Modulation of the interbilayer hydration pressure by the addition of dipoles at the hydrocarbon/water interface

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ABSTRACT The effects of the cholesterol analog 5α-cholestan-3β–ol-6-one (6-ketocholestanol) on bilayer structure, bilayer cohesive properties, and interbilayer repulsive pressures have been studied by a combination of x-ray diffraction, pipette aspiration, and dipole potential experiments. It is found that 6-ketocholestanol, which has a similar structure to cholesterol except with a keto moiety at the 6 position of the B ring, has quite different effects than cholesterol on bilayer organization and cohesive properties. Unlike cholesterol, 6-ketocholestanol does not appreciably modify the thickness of liquid-crystalline egg phosphatidylcholine (EPC) bilayers, and causes a much smaller increase in bilayer compressibility modulus than does cholesterol. These data imply that 6-ketocholestanol has both its hydroxyl and keto moieties situated near the water-hydrocarbon interface, thus making its orientation in the bilayer different from cholesterol's. The addition of equimolar 6-ketocholestanol into EPC bilayers increases the magnitude, but not the decay length, of the exponentially decaying repulsive hydration pressure between adjacent bilayers. Incorporation of equimolar 6-ketocholestanol into EPC monolayers increases the dipole potential by ~300 mV. These data are consistent with our previous observation that the magnitude of the hydration pressure is proportional to the square of the dipole potential. These results mean that 6-ketocholestanol, despite its location in the bilayer hydrocarbon region, ~10 Å from the physical edge of the bilayer, modifies the organization of interlamellar water. We argue that the incorporation of 6-ketocholestanol into EPC bilayers increases the hydration pressure, at least in part, by increasing the electric field strength in the polar head group region.

INTRODUCTION

Colloidal materials in aqueous media will approach each other to short distances under the influence of van der Waals attractive forces. The hydration pressure, P_h, is the major repulsive barrier that hydrated surfaces must overcome to approach within distances on the order of 20 Å or less (LeNeveu et al., 1977; Parsegian et al., 1979). For a variety of systems, including lipid bilayers and macromolecules such as polysaccharides and DNA, P_h has been shown to decay exponentially as $P_h = P_o \cdot \exp(-d_f/\lambda)$, where d_f is the distance between surfaces, λ is the decay length of the pressure and P_o is the magnitude of the hydration pressure (LeNeveu et al., 1977; Parsegian et al., 1979; Rau et al., 1984; McIntosh and Simon, 1986; McIntosh et al., 1989*a*; Rau and Parsegian, 1990). Although most theoretical analyses agree that the hydration pressure arises from the partial orientation or polarization of water by the bilayer (Marcelja and Radic, 1976; Schiby and Ruckenstein, 1983; Gruen and Marcelja, 1983; Kornyshev, 1986; Belaya et al., 1986; Dzhavakhidze et al., 1986; 1988), the physico-chemical principles underlying the hydration pressure are not well understood at present. Because the terms in the above expression for P_h can be determined experimentally, quantitation of the variations in each parameter with respect to changes in solvent and surface electric fields allow current theories of the origins of the hydration pressure to be tested.

With regard to the decay length, measurements of P_h between lipid bilayers in several solvents show that λ decreases with increasing solvent packing density and thus is related to the size of the solvent molecule (McIntosh et al., 1989*b*). That is, the larger the size of the solvent molecule the larger the decay length. With water as the solvent, λ has been found to be ~2 Å for a variety of bilayers (LeNeveu et al., 1977; McIntosh and Simon, 1986; Simon et al., 1988; Rand and Parsegian, 1989; McIntosh et al., 1989*a*, *b*, and *c*; Simon et al., 1991).

With regard to factors that determine the magnitude of the hydration pressure for lipid bilayers, we (Simon et al., 1988; McIntosh et al., 1989*a* and *b*; Simon and McIntosh, 1989; McIntosh et al., 1990; Simon et al., 1991) have shown for a variety of lipid systems, including phospholipids in their gel and liquid crystalline phases, with and without cholesterol, and in nonaqueous solvents, that

$$P_o = 2\chi(V/\lambda)^2 \quad (1)$$

where V is the dipole potential measured in monolayers at a similar packing density as is found in bilayers

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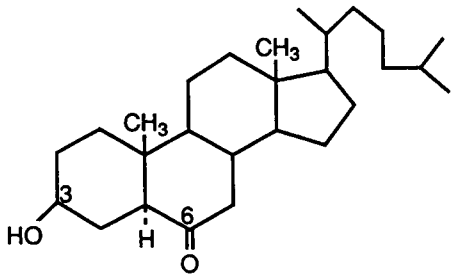
(MacDonald and Simon, 1987), and χ is the orientational susceptibility of the solvent which is equal to $\epsilon_0(\epsilon - 1)/\epsilon$, where ϵ_0 is the permittivity of free space and ϵ is the bulk dielectric constant of the solvent. Eq. 1, which predicts that P_o is proportional to the square of the electric field (V/λ),[2] was obtained from the theoretical analysis of Cevc and Marsh (1985), as modified by us by equating the hydration potential to the dipole potential measured in monolayers in equilibrium with bilayers. Other theoretical treatments (Gruen and Marcelja, 1983; Schiby and Ruckenstein, 1983; Kornyshev, 1986; Belaya et al., 1986; Dzhavakhidze et al., 1986; 1988) also relate P_o to the electric field of the bilayer, although the proportionality constant in each of these other treatments is different than that given in Eq. 1.

Based on their experimental data, Simon and McIntosh (1989) have argued that oriented dipoles in the head group region of the bilayer, including those of both the lipid and chemisorbed solvent molecules, contribute to the dipole potential and that the magnitude of the hydration pressure depends on the size of the dipole potential. They (Simon and McIntosh, 1989) have also argued that the fields produced by these oriented dipoles polarize interbilayer water, giving rise to the hydration pressure. In an independent treatment, Gawrisch et al (1990) have recently raised the important question about membrane potentials and hydration pressure: which is cause and which is effect? That is, they asked whether water is ordered by electric fields arising from charges and dipoles, or by constraints of hydrogen bonding of water molecules adsorbed to the surface. They have argued that "water molecules in the first hydration layer are ordered by hydrogen bonding with the lipid headgroups" and that "the change in water arrangement enforced by these headgroups is the source of the hydration force" and that "the 'dipole potential' can come in good part from oriented water molecules."

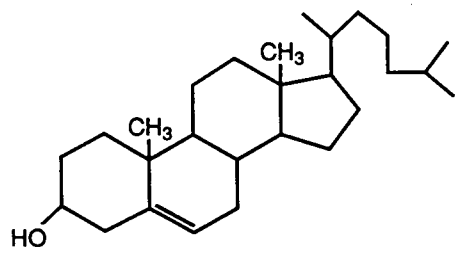
Although it is generally agreed that the presence of adsorbed water molecules must make a major contribution to the surface dipole potential (Simon and McIntosh, 1989; Gawrisch et al., 1990), it is not clear, at the moment, how much of a contribution could arise from intrinsic dipoles located deep in the head group of the bilayer, away from the physical edge of the bilayer. In this paper we focus our attention on the, as yet, ill-specified origin of the polarizing electric fields near the physical edge of the bilayer. Two questions are posed, Can the presence of strong dipoles in the interior of the bilayer influence the dipole potential and, by inference, the polarization of water at the interface? And, if so, does this surface polarization show up as an increased repulsive hydration pressure?

To address these two questions, it would be useful to be able to insert a molecule with a large dipole moment deep in the lipid head group region, away from the physical edge of the bilayer so that direct hydrogen bonding between the inserted molecule and interlamellar water would not be probable. To accomplish this, we added the cholesterol analogue, 5α-cholestan-3β–ol-6-one (6-ketocholestanol, Fig. 1) to phospholipid multilayers. Monolayers of cholesterol analogs containing a keto group have very large dipole potentials (Adam, 1968). In phospholipid bilayers, the keto group of 6-ketocholestanol would be expected to be anchored near the hydrocarbon-water interface (Fig. 2), ~10 Å away from the physical edge of the bilayer (McIntosh and Simon, 1986).

We therefore chose to characterize egg phosphatidylcholine (EPC) bilayers containing equimolar amounts of 6-ketocholestanol. We have used x-ray diffraction of multilayer suspensions, micropipet manipulation of individual lipid vesicles, and dipole potential measurements of monolayers to determine the location of 6-ketocholestanol in the bilayer and its effect on bilayer structure, cohesive properties, and dipolar character. We also used x-ray diffraction data from osmotically stressed lipid multilayers to provide information on how the magnitude (P_o) and decay length (λ) of the hydration pressure depend on the dipolar properties of the lipid bilayer in the presence and absence of 6-ketocholestanol.



5α-cholestan-3β-ol-6-one
(6-ketocholestanol)



cholesterol

FIGURE 1 Structural models of cholesterol and 6-ketocholestanol.

