Temperature Dependence of the Repulsive Pressure Between Phosphatidylcholine Bilayers

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ABSTRACT Bilayer structure and interbilayer repulsive pressure were measured from 5 to 50°C by the osmotic stress/x-ray diffraction method for both gel and liquid crystalline phase lipid bilayers. For gel phase dibehenoylphosphatidylcholine (DBPC) the bilayer thickness and pressure-distance relations were nearly temperature-independent, and at full hydration the equilibrium fluid spacing increased ~1 Å, from 10 Å at 5°C to 11 Å at 50°C. In contrast, for liquid crystalline phase egg phosphatidylcholine (EPC), the bilayer thickness, equilibrium fluid spacing, and pressure-distance relation were all markedly temperature-dependent. As the temperature was increased from 5 to 50°C the EPC bilayer thickness decreased ~4 Å, and the equilibrium fluid spacing increased from 14 to 21 Å. Over this temperature range there was little change in the pressure-distance relation for fluid spacings less than ~10 Å, but a substantial increase in the total pressure for fluid spacings greater than 10 Å. These data show that for both gel and liquid crystalline bilayers there is a short-range repulsive pressure that is nearly temperature-independent, whereas for liquid crystalline bilayers there is also a longer-range pressure that increases with temperature. From analysis of the energetics of dehydration we argue that the temperature-independent short-range pressure is consistent with a hydration pressure due to polarization or electrostriction of water molecules by the phosphorylcholine moiety. For the liquid crystalline phase, the 7 Å increase in equilibrium fluid spacing with increasing temperature can be predicted by an increase in the undulation pressure as a consequence of a temperature-dependent decrease in bilayer bending modulus.

INTRODUCTION

Nonspecific interactions between surfaces in solvent have historically been considered in terms of classical van der Waals attractive interactions and electrostatic double-layer interactions (Derjaguin and Landau, 1941; Verwey and Overbeek, 1948). More recently it has been found that the measured interactions between lipid bilayers (LeNeveu et al., 1977; Parsegian et al., 1979; McIntosh and Simon, 1986b; Simon et al., 1991; McIntosh et al., 1992; Tsao et al., 1993), DNA helices (Rau et al., 1984; Leikin et al., 1991; Rau and Parsegian, 1992a,b), and polysaccharide molecules (Rau and Parsegian, 1990) cannot be fully described in terms of these two pressures, especially at small surface separations. In the case of lipid bilayers, additional repulsive interactions have been invoked to describe the measured pressure-distance relations, namely the hydration pressure, due to the orientation of water by the bilayer surface (Marcelja and Radic, 1976; Parsegian et al., 1979; Gruen and Marcelja, 1983; McIntosh and Simon, 1986b), and steric (entropic) pressures, due to motion of individual lipid molecules (McIntosh et al., 1987; Israelachvili and Wennerström, 1990, 1992) and undulations of the bilayer surface (Harbich and Helfrich, 1984; Evans and Parsegian, 1986; Sonette and Ostrowski, 1986; Servuss and Helfrich, 1987). The question of how much of the pressure can be attributed to hydration repulsion and how much to steric interactions is very difficult and requires additional investigation. Indeed, Parsegian and Rand (1995) state that the ". . . dissection of the measured pressure P into its physically distinct components is a problem almost as difficult as the theoretical explanation of these components themselves." Part of the problem is the absence of a theoretical model of sufficient breadth that can account for all the experimental data (Rand and Parsegian, 1989; Israelachvili, 1991; Lipowsky and Grotehans, 1993; McIntosh and Simon, 1994).

The theoretical or experimental bases for the various interbilayer pressures have been recently reviewed (McIntosh and Simon, 1994). Briefly, for the hydration pressure the pioneering theoretical treatment of Marcelja and Radic (1976) states that the bilayer surface causes an ordering of solvent molecules that decays exponentially with increasing distance from the surface, and several subsequent models (Gruen and Marcelja, 1983; Schiby and Ruckenstein, 1983; Cevc and Marsh, 1985; Kornyshev, 1986; Belaya et al., 1986) theorize that electric fields at the bilayer surface are responsible for the water ordering or polarization. The postulated steric interactions include a very short-range interaction due to lipid headgroup motions (McIntosh et al., 1987), an exponentially decaying protrusion pressure due to extensions or protrusions of individual lipid molecules from the bilayer surface (Israelachvili and Wennerström, 1990, 1992), and an undulation pressure due to thermally driven undulations or out-of-plane fluctuations of the entire bilayer (Harbich and Helfrich, 1984; Evans and Parsegian, 1986; Evans, 1991). The undulation pressure is predicted to have a longer range than the hydration pressure (Harbich and

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Helfrich, 1984; Evans and Parsegian, 1986), and its existence has been experimentally determined in fluid lipid bilayers and micelles (Safinya et al., 1986; McIntosh et al., 1989c, 1995; Safinya, 1989; Faucon et al., 1989; McIntosh and Simon, 1993).

