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Interbilayer Interactions between Sphingomyelin and Sphingomyelin/Cholesterol Bilayers†

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ABSTRACT: Pressure versus fluid spacing relations have been obtained for sphingomyelin bilayers in the gel phase and equimolar sphingomyelin/cholesterol in the liquid-crystalline phase by the use of X-ray diffraction analysis of osmotically stressed aqueous dispersions and oriented multilayers. For interbilayer separations in the range of 5-20 Å, the repulsive hydration pressure decays exponentially with increasing fluid spacing. The decay length (λ) of this repulsive pressure is about 2 Å for both bovine brain and *N*-tetracosanoylsphingomyelin, similar to that previously found for phosphatidylcholine bilayers. However, both the magnitude of the hydration pressure and the magnitude of the dipole potential (V) measured for monolayers in equilibrium with liposomes are considerably smaller for sphingomyelin than for either gel or liquid-crystalline phosphatidylcholine bilayers. Addition of equimolar cholesterol increases both the magnitude of the hydration pressure and the dipole potential. These data suggest that the magnitude of the hydration pressure depends on the electric field at the interface as given by $(V/\lambda)^2$. For sphingomyelin bilayers, there is a sharp upward break in the pressure-fluid spacing relation at an interbilayer spacing of about 5 Å, indicating the onset of steric hindrance between the head groups of apposing bilayers.

The hydration pressure (P_h) is a short-range repulsive interaction which provides a major barrier to the close approach of apposing membranes or hydrated macromolecules (Parsegian et al., 1979; Marra & Israelachvili, 1985; Rand & Parsegian, 1989). For bilayers composed of a variety of neutral, charged, and zwitterionic lipids, the hydration pressure has been shown to decay exponentially with increasing fluid spacing (d_f) such that $P_h = P_o \exp(-d_f/\lambda)$, where the decay length (λ) has been measured to be 1-2 Å (LeNeveu et al., 1977; McIntosh & Simon, 1986; McIntosh et al., 1989a,c; Rand & Parsegian, 1989).

Sphingomyelin (SM) is a common membrane phospholipid that has the same phosphorylcholine head group as phosphatidylcholine (PC), but with a different backbone: SM has a sphingosine backbone and PC has a glycerol backbone. It is of interest to measure the hydration pressure between sphingomyelin bilayers for several reasons. First, although it might be expected that the hydration pressure should be similar for sphingomyelin and other phospholipid bilayers, Tamura-Lis et al. (1986) found a decay length of over 6 Å for sphingomyelin bilayers. Second, comparison of pressure–distance data from SM and PC bilayers should provide insights as to the importance of the lipid backbone in determining the hydration pressure. Third, sphingomyelin bilayers provide another test for our previously observed correlation between the magnitude of the hydration pressure (P_o) and the square of the dipole potential (V) as measured for monolayers in equilibrium with bilayers (Simon et al., 1988; McIntosh et al., 1989a–c; Simon & McIntosh, 1989). Fourth, the recent model of Israelachvili and Wennerstrom (1990) asserts that vertical displacements of the lipid head groups can give rise to a short-range repulsive pressure. This sort of steric repulsion would be expected to be significantly smaller for gel-phase bilayers than for liquid–crystalline phase bilayers, due to reduced molecular motion in the gel phase. Since SM is in the gel phase at room temperature (Calhoun & Shipley, 1979), whereas equimolar SM/cholesterol bilayers are in a liquid-crystalline phase (McIntosh et al., 1991), comparisons of pressure–distance relations between SM and equimolar SM/cholesterol bilayers should provide information on the magnitude of the proposed steric repulsion caused by vertical displacements.

In this paper, we use X-ray diffraction analysis of osmotically stressed lipid multilayers to obtain pressure–distance

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relations for bovine brain sphingomyelin (BSM) and *N*-tetracosanoylsphingomyelin (C24-SM) bilayers in the presence and absence of equimolar concentrations of cholesterol. The magnitudes of the hydration pressures for these bilayers are correlated with dipole potentials measured for monolayers in equilibrium with vesicles.