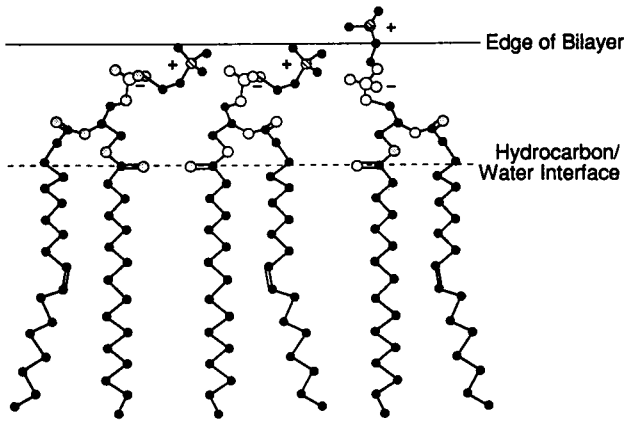


FIGURE 2 Schematic of one monolayer of an SOPC bilayer, showing three SOPC molecules. We place the hydrocarbon-water interface at the position of the deeper carbonyl group of lipid (Simon et al., 1982). Two headgroup conformations are shown (Hauser et al. 1981; McIntosh et al., 1987) and the physical edge of the bilayer is placed at the outer limit of the most prevalent conformation.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (EPC) and 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL), and 5α-cholestan-3β-ol-6-one was purchased from Steraloids Inc. (Wilton, NH). SOPC is a synthetic analog of EPC and the two lipids have similar hydrocarbon chain compositions. These compounds were used without further purification. Poly(vinylpyrrolidone) (PVP) with an average molecular weight of 40,000 was obtained from Sigma Chemical Co. (St. Louis, MO). Triply distilled water was used to make PVP-water solutions in the range of 0–60% w/w.

X-ray diffraction

EPC and 6-ketocholestanol were codissolved in chloroform and the chloroform was removed by rotary evaporation. Osmotic pressures in the range of 1.1×10^5 to 3.2×10^7 dyn/cm^2 were applied to unoriented multiwalled liposomes by the "osmotic stress" method (LeNeveu et al., 1977; Parsegian et al., 1979). In brief, an excess amount of the appropriate PVP solution was added to the dry lipid. The suspensions were covered with nitrogen and incubated for several hours with periodic vortexing above the lipid's main phase transition temperature. Because PVP molecules are too large to enter between the lipid multilayers, they compete for solvent and thereby compress the lipid lattice. Osmotic pressures of the PVP solutions were calculated from the viral coefficients obtained by Vink (1971). These extrapolated pressures are in close agreement with values measured by Parsegian et al. (1986) and by McIntosh et al. (1989*b*). For x-ray diffraction experiments, the lipid-polymer suspensions were sealed in quartz glass capillary tubes and mounted in a point-collimation x-ray diffraction camera.

Vapor pressures in the range of 2.8×10^7 to 5.8×10^8 dyn/cm^2 were applied to oriented lipid multilayers by established procedures (Parsegian et al., 1979; McIntosh et al., 1987). The oriented specimen was formed by placing a small drop of lipid-chloroform solution on a piece of aluminum foil and slowly evaporating the chloroform. The foil substrate was given a convex curvature and mounted in a controlled humidity chamber on a line-focussed single-mirror x-ray camera, where the x-ray beam was oriented at a grazing angle relative to the lipid multilayers. The humidity chamber consisted of a canister with two mylar windows for passage of the x-ray beam. The vapor pressure was controlled by means of a cup of saturated salt solution in the chamber. To speed equilibrium, a gentle stream of nitrogen gas was passed through a flask of the saturated salt solution and through the chamber. For the salt solutions used in these experiments, the ratio of the vapor pressure (p) of the saturated salt solution to the vapor pressure of pure water (p_o) has been measured (O'Brien, 1948; Weast, 1984). The following saturated salt solutions were used to obtain the relative vapor pressures (p/p_o) indicated in parentheses: $CuSO_4$ (0.98), Na_2SO_4 (0.93), KCl (0.87), NH_4Cl (0.80), and $NaNO_2$ (0.66). The applied pressure is given by $P = -(RT/V_w) \cdot \ln(p/p_o)$ where R is the molar gas constant, T is the temperature (293 °K), and V_w is the molar volume of water.

For all specimens, oriented multilayers and unoriented lipid-polymer suspensions, x-ray diffraction patterns were recorded on Kodak DEF x-ray film. X-ray films were densitometered with a Joyce-Loebl microdensitometer as described previously (McIntosh et al., 1989*a* and *b*). After background subtraction, integrated intensities, $I(h)$, were obtained for each order h by measuring the area under each diffraction peak. For unoriented patterns, the structure amplitude $F(h)$ was set equal to $\{h^2 I(h)\}^{1/2}$ (Blaurock and Worthington, 1966; Herbette et al., 1977). For the oriented line-focussed patterns the intensities were corrected by a single factor of h due to the cylindrical curvature of the multilayers (Blaurock and Worthington, 1966; Herbette et al., 1977), so that $F(h) = \{hI(h)\}^{1/2}$.

Estimates for the widths of the bilayer and solvent layer between adjacent bilayers were obtained from the x-ray diffraction data by the use of electron density profiles. Electron density profiles, ρ(x), on a relative electron density scale were calculated from

$$\rho(x) = (2/d) \Sigma \exp\{\phi(h)\} \cdot F(h) \cos(2\pi x h/d), \qquad (2)$$

where x is the distance from the center of the bilayer, d is the lamellar repeat period, $\phi(h)$ is the phase angle for order h, and the sum is over h. Phase angles were determined by a sampling theorem analysis as described in detail previously (McIntosh and Holloway, 1987). The electron density profiles described in this paper are at a resolution of $d/2h_{max} \approx 6$ Å.

Vesicle Membrane Compressibility Modulus

Giant lipid vesicles were made from various mixtures of SOPC and 6-ketocholestanol dissolved in 2:1 chloroform:methanol. SOPC, a synthetic analog of EPC, was used because this laboratory has done considerable work measuring the compressibility of SOPC and SOPC: cholesterol bilayers (Needham and Nunn, 1990). The compressibility modulus of SOPC bilayers (193 dyn/cm, Needham and Nunn, 1990) is somewhat larger than that of EPC bilayers (140 dyn/cm, Kwok and Evans, 1981), probably due to the chain heterogeneity and distribution of double bonds in EPC. The preparation of vesicles followed a slightly modified procedure of the one introduced by Reeves and Dowben (1969) described elsewhere (Needham and Nunn, 1990). Vesicles were harvested and introduced into a temperature-controlled (±0.1 °C) microchamber mounted on the microscope stage. The temperature was monitored by a micro-thermocouple. Only vesicles which appeared most optically transparent (by interference contrast microscopy) were chosen for tests. By subsequent evaluation of membrane elastic modulus, it was possible to discriminate between vesicles with one, two, or more layers, because the elastic moduli group around discrete values; the lowest value is characteristic of a single bilayer (Kowk and Evans, 1981).

Vesicles which we studied were typically 20–30 μm in diameter. For vesicles which had sufficient excess area (over a sphere of the same volume), aspiration by a 10-μm diameter micropipette produced a projection inside the pipet. Micropipettes were produced from a 1-mm glass tube pulled to a fine point and broken by a glass knife to obtain flat tips. Pipette suction pressure was controlled hydrostatically by micrometer-driven displacement of a water reservoir relative to the pipette tip, giving a resolution of microatmospheres. To determine the isothermal compressibility modulus at 15°C, suction pressure was increased and the increase in projection length inside the pipette was measured.

In each experiment, area changes were derived from aspiration lengths in the micropipette as a function of membrane tension at constant temperature. Membrane tension (τ) was uniform over the entire vesicle surface and is given by the pipette suction pressure (P) and the pipette/vesicle geometry (Kwok and Evans, 1981):

$$\tau = PR_p/(2 - 2R_p/R_o), \quad (3)$$

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

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

$$\Delta A = 2PR_p(1 - R_p/R_o)\Delta L. \quad (4)$$

This relationship is valid only if the volume of the vesicle is constant. Changes in volume (due to filtration of water by pipette 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. Because 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. If A_o is the reference area of the vesicle in the pipette at a low (~0.5 dyn/cm) initial membrane tension, then the relative, fractional change in vesicle membrane area ($\Delta A/A_o$) in response to a change in membrane tension at constant temperature yields the compressibility modulus, K.

Monolayer Dipole Potential

For measurements of the dipole potential, monolayers were formed by spreading 10 to 40 μl of solutions of EPC: 6-ketocholestanol in chloroform (25 mg/ml) onto a subphase containing 1 mM KCl. A teflon trough with a surface area of ~30 cm^2 was used. Under these conditions, MacDonald and Simon (1987) have argued that the surface monolayer is in equilibrium with liposomes in the subphase. The dipole potential was measured between a Ag/AgCl electrode in the subphase and a polonium electrode in air which were connected to a Keithly electrometer. The reported values of dipole potential represent the differences in the potential in the presence and absence of the monolayer.

RESULTS

Bilayer structure, cohesion, and dipole potential

X-ray diffraction

Specimens of equimolar EPC:6-ketocholestanol incubated in different concentrations of PVP or at different vapor pressures yielded diffraction patterns that consisted of 2–4 low-angle reflections and a broad wide-angle band with a spacing of ~4.5 Å. For all specimens, the low-angle reflections indexed as orders of a single lamellar repeat period with no indication of a separate ketocholestanol phase. These patterns are typical of bilayers in the L_α or liquid crystalline phase (Tardieu et al., 1973). The lamellar repeat period for these bilayers ranged from 65.0 Å at full hydration (no applied pressure) to 50.1 Å at a relative vapor pressure of 0.66 (applied pressure of 5.8×10^8 dyn/cm^2). Fig. 3 shows structure factors obtained from osmotic stress experiments where 4 orders of diffraction were observed. The solid line is the continuous Fourier transform as calculated from the sampling theorem (Shannon, 1949). All of the observed structure factors fall close to the continuous transform, indicating that the width of the bilayer remains essentially constant for all applied pressures (McIntosh and Simon, 1986).