To dissect the relative contributions of the hydration and steric pressures to the total interaction, we (McIntosh and Simon, 1993) previously measured and compared the interactions between fluid (liquid crystalline) bilayers, where entropic pressures, particularly the undulation pressure, should be appreciable, to the interaction between more ordered (gel or subgel) bilayers, where entropic interactions should be negligible. We found that for liquid crystalline, gel, and sub-gel bilayers the pressure-distance relations decayed exponentially with increasing distance with similar decay lengths for interbilayer distances of 4–8 Å, implying that there is the same underlying pressure for both systems. However, for liquid crystalline bilayers the pressure-distance relation extended to much larger fluid spacings at small applied pressures, implying that the undulation pressure plays an important role in determining the bilayer hydration properties and the range of the total repulsive pressure (McIntosh and Simon, 1993; McIntosh et al., 1995).

Another way to investigate the relative magnitudes of the hydration and entropic interactions is to measure the energetics of hydration as a function of temperature. To this point, Lipowsky and Grotehans (1993) have suggested that these two types of interactions should have different temperature dependencies. If the hydration pressure were due to the polarization of water by the full or partial charges in the polar headgroup, then the energy of dehydration should be nearly independent of temperature, because the work to dehydrate ions is nearly temperature-independent. That is, for ions, the entropy of dehydration is small compared with the total energy of dehydration (Bockris and Reddy, 1973; Marcus, 1991; Israelachvili, 1991). In contrast, theoretical treatments predict that the magnitudes of both the undulation (Harbich and Helfrich, 1984; Evans and Parsegian, 1986; Evans, 1991) and protrusion pressures (Israelachvili and Wennerström, 1990, 1992) should increase with increasing temperature. Previous workers (Elworthy, 1961; Wilkinson et al., 1977) have obtained adsorption isotherms as a function of temperature, and these isotherms have been analyzed in terms of a hydration pressure (Marsh, 1989). However, adsorption measurements are usually performed for lipids on solid substrates, where undulation pressures are depressed (Tsao et al., 1993). Pressure-distance measurements as a function of temperature have been performed on DNA double helices (Leikin et al., 1991; Rau and Parsegian, 1992a). However, the analysis of these data regarding the short-range hydration is complicated by the presence of the charge on the DNA molecules and the effects of ion binding.

In this paper, to obtain a better understanding of the factors that influence the short- and long-range repulsive pressures between membranes, and to estimate the entropy and enthalpy of the work to remove water from between bilayers, we measured pressure-distance relations as a function of temperature for electrically neutral phosphatidylcholine (PC) bilayers in the gel and liquid crystalline phases. Comparisons of the temperature dependence of pressure-distance curves for gel and liquid crystalline bilayers were used to help distinguish the interactions, since the undulation pressure should be suppressed in the gel phase (McIntosh and Simon, 1993, 1994), and König et al. (1995) have found lipid protrusions in the liquid crystalline but not in the gel phase. For the gel phase lipid we chose dibehenoylphosphatidylcholine (DBPC, a saturated lipid with 22 carbons per acyl chain), since its gel-to-liquid crystalline transition temperature is 70°C (Lewis et al., 1987), and thus the temperature dependence of the short-range pressures in the gel phase can be measured over a relatively large (and accessible) temperature range. For the liquid crystalline lipid we chose egg PC (EPC), because it is in the liquid crystalline (L_α) phase from 5 to 50°C, and the pressure-fluid spacing profiles at room temperature have been extensively analyzed (McIntosh and Simon, 1986a; McIntosh et al., 1987).

MATERIALS AND METHODS

Materials

DBPC and EPC were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Dextran of average molecular weight 580,000 was purchased from Sigma Chemical Co. (St. Louis, MO.) and purified polyethylene glycol (PEG) with an average molecular weight of 8,000 was the kind gift of Dr. Barry Lentz of the University of North Carolina. The PEG, from Fisher Scientific (Fairlawn, NJ), was purified by procedures given in Lentz et al. (1992).