Materials and Methods

Materials. Bovine brain sphingomyelin (BSM) was obtained from Avanti Polar lipids, Inc. (Birmingham, AL). Cholesterol and poly(vinylpyrrolidone) (PVP), with an average molecular weight of 40 000, were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Tetracosanoylsphingomyelin (C24-SM) was synthesized as described in the preceding paper in this issue (McIntosh et al., 1992).

X-ray Diffraction of Osmotically Stressed Multilayers. Sphingomyelin and equimolar sphingomyelin/cholesterol bilayers were subjected to osmotic stress as described in detail in the preceding paper in this issue (McIntosh et al., 1992). In brief, osmotic pressures in the range of (0–3.2 × 10^7 dyn/cm^2 were applied to unoriented lipid suspensions by equilibrating dry lipid in 0–60% (w/w) aqueous solutions of PVP. Pressures in the range of 2.8 × 10^7 to 2.7 × 10^9 dyn/cm^2 were applied to oriented multilayers through the vapor phase by equilibration with saturated salt solutions (Parsegian et al., 1979; McIntosh et al., 1987). For each applied pressure, X-ray diffraction patterns were recorded and electron density profiles were calculated as described previously (McIntosh et al., 1987, 1989a,c, 1992).

Dipole Potential Measurements. For measurements of the dipole potential (V), monolayers were formed by spreading 10–40 μL of a lipid/chloroform solution (25 mg/mL) onto a subphase of 1 mM KCl. A teflon trough with a surface area of about 30 cm^2 was used. MacDonald and Simon (1987) have argued that under these conditions the surface monolayer is in equilibrium with liposomes in the subphase. To ensure that the surface was free of surface-active impurities, the KCl was roasted at 600 °C and the subphase surface was vacuum aspirated immediately before the monolayer was spread. The trough was thoroughly cleaned between runs. The dipole potential was measured between a Ag/AgCl electrode in the subphase and a polonium electrode in air which was connected to a Keithly electrometer, as previously described (MacDonald et al., 1987; McIntosh et al., 1989b). The reported values of dipole potential represent the differences in the potential of the subphase surface in the presence and absence of the monolayer.

Results

X-ray Diffraction and Interbilayer Pressure. For all specimens of C24-SM, BSM, equimolar C24-SM/cholesterol, and equimolar BSM/cholesterol, the diffraction patterns consisted of a series of low-angle reflections, which indexed as orders of a lamellar repeat period, and a single wide-angle band. For C24-SM or BSM bilayers, sharp wide-angle reflections centered at 4.15 Å were recorded, typical of lipids in the gel phase (Tardieu et al., 1973). For all samples of equimolar cholesterol and either BSM or C24-SM, the wide-angle pattern consisted of a broad wide-angle band centered at about 4.5 Å, typical of liquid-crystalline phase bilayers (Tardieu et al., 1973). For each type of sample, either SM or SM/cholesterol, the wide-angle pattern was independent of applied osmotic pressure.

The ranges of measured repeat periods for the applied osmotic pressures given in parentheses were 72.8–63.2 Å (4.3 × 10^5 to 1.6 × 10^9 dyn/cm^2) for C24-SM, 75.6–63.4 Å (1.1 × 10^5 to 2.7 × 10^9 dyn/cm^2) for BSM, 68.1–66.3 Å (2.3 × 10^6 to 6.9 × 10^6 dyn/cm^2) for equimolar C24-SM/cholesterol, and 66.3–59.3 Å (1.1 × 10^5 to 1.9 × 10^7 dyn/cm^2) for equimolar bovine brain SM/cholesterol. Structure factors for these osmotic stress experiments with bilayers of C24-SM, BSM, equimolar C24-SM/cholesterol, and equimolar bovine brain SM/cholesterol have been presented in Figure 4A–D of the preceding paper (McIntosh et al., 1992). For each of these samples, the structure factors fell close to the continuous transform, indicating that the thickness of each type of bilayer does not change appreciably over the entire range of pressure applied (McIntosh et al., 1986, 1989a,b).