Fig. 4 shows electron density profiles for bilayers of equimolar EPC:6-ketocholestanol, along with profiles of EPC and equimolar EPC:cholesterol taken from McIntosh et al. (1989*a*). Each profile was obtained under the same applied osmotic pressure (2×10^8 dyn/cm^2). For each profile, the geometric center of the bilayer is at the origin (0 Å). The low-density trough in the center of each profile corresponds to the localization of the terminal methyl groups of the lipid acyl chains, and the highest density peaks, located at approximately ±20 Å, correspond to the lipid headgroups. The methylene groups of the lipid chains are located in the medium density regions between each head group peak and the terminal methyl trough. The distance between head group peaks across the bilayer is different for the three bilayers, as is the shape of the hydrocarbon chain region. The distance between head group peaks across the

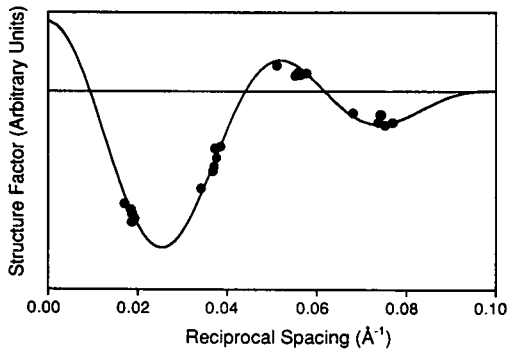


FIGURE 3 Structure factors for EPC:6-ketocholestanol plotted versus reciprocal space coordinate. The circles represent structure factors for osmotic pressure experiments. The solid line is a continuous Fourier transform calculated using the sampling theorem for one data set.

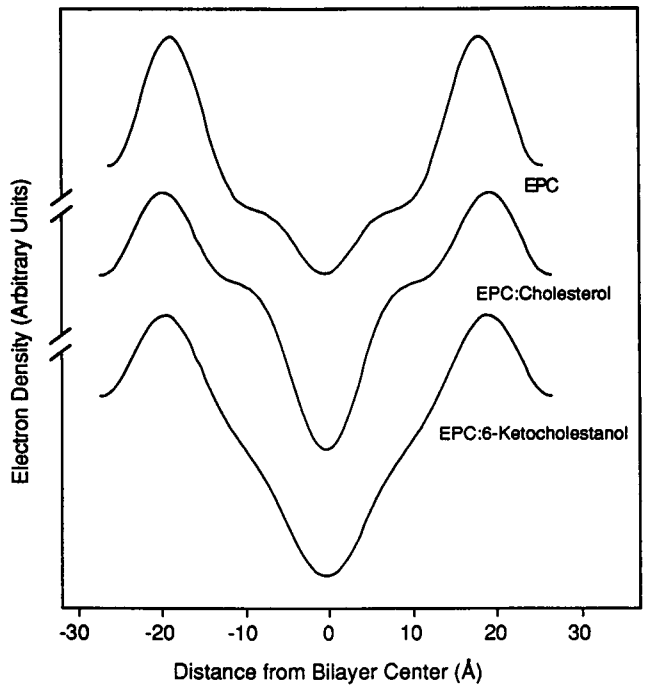


FIGURE 4 Electron density profiles for EPC, equimolar EPC: cholesterol and equimolar EPC:6-ketocholestanol at an applied osmotic pressure of 2×10^8 dyn/cm^2.

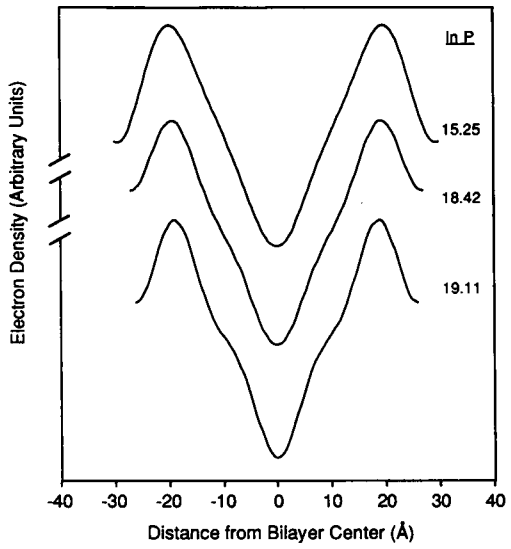


FIGURE 5 Electron density profiles for EPC:6-ketocholestanol at three different osmotic pressures.

bilayer was found to be 37.8 Å ± 0.4 Å (n = 6) for equimolar EPC:6-ketocholestanol, compared to 37.8 Å ± 0.8 Å (mean ± standard deviation, n = 10 experiments) for EPC (McIntosh and Simon, 1986), 39.7 Å ± 0.7 Å (n = 12) for equimolar EPC:cholesterol bilayers (McIntosh et al., 1989*a*). The addition of cholesterol to EPC bilayers raises the electron density of the methylene chain region of the profile relative to the terminal methyl trough (Fig. 4). This locates the more electron dense ring structure of cholesterol (Fig. 1) in the outer chain regions of the bilayer (McIntosh, 1978). The addition of 6-ketocholestanol to EPC bilayers also raises the electron density of the methylene chain region relative to the terminal methyl region. However, in contrast to cholesterol, there is not a distinct shoulder of high electron density adjacent to the head group peak, as the electron density decreases more smoothly from the headgroup region to the bilayer center.

Fig. 5 shows electron density profiles of equimolar EPC:6-ketocholestanol bilayers obtained at applied pressures of 4.2×10^6, 1.0×10^8, and 2.0×10^8 dyn/cm^2. The shape of each profile and the distance between head group peaks across the bilayer (see above) is nearly constant for all applied pressures.

The electron density profiles can be used to estimate the fluid spacing, d_f, between bilayers at each pressure based on the following considerations. Since at this resolution, the high density head group peaks in the electron density profile are known to be located between the phosphate moiety and the glycerol backbone of the lipid (Hitchcock et al., 1974; Lesslauer et al., 1972), the profiles in Fig. 5 can be used to estimate the location of the edge of the bilayer. As noted previously (McIntosh and Simon, 1986; McIntosh et al., 1987; 1989*a* and *b*), the definition of the lipid/water interface is somewhat arbitrary, because the bilayer surface is not smooth, the lipid head groups are mobile (Hauser et al., 1981), and water penetrates into the head group region of the bilayer (Worcester and Franks, 1976; Simon et al., 1982). We operationally define the bilayer width as the total thickness of the bilayer assuming that the head group conformation is the same as it is in single crystals of dimyristoylphosphatidylcholine (Pearson and Pascher, 1979). That is, we assume that the phosphocholine group is, on average, oriented approximately parallel to the bilayer plane (see Fig. 2), so that the edge of the bilayer lies ~ 5 Å outward from the center of the high density peaks in the electron density profiles (McIntosh and Simon, 1986; McIntosh et al., 1987; 1989*a* and *b*). Thus, we estimate the total bilayer thickness (d_b) to be the distance between head group peaks in the profiles plus 10 Å (Table 1). With these assumptions, the fluid thickness (d_f) can be calculated for each osmotic stress experiment by subtracting the bilayer thickness of 47.8 Å from the lamellar repeat period (Table 1).

TABLE 1 **Hydration pressure, structural, and cohesive properties of bilayers in presence and absence of 6-ketocholestanol**

	P_o	λ	V	$2_x(V/\lambda)^2$	K*	d_b	d_{fe}
	(dyn/cm² × 10^{-8})	(Å)	(mV)	(dyn/cm² × 10^{-8})	(dyn/cm)	(Å)	(Å)
EPC	4.0	1.7	415	10.4	192	47.8	15.4
EPC:6-keto-cholestanol	17.6	1.7	703	29.4	370	47.8	17.2

*Compressibility measurements were made with SOPC and SOPC:6-ketocholestanol bilayers.

Membrane compressibility

Compressibility moduli (K) were measured for concentrations of 6-ketocholestanol in SOPC vesicles ranging from 25 to 74 mol%. Fig. 6 shows a typical plot of the applied bilayer tension versus the relative area changes of single unilamellar vesicles formed from 55:45 SOPC:6-ketocholestanol. From measurements taken at 55:45 and 45:55 SOPC:6-ketocholestanol the compressibility modulus of equimolar SOPC:6-ketocholestanol was interpolated to be 370 dyn/cm (Table 1).

Monolayer dipole potential

The dipole potential, V, of equimolar EPC:6-ketocholestanol monolayers spread over 1 mM KCl was 703 ± 32.7 mV (n = 8), which, as shown in Table 1, is considerably larger than the V = 415 mV measured for EPC monolayers (McIntosh et al., 1989*a*).

