its existence (Leikin et al., 1996), a pivotal plane has been observed in all systems investigated to date, including these lipid mixtures. It represents a position within the lipid monolayer that remains practically constant in molecular area, both through lamellar-hexagonal phase transitions and during deformation of the hexagonal phase (Rand and Fuller, 1994). Otherwise, molecular area can nearly double at the chain terminals and decrease considerably toward the water layer. The position of constant area is always very close to the interface separating hydrocarbon chains and polar groups.

The spontaneous curvatures derived for individual lipids are shown in Table 1. Although cholesterol and diacylglycerol (Leikin et al., 1996) both induce the hexagonal phase, cholesterol's own apparent spontaneous radius of curvature is considerably larger than that of diacylglycerol and more like DOPE. DOPC has a very large spontaneous radius of curvature, accounting for its preference for the lamellar phase; our value is very close to that approximated by Keller et al. (1993). We have recently shown in similar studies that lysophosphatidylPC's contribute the opposite curvature when added to diacylphospholipids. We see such curvatures as providing one measure of the frustration of a lipid when constrained to a flat bilayer, frustrations invoked in thinking about lipid-membrane protein interactions and about membrane fusion mechanisms (Chernomordik et al., 1995).

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