Electron density profiles for C24-SM, BSM, 1:1 C24-SM/cholesterol, and 1:1 bovine brain SM/cholesterol at various applied pressures, P, are shown in Figures 1, 2, 3, and 4, respectively. For each profile, the high-density peaks, located at about ± 25 Å from the bilayer center, correspond to the high-density lipid head groups, the low-density region

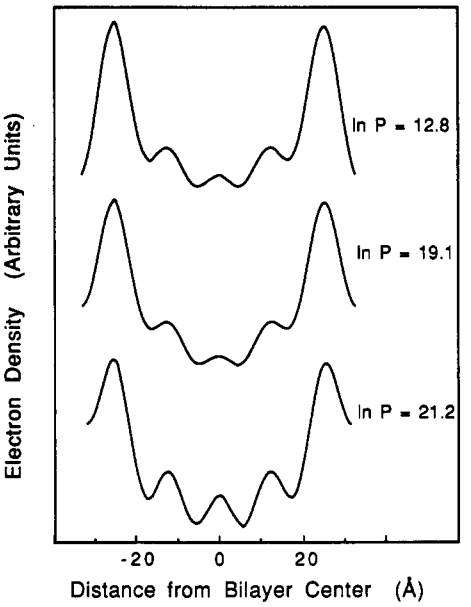

FIGURE 1: Electron density profiles for C24-SM bilayers at three osmotic pressures.

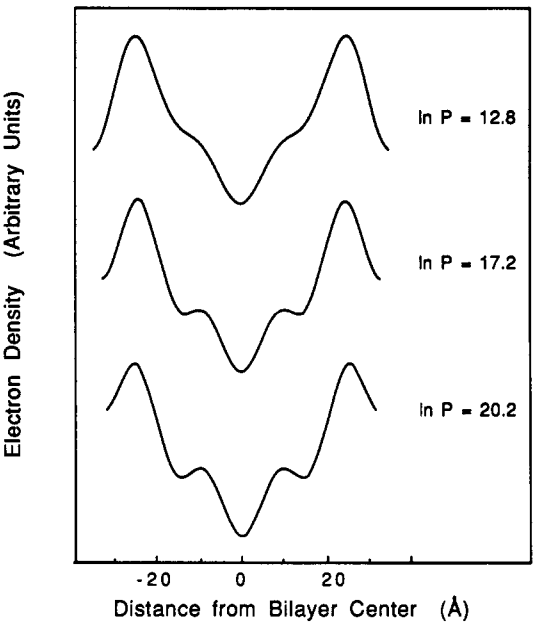

FIGURE 2: Electron density profiles for BSM bilayers at three osmotic pressures.

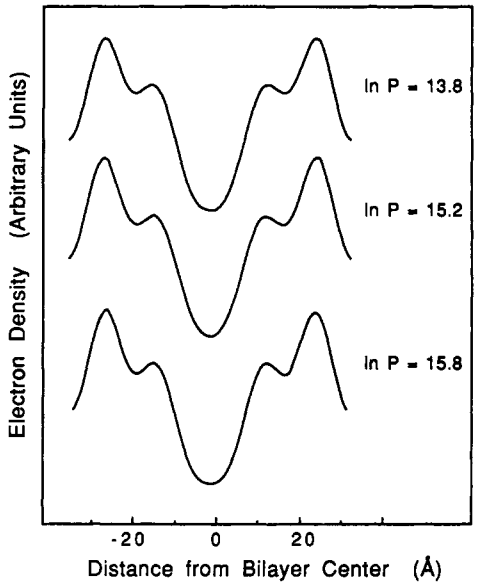

FIGURE 3: Electron density profiles for equimolar C24-SM/cholesterol bilayers at three osmotic pressures.