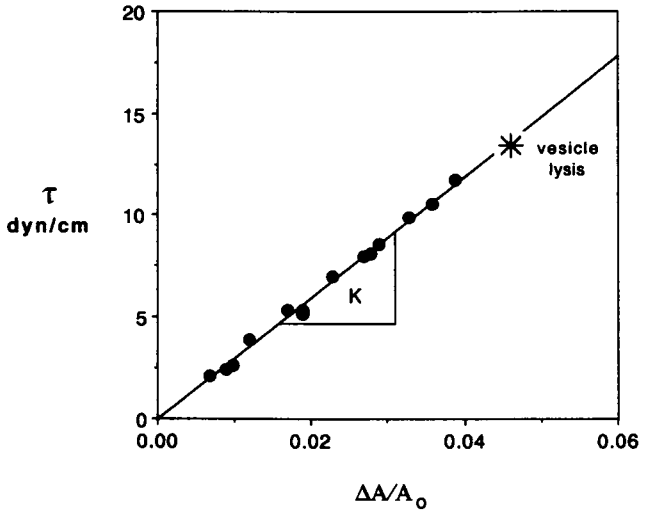


FIGURE 6 Stress (τ) versus straint ($\Delta A/A_0$) relation for unilamellar vesicles composed of 55:45 SOPC:6-ketocholestanol. Increasing the membrane tension causes a linear increase in vesicle membrane area. The slope of this line is the elastic area expansion modulus, K. Subsequent increases in membrane tension produced failure of the membrane and lysis of the vesicle at some critical areal strain and critical tension or tensile strength.

Pressure-distance relations

A plot of the natural logarithm of applied pressure (ln P) versus the distance between bilayer surfaces for equimolar EPC:6-ketocholestanol bilayers is shown in Fig. 7. For this entire range of pressures and bilayer separations these data points can be fit closely (r^2 = 0.98) with a straight line whose slope gives λ = 1.7 Å. For comparison, pressure-distance plots of EPC bilayers are also shown (McIntosh and Simon, 1986). For bilayer separations greater than ~5 Å, the EPC data points are parallel to the EPC:6-ketocholestanol data but displaced to the left by 2–3 Å. However, a sharp upward break is observed at ln $P \approx 18$ and $d_f \approx 4$ Å for EPC bilayers, but not for equimolar EPC:6-ketocholestanol bilayers. This break arises from steric interactions between the polar head groups from apposing bilayers (see Appendix II and McIntosh et al., 1987). For the sake of clarity, the pressure-distance data are replotted in Fig. 8, omitting the data points for $d_f < 4$ Å. For each lipid system, the data points in Fig. 8 can be fit quite closely ($r^2 > 0.96$ for each data set) with a single straight line, indicating that for each system, for this range of d_f, the repulsive pressure decays exponentially with increasing bilayer separation (Appendix II). The two straight lines are nearly parallel, with the EPC:6-ketocholestanol data offset to larger bilayer separations by ~3 Å. For both bilayers the decay constant of the exponential decay is 1.7 Å, showing that the interaction has the same form and that the decay length is not dependent on the geometry of the interface. The P_o values, the intercepts of the straight lines with the ordinate, are 1.8 × 10^9

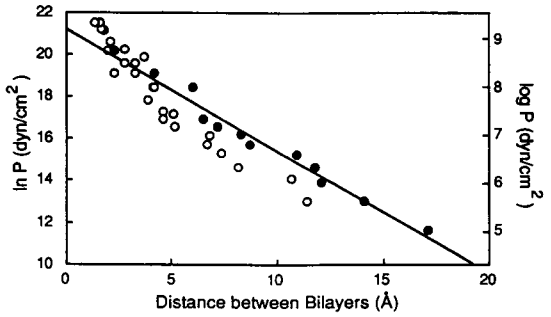


FIGURE 7 Natural logarithm (*left-hand scale*) and common logarithm (*right-hand scale*) of applied pressure plotted versus the fluid spacing between bilayers and EPC (*open circles*, taken from McIntosh et al., 1987) and equimolar EPC:6-ketocholestanol (*solid circles*).

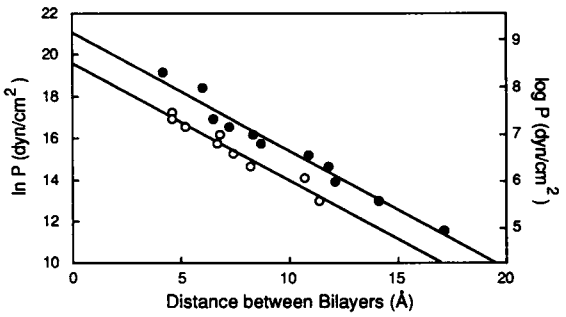


FIGURE 8 Natural logarithm (*left-hand scale*) and common logarithm (*right-hand scale*) of applied pressure plotted versus the fluid spacing between bilayers of EPC (*open circles,* taken from McIntosh et al., 1987) and equimolar EPC:6-ketocholestanol (*solid circles*). This figure differs from the data in Fig. 7 in that the points have been omitted at smaller bilayer separations, where steric hindrance between apposing bilayers is significant.

dyn/cm^2 and 4.0 × 10^8 dyn/cm^2 for equimolar EPC:6-ketocholestanol and EPC, respectively (Table 1).

The equilibrium fluid spacing (d_{fe}), the fluid spacing in excess water with no applied pressure, can be obtained by subtracting d_b from the lamellar repeat period. The equilibrium fluid spacing of 17.2 Å for equimolar EPC:6-ketocholestanol (Table 1), is larger than the equilibrium fluid spacing for either EPC (15.4 Å) or equimolar EPC:cholesterol (16.2 Å) bilayers (McIntosh et al., 1989*a*).

DISCUSSION

Structural and mechanical changes caused by 6-ketocholestanol

Structural information from x-ray diffraction can be combined with mechanical measurements on single lipid bilayer vesicles to characterize the interaction of 6-ketocholestanol with phosphatidylcholine and to determine the likely orientation of the molecule and its dipole in the bilayer.

The incorporation of equimolar concentrations of 6-ketocholestanol into liquid-crystalline phoshpatidylcholine (EPC or SOPC) bilayers does not detectably increase the bilayer thickness and increases the bilayer compressibility modulus by only ~180 dyn/cm (Table 1). These relatively small changes contrast with the significant increases in both membrane thickness (2 Å) and compressibility modulus (500 dyn/cm) caused by the incorporation of equimolar cholesterol into phosphatidylcholine bilayers (McIntosh et al., 1989*a*; Needham and Nunn, 1990). These data show that, unlike cholesterol, 6-ketocholestanol does not markedly change the bilayer thickness or area per PC molecule. That is, 6-ketocholestanol, like other keto steroids (Demel et al., 1972), does not appreciably condense PC bilayers. In addition, the hydrocarbon regions of electron density profiles of EPC:cholesterol and EPC:6-ketocholestanol (Fig. 4) are significantly different in shape.

These differences in structure and cohesive properties can be explained by a simple model where the orientation in the bilayer is different for 6-ketocholestanol than for cholesterol. First, consider the case of cholesterol. Cholesterol is anchored to the hydrocarbon-water interface by virtue of its hydroxyl group and has been shown by x-ray and neutron diffraction to be oriented approximately perpendicular to the plane of the membrane (Franks, 1976; McIntosh, 1978). In this location, cholesterol increases the density of the methylene chain region of the bilayer as seen in electron density profiles (Fig. 4 and Franks, 1976; McIntosh, 1978) and increases the order parameter of the methylene groups in contact with the steroid nucleus (Demel and DeKruyff, 1976; Brown and Seelig, 1978). NMR measurements have shown that the incorporation of cholesterol in EPC bilayers increases the probability of trans conformations in the acyl chains (Brown and Seelig, 1978). The formation of, on average, ~1 additional *trans* conformation per lipid could explain the 2 Å increase in bilayer thickness caused by the addition of equimolar cholesterol to EPC bilayers (Table 1). The comparative sharpness of the terminal methyl trough seen in electron density profiles of EPC:cholesterol bilayers (Fig. 4) is also consistent with cholesterol restricting acyl chain motion and spatially localizing the terminal methyl groups. This condensation and restriction of motion of the acyl chains by cholesterol would increase the cohesive energy of the hydrocarbon region, by increasing the van der Waals interactions, and thus increase the bilayer compressibility modulus (Needham and Nunn, 1990).

The major structural difference between 6-ketocholestanol and cholesterol is the presence of the keto moiety at the 6 position in the B ring of the cyclopentanophenthrene nucleus (Fig. 1). If 6-ketocholestanol were anchored to the interface by its hydroxyl group and oriented perpendicular to the bilayer plane in a similar manner to cholesterol, then the keto group would be inside the hydrocarbon region, about 6 Å from the hydrocarbon-water interface. However, from energy considerations (see Appendix I), one would expect that the keto group would prefer to be closer to the hydrocarbon-water interface. This estimate suggests that it is energetically unfavorable for the keto group to be completely buried in the hydrocarbon region and therefore it would tend to be in a region of higher dielectric constant, near or at the hydrocarbon-water interface. Based on this energy calculation, we argue that the

ketocholestanol molecule is oriented with both its hydroxy and keto groups near the hydrocarbon-water interface. In this orientation, the molecule would be tilted relative to the bilayer plane and extend ~6 Å less into the membrane than cholesterol does. Thus, compared to cholesterol, the keto group of 6-ketocholestanol would be expected to orient the molecule such that it does not extend as deeply into the bilayer as does cholesterol. The electron density profiles in Fig. 4 are consistent with the idea that 6-ketocholestanol does not extend as deeply into the bilayer as does cholesterol. Although the profiles are not at high enough resolution to determine the precise orientation of 6-ketocholestanol in the bilayers, the electron density profile of equimolar EPC:cholesterol contains a prominent increase in density which extends wll into the methylene chain region of the bilayer, whereas the electron density profile of equimolar EPC:6-ketocholestanol does not show this wide region of increased electron density (Fig. 4). In addition, the broader terminal methyl dip in the EPC:6-ketocholestanol profile compared to the EPC:cholesterol profile (Fig. 4) suggests that there is more disorder in the center of the hydrocarbon region in the bilayers containing 6-ketocholestanol.