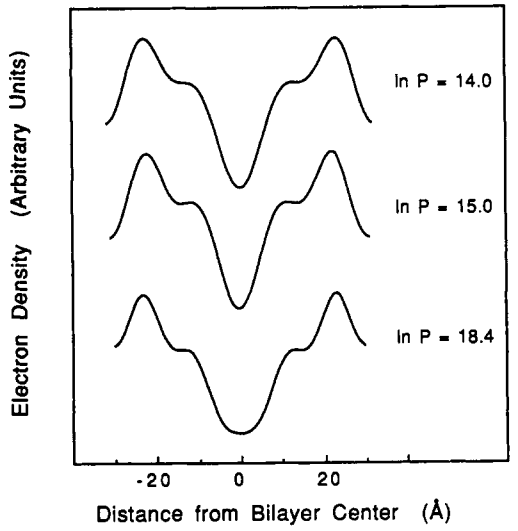

FIGURE 4: Electron density profiles for equimolar BSM/cholesterol bilayers at three osmotic pressures.

between the head-group peaks corresponds to the hydrocarbon interior of the bilayer, and the low-density regions at the outer edges of the profile correspond to half of the fluid space between adjacent bilayers. The addition of cholesterol changes the shape of the hydrocarbon interior of the profile, as discussed in the preceding paper (McIntosh et al., 1992). The distances between head-group peaks across the bilayer for the range of applied pressures were found to be 51.0 ± 0.4 Å (mean ± standard deviation, $N = 7$ experiments), 50.1 ± 0.8 Å ($N = 21$), 50.9 ± 0.7 Å ($N = 4$), and 45.5 ± 0.6 Å ($N = 7$) for C24-SM, BSM, 1:1 C24-SM/cholesterol, and 1:1 BSM/cholesterol, respectively. Thus, for each of these bilayer systems, the distance between head-group peaks across the bilayer remained approximately constant for all applied pressures.

The osmotic stress data described above can be used to obtain information on the repulsive pressures between the surfaces of SM bilayers. As noted previously (McIntosh et al., 1986, 1987, 1989a,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

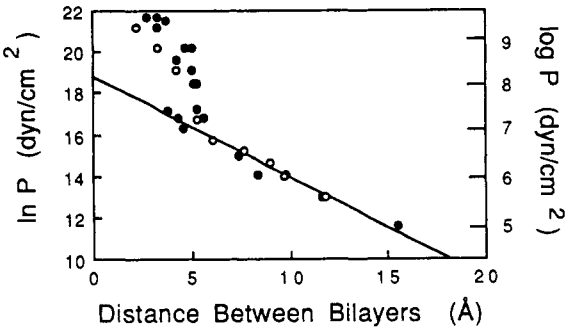

FIGURE 5: Natural logarithm (left-hand scale) and common logarithm (right-hand scale) of applied osmotic pressure versus the distance between bilayer surfaces for bilayers of BSM (solid circles) and C24-SM (open circles).

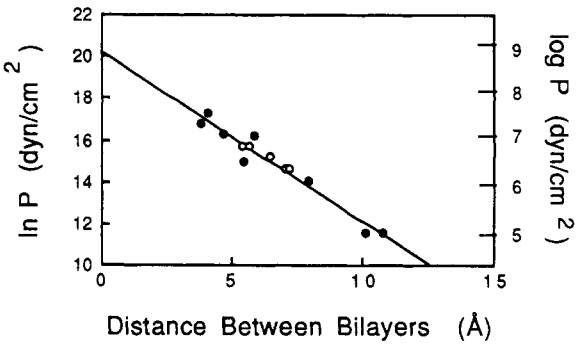

FIGURE 6: Natural logarithm (left-hand scale) and common logarithm (right-hand scale) of applied osmotic pressure versus the distance between bilayer surfaces for bilayers of equimolar BSM/cholesterol (solid circles) and equimolar C24-SM/cholesterol (open circles).

bilayer (Worcester & Franks, 1976; Simon & McIntosh, 1986). We operationally define the bilayer width as the total thickness of the bilayer, assuming that the conformation of the phosphorylcholine head group in SM is the same as it is in single crystals of phosphatidylcholine (Pearson & Pascher, 1979). In that case, the high-density head-group peak in the profiles would be located between the phosphate group and the sphingosine backbone. We assume that the phosphorylcholine group is, on average, oriented approximately parallel to the bilayer plane, so that the edge of the bilayer lies about 5 Å outward from the center of the high-density peaks in the electron density profiles (McIntosh et al., 1986, 1987, 1989a,b). Therefore, for each osmotic pressure, we calculate the bilayer thickness as the distance between head-group peaks across the bilayer in the profiles plus 10 Å. The distance between bilayer surfaces (d_f) is calculated as the difference between the lamellar repeat period and this bilayer thickness (McIntosh et al., 1986, 1987, 1989a,b), which is 61.0, 60.1, 60.9, and 55.5 Å for C24-SM, BSM, 1:1 C24-SM/cholesterol, and 1:1 BSM/cholesterol, respectively.

Using this definition of the lipid/water interface, we plot the natural logarithm of applied pressure (ln P) versus the distance between bilayers for SM and equimolar SM/cholesterol bilayers in Figures 5 and 6, respectively. Both with and without cholesterol, the values for BSM were similar to those of C24-SM. For the two gel-phase sphingomyelin bilayers, there were two distinct regions in the pressure–distance relation (Figure 5). For applied pressures less than 2.5×10^7 dyn/cm^2 (ln $P < 17$), the points could be fit closely ($r^2 = 0.96$) with a straight line, indicating that for these pressures the repulsive pressure decayed exponentially with increasing fluid spacing (d_f) between bilayer surfaces such that $P = P_o \exp(-d_f/\lambda)$, with $P_o = 1.6 \times 10^8$ dyn/cm^2 and $\lambda = 2.0$ Å (Table I). However, for bilayer separations of less than 5 Å, there was a sharp

Table I: Hydration Pressure and Dipole Potential Measurements[a]

lipid	phase	P_0 (dyn/cm^2 × 10^{-8})	V (mV)	$2\chi(V/\lambda)^2$ (dyn/cm^2 × 10^{-8})
SM	$L_{\beta'}$	1.6	328	4.7
EPC/cholesterol (1:1)	L_α	3.2	493	9.6
EPC	L_α	4.0	415	10.4
SM/cholesterol (1:1)	L_α	6.2	412	19.2
DPPC	$L_{\beta'}$	47.0	575	34.6

[a] For SM and SM/cholesterol, values of P_0 were obtained from pressure–distance relationships shown in Figures 5 and 6. Data for EPC, EPC/cholesterol, and DPPC were taken from Simon and McIntosh (1989).

upward break in the pressure–distance relationship. These results at high applied pressures were in agreement with the experiments of Khare and Worthington (1978), who found that the lamellar repeat period of bovine brain sphingomyelin did not change appreciably as a function of relative vapor pressure. For equimolar BSM/cholesterol or C24-SM/cholesterol bilayers in the liquid-crystalline phase (Figure 6), the data points for ln $P < 17$ could be fit ($r^2 = 0.95$) to a straight line, such that $P = P_0 \exp(-d_f/\lambda)$, where $P_0 = 6.2 \times 10^8$ dyn/cm^2 and $\lambda = 1.2$ Å. For equimolar SM/cholesterol bilayers, there was lipid phase separation in the high-pressure region (ln $P > 17$) as evidenced by additional reflections at 34 and 17 Å, characteristic of a cholesterol phase (McIntosh et al., 1992).

Dipole Potential. Dipole potentials were measured to be 328 ± 14 mV (mean ± standard deviation, $n = 8$ experiments) for BSM and 412 ± 17 mV ($n = 4$) for equimolar BSM/cholesterol monolayers.