The differences in bilayer thickness and compressibility between PC bilayers containing equimolar cholesterol and 6-ketocholestanol can also be rationalized in terms of a different orientation of the steroid molecules. If 6-ketocholestanol did not extend as far into the membrane as cholesterol, the acyl chains located beyond the steroid nucleus of 6-ketocholestanol would have a larger "free volume" (additional gauche configurations and more disorder) that would tend to decrease the bilayer thickness compared with EPC:cholesterol bilayers. Moreover, the shorter distance of penetration of 6-ketocholestanol into the bilayer would imply that the total van der Waals attraction (and hence K) would be greater between the acyl chains and cholesterol than between acyl chains and 6-ketocholestanol. Also the keto group might sterically prevent the acyl chains near the interface from approaching as close to the 6-ketocholestanol as they do to cholesterol, thus, further reducing van der Waals interactions. Both of these effects would tend to make the compressibility modulus smaller for SOPC:6-ketocholestanol bilayers than for SOPC:cholesterol bilayers. However, the observation that K is larger for SOPC:6-ketocholestanol than for SOPC bilayers (Table 1) indicates that the 6-ketocholestanol molecule is located in the bilayer hydrocarbon region and suggests that the van der Waals attraction between 6-ketocholestanol and lipid acyl chains is larger than the attraction between the acyl chains themselves.

Therefore, the x-ray diffraction and compressibility results, along with energy considerations (Appendix I), indicate that 6-ketocholestanol is located in the bilayer hydrocarbon region with both the keto and hydroxy groups located near the hydrocarbon-water interface.

Dipole potential of EPC:6-ketocholestanol monolayers

The larger dipole potential observed for monolayers for EPC:6-ketocholestanol (703 mV) compared with monolayers of EPC (415 mV) or EPC:cholesterol (493 mV) (McIntosh et al., 1989*a*) is also consistent with the structural and mechanical data that place the keto moiety at, or near, the hydrocarbon-water interface. The dipole potential of uncharged lipid monolayers (and bilayers) arises from the sum of the perpendicular components of the dipoles (and multipoles) of water, the lipid polar groups, and the lipid acyl chains (Bockris and Reddy, 1973; Flewelling and Hubbell, 1986). The dipole potentials of steroids having a keto group in the 3 position are greater than those having hydroxyl groups in the 3 position. For example, at a constant area per molecules of 40 Å^2, the dipole potential for monolayers of pure cholestane-3-one and cholesterol are ~700 mV and 400 mV, respectively (Adam, 1968). This difference can be rationalized by having the dipole of the keto group (2.3–2.7 D, Israelachvili, 1985) of 6-ketocholestanone monolayers pointing with its positive end towards the bilayer interior. This orientation of the keto group, together with the presence of the hydroxyl group in the 3 position, could result in a dipole potential larger than measured for cholesterol monolayers since the dipole moment of the hydroxyl group is about 1.5 D (Israelachvili, 1985).

The simplest model to explain the differences in V between EPC and EPC:6-ketocholestanol bilayers is the parallel plate capacitor model of the dipole potential (Flewelling and Hubbell, 1986; Martin et al., 1990). In this model V (in millivolts) can be expressed as

$$V = 38\Sigma\mu_p/\epsilon A, \quad (5)$$

where $\Sigma\mu_p$ represents the sum of the perpendicular components of the dipoles of the solvent and amphiphile (in millidebyes), ϵ is the effective dielectric constant, and A is the molecule area of the amphiphile (in Å^2). We will compare monolayers of equimolar EPC:6-ketocholestanol (V = 703 mV) and EPC:cholesterol (V = 493 mV, (McIntosh et al., 1989*a*), and use Eq. 3 to estimate roughly the contribution of the keto group to the measured dipole potential. Assuming an effective dielectric constant of 10 for the head group region (Flewelling and Hubbell, 1986), an area per EPC head group of 100 Å^2 for an equimolar EPC:6-ketocholestanol monolayer, and that the keto group is oriented approximately perpendicular to the air-water interface (μ_p = 2,500

mD), we calculate from Eq. 5 that the keto group itself could contribute ~100 mV to the dipole potential. The rest of the 210 mV difference in dipole potential between EPC:ketocholestanol and EPC:cholesterol monolayers could arise from the contributions of water molecules or slight reorientation of the lipid dipole (Bechinger and Seelig, 1991). Although this simple calculation makes a number of assumptions, it does indicate that 6-ketocholestanol can give rise to an appreciable electric potential.

Effect of 6-ketocholestanol on hydration pressure

The incorporation of 6-ketocholestanol into EPC bilayers modifies the pressure-distance relation for $d_f > 4$ Å (Fig. 8) as well as the equilibrium fluid separation (Table 1). Based on considerations presented in Appendix II, we conclude that the incorporation of 6-ketocholestanol increases the fluid space between apposing bilayers by increasing the repulsive hydration pressure, rather than by modifying other interbilayer pressures.

How could the incorporation of 6-ketocholestanol increase the magnitude of the hydration pressure for EPC bilayers? As noted in the Introduction, for a number of bilayer systems we have previously found a correlation between P_o and V as given by Eq. 1 and have argued that the fields produced by oriented dipoles in the head group region polarize interbilayer water and give rise to the hydration pressure. The data in Table 1 show that the incorporation of 6-ketocholestanol into EPC also significantly increases both P_o and the quantity $2\chi (V/\lambda)^2$. However, for both EPC and EPC:6-ketocholestanol bilayers the measured values for P_o are lower than the quantity $2\chi (V/\lambda)^2$. There are several possible factors that might contribute to this discrepancy as discussed in Appendix III.

The correlation between P_o and $(V/\lambda)^2$ has been observed for a number of bilayer systems for a range V from 223 mV to 612 mV (Simon and McIntosh, 1989; Simon et al., 1991). However, there does not appear to be a correlation between dipole potential and P_o for ether-linked phosphatidylcholines as Gawrisch et al. (1990) found no detectable difference in hydration pressure between liquid-crystalline ether- and ester-linked phosphatidylcholines that differed in dipole potential by ~100 mV. The reasons for their result with ether linked lipids are not fully understood at present, although we note that Eq. 1 predicts that a change in V of 100 mV would produce a relatively small change in P_o that would be difficult to detect experimentally.

Modification of interlamellar water by 6-ketocholestanol: possible mechanisms

The observation that 6-ketocholestanol increases the magnitude of the hydration pressure for EPC bilayers (Table 1) indicates that 6-ketocholestanol, in its location at the hydrocarbon-water interface, modifies the organization and properties of interlamellar water. Because the hydrocarbon-water interface is separated from interlamellar water by the lipid head group region of the bilayer (Fig. 2), which is about 10 Å thick (McIntosh and Simon, 1986), 6-ketocholestanol could not directly hydrogen bond with water molecules in the interlamellar space.

There are at least three possible mechanisms to explain how 6-ketocholestanol could modify interlamellar water: (*a*) through an extended hydrogen bonded network of water molecules, (*b*) through constraints of hydrogen bonding imposed by the geometry of the bilayer surface (Gawrisch et al., 1990), and (*c*) by electric fields produced by 6-ketocholestanol and its chemisorbed water molecules.

For the first mechanism to operate, a hydrogen bond network would entail a relatively extensive string of water molecules. As discussed above, for fully hydrated bilayers the keto group is at least 10 Å from the edge of the bilayer and ~18 Å from the middle of the fluid space between adjacent bilayers. Because a water molecule is ~3 Å in diameter, this mechanism would take a linear array of at least three correlated water molecules to reach from the keto group to interlamellar water at the edge of the bilayer and a string of 6 correlated water molecules to reach from the keto group to water in the middle of the fluid space.

With regard to the second mechanism, it is undoubtedly true that water molecules are oriented by hydrogen bonding to the lipid head group and that their orientation is affected by surface topography. However, at present it is not clear whether this second mechanism, by itself, can accurately predict the changes in P_o that accompany changes in area and surface topography caused by the addition of 6-ketocholestanol. Moreover, it is not clear whether this mechanism would predict the observed invariance of the decay length (λ) upon incorporation of 6-ketocholestanol (Table 1).