Discussion

First, we consider the low-pressure region (ln $P < 17$) of the pressure–distance relations. For all SM and SM/cholesterol bilayers studied, the total repulsive pressure decayed exponentially with increasing fluid separations over 5 Å (Figures 5 and 6). This type of exponential pressure–distance relationship has been previously found for a variety of lipid systems and attributed to the repulsive hydration pressure (Parsegian et al., 1979; McIntosh et al., 1986; Rand & Parsegian, 1989). The decay lengths of 2.0 Å for SM bilayers and 1.2 Å for equimolar SM/cholesterol bilayers are similar to those previously found for phospholipid bilayers (McIntosh et al., 1986, 1989a; Rand & Parsegian, 1989). The reason is not known for the difference in decay length between SM and SM/cholesterol bilayers. However, both of these values are significantly smaller then the value of $\lambda = 6.3$ Å for BSM determined by Tamura-Lis et al. (1986). The reason for the difference between our results and those of Tamura-Lis et al. (1986) is that, by gravimetric methods, Tamura-Lis et al. found a relatively large change (on the order of 15 Å) in the thickness of SM bilayers on partial dehydration, whereas we found no appreciable change in bilayer thickness from analysis of electron density profiles (Figure 2). Since the fluid separation is determined by subtracting the bilayer thickness from the repeat period, a large change in bilayer thickness upon dehydration would change the slope of the plot of ln P versus fluid separation and increase the calculated value of decay length. The invariance of the wide-angle diffraction pattern upon dehydration and the sampling theorem analysis (McIntosh et al., 1992) provide strong supporting evidence indicating that there is little change in area per lipid molecule or bilayer thickness upon partial dehydration. Moreover, as shown previously (McIntosh & Simon, 1986; Simon et al., 1988), a large change in membrane thickness over this range of applied pressures (11 < ln P < 17) is not consistent with the large compressibility modulus found for gel-phase lipids (Needham & Evans, 1988).

Table I shows values of P_0 for SM and equimolar SM/cholesterol bilayers obtained from Figures 5 and 6, respectively, along with values of P_0 taken from Simon and McIntosh (1989) for egg phosphatidylcholine (EPC) and equimolar EPC/cholesterol in the liquid-crystalline phase and DPPC bilayers in the gel phase. The magnitude of the hydration pressure for SM bilayers is smaller than that of the other bilayers, either gel or liquid-crystalline phase. Since both SM and DPPC are in their gel phase ($L_{\beta'}$), the contributions of the undulation pressure (Helfrich, 1973; Evans & Parsegian, 1986) to the total repulsive pressure of these lipids must be very small and can be ignored. In addition, given the similarities in the chemical compositions of SM and DPPC, the attractive van der Waals pressure for these two lipids should be similar. Thus, the observed difference in P_0 between SM and DPPC bilayers must be due to differences in the hydration pressure.

A possible reason for the relatively low value of P_0 for gel-phase SM bilayers compared to other bilayers (Table I) can be obtained from consideration of the relatively low dipole potential of SM monolayers. The theoretical analysis of Cevc and Marsh (1985) predicts that $P_0 = 2\chi(\Psi/\lambda)^2$, where χ is the dielectric susceptibility and Ψ is the bilayer hydration potential. Simon and McIntosh (1989) have argued that V, as measured in a monolayer with the same area per molecule as a bilayer (MacDonald et al., 1987), provides a reasonable estimate for Ψ. Simon and McIntosh (1989) have found a correlation between the magnitude of the hydration pressure P_0, as measured by X-ray diffraction, and the quantity $2\chi(V/\lambda)^2$. That is, this analysis relates P_0 to the square of the electric field of the bilayer $(V/\lambda)^2$. Other theoretical analyses of the hydration pressure (Gruen & Marcelja, 1983; Schiby & Ruckenstein, 1983; Belaya et al., 1986) also predict that P_0 should be proportional to the square of the electric field, although the proportionality constant is different for each analysis. The data in table I demonstrate that the observed values of P_0, V, and the quantity $2\chi(V/\lambda)^2$ are all lower for sphingomyelin than for either liquid-crystalline EPC or gel-phase dipalmitoylphosphatidylcholine (DPPC). Because DPPC and SM differ only in their lipid backbones (glycerol for DPPC and sphingosine for SM), differences in P_0 must arise from differences in this region of the lipid. One major difference in the dipole composition between SM and DPPC [see Figure 1 of McIntosh et al. (1992)] is that DPPC has a carbonyl group on the 1 chain that points approximately perpendicular to the interface, so that it would yield a positive dipole potential, whereas BSM has a hydroxyl group in that position. The simplest model for the dipole potential is the parallel plate capacitor model where $V = n\mu_p/A\epsilon$, where μ_p is the effective dipole moment perpendicular to the interface of each water and lipid dipole at the interface, A is the area per lipid molecule, n is the number of dipoles, and ϵ is the effective dielectric constant (Flewelling & Hubbell, 1986). The ratio of the dipole moment of hydroxyl and carbonyl groups is 1.5 D/2.5 D = 0.6 (Israelachvili, 1985), which is similar to the ratio of dipole potential of BSM and DPPC taken from Table I (328 mV/575 mV = 0.57). Thus, the difference in dipole potential between SM and DPPC can be largely explained in terms of the difference in dipole moments of the hydroxyl and carbonyl groups of their respective backbone regions.

For SM and DPPC, as well as for the other lipid bilayers presented in Table I, the observed magnitude of P_0 appears

to be correlated with the square of electric field, as P_o increases monotonically with $(V/\lambda)^2$. In particular, addition of cholesterol to SM bilayers, raises P_o and $(V/\lambda)^2$ by parallel amounts (Table I). As discussed in detail previously (Simon & McIntosh, 1989), there are several possible reasons for the differences in magnitude between P_o and $2\chi(V/\lambda)^2$. In particular, the largest source of experimental uncertainty is in the location of the plane where $d_f = 0$. In order to obtain P_o from the X-ray measurements, it is necessary to extrapolate the pressure–distance relationships to $d_f = 0$, and a relatively small change in the location of this plane of origin can cause a significant shift in the values of P_o.

Some of the data presented in Table I do not seem to be consistent with the model developed by Israelachvili and Wennerstrom (1990), which states that the repulsive pressure originates from the entropic repulsion of molecular groups that are thermally excited to protrude from the bilayer surface. This lipid protrusion model cannot explain the relatively large differences in P_o between gel-phase SM and DPPC (Table I) for two reasons. First, in the gel-phase vertical fluctuations of the entire lipid molecule would be minimal. Second, vertical out of plane fluctuations involving only the polar head group should be similar for SM and DPPC since these lipids have the same phosphorylcholine head group. Moreover, the lipid protrusion model would predict that the magnitude of the repulsive pressure should be greater for liquid–crystalline than for gel-phase bilayers, because the free energy of transfer of a methylene group from a gel phase to water is greater (by the heat of fusion) than the free energy of transfer of a methylene group from a liquid-crystalline phase to water. However, the magnitude of P_o is greater for DPPC in the gel phase then for EPC or SM/cholesterol in the liquid-crystalline phase (Table I).

For SM bilayers a sharp upward break in the pressure–distance relation occurs at about 5 Å (Figure 5). A similar upward break at this same fluid spacing has previously been observed for EPC bilayers (McIntosh et al., 1987, 1989a). For EPC bilayers, we attributed this break to the onset of steric hindrance between the head groups from apposing bilayers. We also found that an appreciable fraction of the measured steric energy could be ascribed to a decrease in the configurational entropy due to restricted head-group motion as adjacent bilayers come together (McIntosh et al., 1987). Evidently a similar steric pressure operates between SM bilayers.

Registry No. Cholesterol, 57-88-5.

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