We argue that the third mechanism is attractive because this large field could propagate through the head group and modify the orientation of interlamellar water molecules. Two further pieces of evidence support the idea that fields from oriented dipoles are at least in part responsible for the hydration pressure. First, because it has been shown experimentally that dipole

potential is inversely proportional to the area per lipid molecule (A), the dipole potential model would predict from Eq. 1 that P_o should be inversely proportional to the A^2. In fact, such a relationship has been found experimentally (Simon et al., 1988). That is, as A decreases and intercalated water is removed from the head group region of the bilayer, both P_o and V increase. Secondly, P_o has been found to be proportional to V^2 even when the shape or topography of the bilayer surface is modified, either by changing from the gel to the liquid-crystalline phase (Simon et al., 1988) or by the addition of cholesterol (McIntosh et al., 1989*a*) or 6-ketocholestanol to the bilayer.

Thus, we argue that our data from EPC:6-ketocholestanol bilayers indicate that electric fields contribute, along with other mechanisms such as hydrogen bonding, to the polarization of interlamellar water by the bilayer and to the magnitude of the hydration pressure.

Spatial dependence and magnitude of the dipole potential

Given that the hydration pressure depends, at least in part, on fields produced by oriented dipoles, several important questions remain. First of all, what is the spatial dependence of the dipole potential? That is, how steeply does the dipole potential drop across the lipid head group and the interlamellar fluid space? Are the fields in fact large enough to orient water molecules in the head group region and in the interlamellar space?

Spatial dependence of dipole potential

Although a computer simulation of the dipole potential at a water-platinum interface has been performed (Spohr, 1989), we know of no simulation of the potential profile near the lipid-water interface based on the correct number of waters and orientation of lipid and water dipoles. In the absence of such a computer simulation, we will analyze the form of the potential drop predicted by simpler models of V. The parallel plate model (Eq. 5) predicts that the potential drops linearly across the plates of the capacitor (Martin et al., 1990). In this model, the distance between the plates is not specified nor is the location of the plates relative to the lipid bilayer. Flewelling and Hubbell (1986) developed a model for V based on point dipoles arranged on a lattice in contact with a variable dielectric media. In this model, V decays very rapidly, but the location of the lattice of point dipoles in the bilayer is not evident. Theoretical analyses of the range of the electric field responsible for polarizing solvent dipoles by Schiby and Ruckenstein (1983) and of the hydration potential by Cevc and Marsh (1985) predict that the field (potential) decreases exponentially from the plane of origin with a decay constant λ. In these models, as in previous ones, the position of the plane of origin relative to the lipid head group is not specified.

One constraint on the position of the plane of origin for any of these models comes from experiments by McLaughlin and colleagues (Andersen et al., 1978; Eisenberg et al., 1979; Lau et al., 1981; Langer et al., 1990). Calculations of the zeta potential of EPC liposomes show that at the "plane of shear," the zeta potential is 0.0 ± 0.5 mV (Eisenberg et al., 1979; Lau et al., 1981). From these measurements, together with similar measurements using charged lipids, analysis of ion transport across bilayers, and ^{13}C and ^{31}P NMR measurements of ion binding, Lau et al. (1981) concluded that the plane of shear is ~2 Å from the fixed charges of the bilayer. Moreover, they found that classical Gouy-Chapman or double-layer theory, together with a parameter that accounts for ion binding, accurately describes the potential profile arising from fixed or adsorbed charges on the lipids. Their data show that in the absence of ion binding, small ions at the plane of shear do not experience the effects of the dipole potential. In contrast, hydrophobic ions such as tetraphenylboron, which partition into the interfacial region (Andersen et al., 1978), do experience the effects of the dipole potential (Flewelling and Hubbell, 1986).

The following problem is raised by the results of these experiments. If the hydration pressure arises from the polarization of interlamellar water (Marcelja and Radic, 1976; Gruen and Marcelja, 1983; Schiby and Ruckenstein, 1983; Cevc et al., 1985; Dzhavakhidze et al., 1986, 1988), why don't these polarized water molecules give rise to a dipole potential that can be detected by zeta potential measurements? One possible factor, as discussed in detail by Simon and McIntosh (1989), is that the plane of shear might be located further than 2 Å from the fixed or adsorbed charges at the bilayer surface. Another factor that would tend to delocalize the plane of shear from a specific location is that ions or nonelectrolytes that adsorb to the lipid surface and change the dipole potential also cause a conformational change of the lipid polar head group (Brown and Seelig, 1977; Hauser et al., 1976; Seelig et al., 1987; Bechinger and Seelig, 1991). However, regardless of the precise location of the "plane" of shear, the data of Eisenberg et al. (1979) and Lau et al. (1981) suggest that the dipole potential decays to near 0 mV at a distance not far from the physical edge of the bilayer.

Magnitude of dipole potential

Although the spatial dependence of the dipole potential across the head group region of the bilayer and into the

fluid space is not known, we can use the above information to give a rough estimate for the magnitude of the electric field near the edge of the bilayer. For the case of equimolar EPC:6-ketocholestanol, we use the parallel capacitor model and assume that the dipole potential drops linearly from 703 mV at the location of the keto group near the hydrocarbon-water interface to 0 mV at a location 2 Å from the edge of the bilayer. That is, the potential would drop 703 mV in a distance of ~12 Å (Fig. 2) so that the electric field near the edge of the bilayer would be approximately 703 mV/12 Å = 5.9×10^6 V/cm (see Appendix III). This is a very large field capable of aligning dipoles (Gurney, 1953; Israelachvili, 1985; Partenskii et al., 1991). This calculation indicates that the electric field near the edge of the bilayer could be sufficiently large to orient the first layer of interlamellar water. The rest of the interlamellar water could respond to this field by arranging in a manner to maximize the number of hydrogen bonds (Attard and Batchelor, 1988).

APPENDIX I

Energy consideration for the keto group in the hydrocarbon region of the bilayer

The free energy to transfer a C=O group from the hydrocarbon region of a bilayer into water is approximately $-N\mu^2/4\pi\epsilon_o\epsilon l^3 = -5.6$ Kcal/mol, where μ is the dipole moment (~2.5 D), l is the length of the dipole (~2 Å), ϵ is the dielectric constant of hydrocarbon (2), and N is Avogadro's number (Israelachvili, 1985). This free energy would be opposed by the energy required to expose of some of the hydrocarbon on the A ring of 6-ketocholestanol to water. Using 47 cal/mol/Å^2 as the energy to insert hydrocarbons into water (Sharp et al., 1991), we calculate that exposure of 45 Å^2 (the approximate area per molecule of 6-ketocholestanol) of hydrocarbon to water would require an energy of ~2 Kcal/mol. Therefore, it is energetically unfavorable for C=O group to be buried in the hydrocarbon region of the bilayer.

APPENDIX II

Component pressures

For uncharged bilayers, four nonspecific interactions are thought to exist: van der Waals attraction, steric repulsion between head groups from apposing bilayers (McIntosh et al., 1987), repulsion due to thermally driven undulations or fluctuations (Helfrich, 1973; Evans and Parsegian, 1986), and hydration repulsion (LeNeveu et al., 1977; Parsegian et al., 1979; McIntosh and Simon, 1986; Rand and Parsegian, 1989).

The increase in the equilibrium fluid separation (Table 1) and the shift in the pressure-distance data for $d_f > 4$ Å (Fig. 8) potentially could be explained by either a decrease in van der Waals attraction or by an increase in one or more of the repulsive pressures. However, the incorporation of 6-ketocholestanol into bilayers should increase, not decrease, the van der Waals pressure because the steroid nucleus is more polarizable than the acyl chains (Nir, 1976). The increase in fluid spacing caused by the incorporation of 6-ketocholestanol into EPC bilayers therefore cannot be accounted for by a reduction in attractive pressure and must originate from an increased repulsion between apposing membranes. In the following paragraphs, we consider the relative contributions of the three nonspecific repulsive pressures.

Steric pressure ($d_f < 4$ Å).

The steric pressure between head groups from apposing bilayers has been shown to contribute to the total pressure of EPC bilayers for bilayer separations of less than 4 Å, but not contribute significantly to the total pressure for equimolar EPC:cholesterol bilayers (McIntosh et al., 1987). For EPC bilayers, the presence of steric hindrance between the head groups from apposing bilayers is indicated by a sharp upward break in the plot of ln P versus fluid separation for separations of less than 4–5 Å (Fig. 7 and McIntosh et al., 1987). No such upward break is observed in plots of ln P versus bilayer separation for equimolar EPC:cholesterol bilayers (McIntosh et al., 1989*a*) or for equimolar EPC:6-ketocholestanol bilayers (Fig. 7). This indicates that steric pressure is negligible for these bilayer systems for the range of pressures applied. The reduction of steric pressure by the incorporation of cholesterol has been explained by cholesterol separating the PC head groups in the plane of the bilayers, and thereby decreasing the volume fraction of PC head groups at the interface (McIntosh et al., 1989*a*). A similar reduction of steric interaction is evidently occurring with the incorporation of 6-ketocholestanol into EPC bilayers. Thus, in our analysis we have ignored the effects of steric pressure, and analyzed the pressure-distance relationships for EPC and equimolar EPC:6-ketocholestanol bilayers only for bilayer separations >4 Å (Fig. 8).

Fluctuation and hydration pressure ($d_f > 4$ Å)

Because steric interactions are negligible for $d_f > 4$ to 5 Å (McIntosh et al., 1987), the differences in the pressure-distance plots of Fig. 8 must be rationalized in terms of modifications in either the repulsive fluctuation or hydration pressures. The fluctuation pressure has been predicted to be inversely proportional to the bilayer bending modulus (Harbich and Helfrich, 1984), which, for a given bilayer thickness, is directly proportional to the area compressibility modulus (K). Given that K is larger for equimolar EPC:6-ketocholestanol than for EPC bilayers (Table 1), the fluctuation pressure should be larger for EPC bilayers than equimolar EPC:6-ketocholestanol bilayers. However, because the total pressure is larger for EPC:6-ketocholestanol bilayers than for EPC bilayers, it follows that 6-ketocholestanol's effects on the fluctuation pressure can not be responsible for the observed differences in the pressure-distance relationships (Fig. 8).

We therefore conclude that, for fluid spacing greater than 4 Å (Fig. 8), the increases in bilayer separation induced by the addition of 6-ketocholestanol must arise from an increase in the hydration pressure.

APPENDIX III

Comparison between measured and predicted P_o values

Although the measured and predicted values for P_o both increase upon the incorporation of 6-ketocholestanol, the values for P_o obtained by x-ray diffraction are lower than the values for both EPC and 6-ketocholestanol calculated from Eq. 1. There are several possible factors that might contribute to this discrepancy. First, by far the largest source of experimental uncertainty is in the location of the "plane of origin" (the plane where $d_f = 0$). In order to obtain P_o from the x-ray measurements it is necessary to extrapolate the expontential pressure-

distance relationships to $d_f = 0$. We (McIntosh and Simon, 1986; McIntosh et al., 1987; Simon and McIntosh, 1989; McIntosh et al., 1989*a*; McIntosh et al., 1990) have chosen this "plane" to be at the physical edge of the bilayer to take into account all of the oriented dipoles at the lipid-water interface. A small change in the location of this plane of origin can cause a significant shift in the values of P_o. For example, if the plane of origin were shifted only 1 Å into the head group region of the bilayer, then the measured and calculated values of P_o would be in very close agreement. Moreover, because plots of ln P versus d_f for EPC and equimolar EPC:6-ketocholestanol are parallel but displaced by a constant fluid spacing, it follows that P_o must be larger for EPC:6-ketocholestanol than EPC, regardless of the choice of the plane of origin.

A second source of experimental uncertainty in the measured values of P_o concerns possible bilayer deformations or changes in bilayer thickness upon partial dehydration with applied osmotic pressure. The electron density profiles of equimolar EPC:6-ketocholestanol (Fig. 5) do not show a systematic change in bilayer thickness upon partial dehydration. Consequently, the pressure-distance relationships shown in Fig. 8 were obtained assuming a constant bilayer thickness for all applied pressures. However, the electron density profiles are at limited resolution ($d/2h_{max} \approx 6$ Å), so that small changes (on the order of 1 Å) might not be detectable. To esimate the possible effects of bilayer deformation on the pressure-distance relationships, we use the procedure developed by Rand and Parsegian (1989) (see pages 372–374). That is, we use the measured compressibility moduli (Table 1) to calculate the changes in bilayer thickness expected for the range of applied pressures shown in Fig. 8. This calculation yields a maximum increase in bilayer thickness upon partial dehydration of 0.8 Å and 0.9 Å for EPC and EPC:6-ketocholestanol, respectively. The increase for EPC:6-ketocholestanol is slightly higher since the range of applied pressure was greater for that system (Fig. 8). Using these adjusted values of bilayer thickness for EPC and EPC:6-ketocholestanol bilayers, we obtain decay constants of 1.8 Å and 1.9 Å, respectively, and values of P_o of 2.9×10^8 dyn/cm^2 and 1.1×10^9 dyn/cm^2, respectively. Thus, although this correction for deformation changes the slopes and intercepts of the pressure-distance relationships, it does not modify the central finding that the incorporation of 6-ketocholestanol into EPC bilayers increases P_o.

We also note that the dipole potential as calculated from conductance measurements of lipophilic ions in bilayers differs from direct measurements of dipole potential using monolayers at the air-water interface by 100–150 mV for various phosphatidylcholines and monoglycerides (Flewelling and Hubbell, 1986; Smaby and Brockman, 1990). Possible reasons for this difference have been discussed previously (Simon and McIntosh, 1989).

Finally, another possible source for the discrepancy between the measured values of P_o and those calculated from Eq. 1 is that, as discussed in the Introduction, the proportionality factor 2χ in Eq. 1 is model dependent.

We thank Drs. O. Andersen, D. Cafiso, E. A. Disalvo, A. McLaughlin, S. McLaughlin, and V. Petrow for helpful discussions. We also are grateful to Drs. K. Gawrisch, J. Zimmerberg, D. Ruston, V. A. Parsegian, R. P. Rand, and N. Fuller for sending us a draft of their manuscript comparing the hydration properties of ether and ester linked phosphatidylcholines, which has been presented in abstract form (Gawrisch et al., 1990).

This work was funded by National Institutes of Health grant GM-27278.

Received for publication 14 August 1991 and in final form 24 October 1991.

REFERENCES

Adam , N. K. 1968. The Physics and Chemistry of Surfaces. Dover Publications, New York. 75–84.

Andersen, O. S., S. Feldberg, H. Nakadomari, S. Levy, and S. McLaughlin. 1978, Electrostatic interactions among hydrophobic ions in bilayer membranes. *Biophys. J.* 21:35–70.

Attard, P., and M. T. Batchelor. 1988. A mechanism for the hydration force demonstrated in a model system. *Chem. Phys. Lett.* 149:206–211.

Bechinger, B., and J. Seelig. 1991. Interaction of electric dipoles with phospholipid head groups. A ^{2}H and ^{31}P NMR study of phloretin and phloretin analogues in phosphatidylcholine membranes. *Biochemistry.* 30:3923–3929.

Belaya, M. L., M. V. Feigel'man, and V. G. Levadny. 1986. Hydration forces as a result of non-local water polarizability. *Chem. Phys. Lett.* 126:361–364.

Blaurock, A. E., and C. R. Worthington. 1966. Treatment of low angle x-ray data from planar and concentric multilayered structures. *Biophys. J.* 6:305–312.

Bockris, J. O'M., and A. K. N. Reddy. 1973. Modern Electrochemistry. Vol. 2. Plenum Press, New York. 779–792.

Brown, M. F., and J. Seelig. 1977. Ion-induced changes in head group conformation of lecithin bilayers. *Nature, (Lond.).* 269:721–723.

Brown, M. F., and J. Seelig. 1978. Influence of cholesterol on the polar region of phosphatidylcholine and phosphatidylethanolamine bilayers. *Biochemistry.* 17:381–384.

Cevc, G., and D. Marsh. 1985. Hydration of noncharged lipid bilayer membranes. Theory and experiments with phosphatidylethanolamine. *Biophys. J.* 47:21–32.

Cevc, G., J. M. Seddon, and D. Marsh. 1985. Thermodynamics and structural properties of PS bilayer membranes in the presence of lithium ions and protons. *Biochim. Biophys. Acta.* 813:343–346.

Demel, R. A., K. R. Bruckdorfer, and L. L. M. van Deenen. 1972. Structural requirements of sterols for the interaction with lecithin at the air-water interface. *Biochem. Biophys. Acta.* 255:311–320.

Demel, R. A., and B. DeKruyff. 1976. The function of sterols in membranes. *Biochim. Biophys. Acta.* 457:109–132.

Dzhavakhidze, P. G., A. A. Kornyshev, and V. G. Levadny. 1986. The role of the interface in the nonlocal electrostatic theory of hydration force. *Phys. Lett. A.* 118:203–208.

Dzhavakhidze, P. G., A. A. Kornyshev, and V. G. Levadny. 1988. The structure of the interface in the solvent-mediated interaction of dipolar surfaces. *Il Nuovo Cimento.* 10D:627–654.

Eisenberg, M., T. Gresalfi, T. Riccio, and S. McLaughlin. 1979. Adsorption of monovalent cations to bilayer membranes containing negative phospholipids. *Biochemistry.* 18:5213–5223.

Evans, E. A., and V. A. Parsegian. 1986. Thermal-mechanical fluctuations enhance repulsion between bimolecular layers. *Proc. Natl. Acad. Sci. USA. 83:7132–7136.*

Flewelling, R. F., and W. L. Hubbell. 1986. The membrane dipole potential in a total membrane potential model applications to hydrophobic ion interactions with membranes. *Biophys. J.* 541–552.

Franks, N. P. 1976. Structural analysis of hydrated egg lecithin and cholesterol bilayers I. X-ray diffraction. *J. Mol. Biol.* 100:345–358.

Gawrisch, K., J. Zimmerberg, V. A. Parsegian, R. P. Rand, and N. A. Fuller. 1990. Membrane dipole potentials and hydration forces: cause or effect: *Biophys. J.* 57:35*a* (Abstr.)

Gurney, R. W. 1953. Ionic Processes in Solution. Dover Publications, New York. 1–51.

Gruen, D. W. R. and S. Marcelja. 1983. Spatially varying polarization in water. *J. Chem. Soc. Faraday Trans.* 79:225–242.

Harbich, W., and W. Helfrich. 1984. The swelling of egg lecithin in water. *Chem. Phys. Lipids.* 36:39–63.

Hauser, H. 1981. The polar group conformation of 1,2-dialkyl phosphatidylcholines. An NMR study. *Biochim. Biophys. Acta.* 646:203–210.

Hauser, H., I. Pascher, R. H. Pearson, and S. Sundell. 1981. Preferred conformation and molecular packing of phosphatidylethanolamine and phosphatidylcholine. *Biochim. Biophys. Acta.* 650:21–51.

Helfrich, W. 1973. Elastic properties of lipid bilayers: theory and possible experiments. *Z. Naturforsch.* 28C:693–703.

Herbette, L., J. Marquardt, A. Scarpa, and J. K. Blasie. 1977. A direct analysis of lamellar x-ray diffraction from hydrated oriented multilayers of fully functional sarcoplasmic reticulum. *Biophys. J.* 20:245–272.

Hitchcock, P. B., R. Mason, K. M. thomas, and G. G. Shipley. 1974. Structural chemistry of 1,2 dilauroyl-DL-phosphatidylethanolamine: molecular conformation and intermolecular packing of phospholipids. *Proc. Natl. Acad. USA.* 71:3036–3040.

Israelachvili, J. N. 1985. Intermolecular and Surface Forces. Academic Press, London, 37–51.

Kornyshev, A. A. 1986. On the non-local electrostatic theory of hydration force. *J. Electroanal. Chem.* 204:79–84.

Kwok, R., and E. Evans. 1981. Thermoelasticity of large lecithin vesicles. *Biophys. J.* 35:637–652.

Langer, M., D. Cafiso, S. Marcelja, and S. McLaughlin. 1990. Electrostatics of phosphoinositide bilayer membranes. Theoretical and experimental results. *Biophys. J.* 57:335–349.

Lau, A., A. McLaughlin, and S. McLaughlin. 1981. The adsorption of divalent cations to phosphatidylglycerol bilayers. *Biochim. Biophys. Acta.* 645:279–292.

LeNeveu, D. M., R. P. Rand, V. A. Parsegian, and D. Gingell. 1977. Measurement and modification of forces between lecithin bilayers. *Biophys. J.* 18:209–230.

Lesslauer, W., J. E. Cain, and J. K. Blasie. 1972. X-ray diffraction studies of lecithin bimolecular leaflets with Incorporated fluorescent probes. *Proc. Natl. Acad. Sci. USA.* 69:1499–1503.

MacDonald, R. C., and S. A. Simon. 1987. Lipid monolayer states and their relationship to bilayers. *Proc. Natl. Acad. Sci. USA.* 84:4089–4094.

Marcelja, S., and N. Radic. 1976. Repulsion of interfaces due to boundary water. *Chem. Phys. Lett.* 42:129–130.

Martin, D., O. N. Oliveira, Jr., and H. Morgan. 1990. Models for interpreting surface potential measurements and their application to phospholipid monolayers. *J. Colloid Interface Sci.* 139:508–518.

McIntosh, T. J. 1978. The effect of cholesterol on the structure of phosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 513:43–58.

McIntosh, T. J., and P. W. Holloway. 1987. Determination of the depth of bromine atoms in bilayers formed from bromolipid probes. *Biochemistry.* 26:1783–1788.

McIntosh, T. J., A. D. Magid, and S. A. Simon. 1987. Steric repulsion between phosphatidylcholine bilayers. *Biochemistry.* 26:7325–7332.

McIntosh, T. J., A. D. Magid, and S. A. Simon. 1989a. Cholesterol modifies the short-range repulsive interactions between phosphatidylcholine membranes. *Biochemistry.* 28:17–25.

McIntosh, T. J., A. D. Magid, and S. A. Simon. 1989b. Range of the solvation pressure between lipid membranes: dependence on the packing density of solvent molecules. *Biochemistry.* 28:7904–7912.

McIntosh, T. J., A. D. Magid, and S. A. Simon. 1989c. Repulsive interactions between uncharged bilayers. Hydration and fluctuation pressures for monoglycerides. *Biophys. J.* 55:897–904.

McIntosh, T. J., A. D. Magid, and S. A. Simon. 1990. Interactions between charged, uncharged, and zwitterionic bilayers containing phosphatidylglycerol. *Biophys. J.* 57:1187–1197.

McIntosh, T. J., and S. A. Simon. 1986. The hydration force and bilayer deformation: a reevaluation. *Biochemistry* 25:4058–4066.

Needham, D., and R. S. Nunn. 1990. Elastic deformation and failure of lipid bilayer membranes containing cholesterol. *Biophys. J.* 58:997–1010.

Nir, S. 1976. Van der Waals interactions between surfaces of biological interest. *Prog. Surface Sci.* 8:1–58.

O'Brien, F. E. M. 1948. Control of humidity by saturated salt solutions. *J. Sci. Instrum.* 25:73–76.

Paltauf, F., H. Hauser, and M. C. Phillips. 1971. Monolayer characteristics of some 1,2-diacyl, 1-alkyl-2-acyl and 1,2-diacyl phospholipids at the air-water interface. *Biochim. Biophys. Acta.* 249:539.

Parsegian, V. A., N. Fuller, and R. P. Rand. 1979. Measured work of deformation and repulsion of lecithin bilayers. *Proc. Natl. Acad. Sci. USA.* 76:2750–2754.

Parsegian, V. A., R. P. Rand., N. L. Fuller, and R. C. Rau. 1986. Osmotic stress for the direct measurement of intermolecular forces. *Methods Enzymol.* 127:400–416.

Partenskii, M. B., M. Cai, and P. C. Jordan. 1991. The dipolar chain model for the electrostatics of transmembrane ion channels. *Chem. Phys.* 53:125–129.

Pearson, R. H., and I. Pascher. 1979. The molecular structure of lecithin dihydrate. *Nature, (Lond.).* 281:499–501.

Rand, R. P., and V. A. Parsegian. 1989. Hydration forces between phospholipid bilayers. *Biochim. Biophys. Acta.* 988:351–376.

Rau, D. C., B. Lee, and V. A. Parsegian. 1984. Measurement of the repulsive force between polyelectrolyte molecules in ionic solution: hydration forces between parallel DNA double helices. *Proc. Natl. Acad. Sci. USA.* 81:2621–2625.

Rau, D. C., and V. A. Parsegian. 1990. Direct measurement of forces between linear polysaccharides xanthan and schizophylallan. *Science (Wash. DC).* 249:1278–1281.

Reeves, J. P., and R. M. Dowben. 1969. Formation and properties of thin-walled phospholipid vesicles. *J. Cell Physiol.* 73:49–60.

Schiby, D., and E. Ruckenstein. 1983. The role of the polarization layers in hydration forces. *Chem. Phys. Lett.* 95:435–438.

Seelig, J., P. M. Macdonald, and P. G. Scherer. 1987. Phospholipid head groups as sensors of electric charge in membranes. *Biochemistry.* 26:7535–7541.

Shannon, C. E. 1949. Communication in the presence of noise. *Proc. Inst. Radio Engrs. N. Y.* 37:10–21.

Sharp, K. A., A. Nicholls, R. F. Fine, and B. Honig. 1991. Reconciling the Magnitude of the Microscopic and Macroscopic Hydrophobic Effects. *Science (Wash. DC).* 252:106–109.

Simon, S. A., C. A. Fink, A. K. Kenworthy, and T. J. McIntosh. 1991. Hydration pressure between lipid bilayers: A comparison of measurements using x-ray diffraction and calorimetry. *Biophys. J.* 59:538–546.

Simon, S. A., and T. J. McIntosh. 1986. The depth of water penetration into lipid bilayers. *Methods Enzymol.* 127:511–521.

Simon, S. A., and T. J. McIntosh. 1989. Magnitude of the solvation pressure depends on dipole potential. *Proc. Natl. Acad. USA.* 86:9263–9267.

Simon, S. A., T. J. McIntosh, and R. Latorre. 1982. Influence of cholesterol on water penetration into bilayers. *Science (Wash. DC).* 216:65–67.

Simon, S. A., T. J. McIntosh, and A. D. Magid. 1988. Magnitude and range of the hydration pressure between lecithin bilayers as a function of head group density. *J. Colloid Interface Sci.* 126:74–83.

Smaby, J. M., and H. L. Brockman. 1990. Surface Dipole Moments of Lipids at the Argon-Water Interface. Similarities Among Glycerol-Ester-Based Lipids. *Biophys. J.* 58:195–204.

Spohr, E. 1989. Computer simulation of the water/platinum interface. *J. Phys. Chem.* 93:6171–6180.

Tanford, C. 1980. The Hydrophobic Effect, Second Edition. John Wiley and Sons, Inc. New York. 5–13.

Tardieu, A., V. Luzzati, and F. C. Reman. 1973. Structure and polymorphism of the hydrocarbon chains of lipids: a study of lecithin-water phases. *J. Mol. Biol.* 75:711–733.

Vink, H. 1971. Precision measurements of osmotic pressure in concentrated polymer solutions. *Eur. Polymer J.* 7:1411–1419.

Weast, R. C. 1984. Handbook of chemistry and physics. CRC Press, Boca Raton, Florida.

Worcester, D. L., and N. P. Franks. 1976. Structural analysis of hydrated egg lecithin and cholesterol bilayers II. Neutron diffraction. *J. Mol. Biol.* 100:359–378.