Methods

X-ray diffraction/osmotic stress

X-ray diffraction analysis was performed on both unoriented suspensions of multiwalled vesicles and oriented multilayers for three temperatures, 5, 23, and 50°C. Known osmotic pressures were applied to each of these systems by published procedures (LeNeveu et al., 1977; Parsegian et al., 1979; McIntosh and Simon, 1986b; McIntosh et al., 1987). Osmotic stress was applied to the liposomes by incubating them in aqueous solutions of dextran or PEG. Because these polymers are too large to enter the lipid lattice, they compete for water with the lipid multilayers, thereby applying an osmotic pressure (LeNeveu et al., 1977; Parsegian et al., 1979). Osmotic pressures for dextran and PEG solutions obtained as a function of temperature have been published (Parsegian et al., 1986). Pressure was applied to oriented multibilayers by incubating them in constant relative humidity atmospheres maintained with a saturated NaCl solution, which has a nearly constant ratio (0.75–0.77) of vapor pressure (p) to the vapor pressure of pure water (p_o) over the temperature range used in our experiments (O'Brien, 1948). The applied pressure is given by

$$P = -(RT/V_w) \cdot \ln(p/p_o) \quad (1)$$

where R is the molar gas constant, T is the temperature (K), and V_w is the partial molar volume of water (Parsegian et al., 1979).

Unoriented suspensions of either DBPC or EPC were prepared by adding excess buffer (100 mM NaCl, 20 mM Hepes, pH 7) or polymer solution and incubating with extensive vortexing above the phase transition

temperature of the lipid. To ensure equilibration of the salt across the multilayers, several freeze-thaw cycles were used. In control experiments, similar x-ray results were obtained for specimens made in either excess buffer or excess water. All suspensions were pelleted by centrifugation, sealed in quartz glass x-ray capillary tubes, and mounted in a temperature controlled specimen holder in a point collimation x-ray camera.

Oriented multilayers of DBPC or EPC were formed by placing a small drop of lipid/water suspension onto a curved glass substrate and allowing it to equilibrate at 75% relative humidity. The multilayers on the glass substrate were mounted in a controlled humidity chamber on a single-mirror (line-focused) x-ray camera such that the x-ray beam was oriented at a grazing angle relative to the multilayers (McIntosh et al., 1987, 1989b). The humidity chamber, which contained a cup of the saturated salt solution, consisted of a hollow walled copper canister with two Mylar windows for passage of the x-ray beam. Constant temperature was maintained in the chamber by means of water circulating in the wall of the canister. To speed equilibration, a gentle stream of nitrogen was passed through a flask of the saturated salt solution and then through the chamber.

For both oriented and unoriented specimens, x-ray diffraction patterns were recorded on Kodak DEF x-ray film. The films were processed by standard techniques and densitometered with a Joyce-Loebl microdensitometer as described previously (McIntosh et al., 1987, 1989b; McIntosh and Simon, 1986b). 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 $\{h^2I(h)\}^{1/2}$ (Blaurock and Worthington, 1966; Herbette et al., 1977). For the oriented line-focused 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 $F(h) = \{hI(h)\}^{1/2}$.

Electron density profiles, $\rho(x)$, on a relative electron density scale were calculated from

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

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 the use of the sampling theorem (Shannon, 1949) as described in detail previously (McIntosh and Holloway, 1987; McIntosh et al., 1989a). All electron density profiles were calculated at a resolution of $d/2h_{max} \approx 7$ Å.

Dipole potential

Dipole potentials, V, were measured as described previously (MacDonald and Simon, 1987). Briefly, monolayers of DBPC or EPC were formed by spreading 10 μl of a 10 mg/ml lipid in hexane:ethanol (9:1 v:v) solution onto a subphase of 0.1 M KCl (roasted at 600°C) in a trough having a surface area of ~70 cm^2. Under these conditions a lipid monolayer forms in equilibrium with liposomes in the subphase, and it has been shown that the packing of the lipid molecules in the monolayer is approximately the same as it is in a bilayer (MacDonald and Simon, 1987). The trough was placed on a Peltier device, and the subphase was stirred using a magnetic stirring bar to produce a uniform temperature throughout the chamber. To measure the temperature a small thermistor was placed near the surface. Dipole potential measurements were performed at 5, 23, and 50°C. The dipole potential was measured between an Ag/AgCl electrode in the subphase and a ^{241}Am electrode in air using a Keithley electrometer (model 602; Keithley Instruments Co., Cleveland, OH). The reported values of V represent the difference in the potential of the subphase surface in the presence and absence of the monolayer.

RESULTS

X-ray diffraction/osmotic stress measurements

For all specimens of DBPC, the diffraction patterns contained four or five orders of a lamellar repeat period and two wide-angle reflections, one sharp and one broad. The spacing of the sharp wide-angle reflection was 4.35 Å at 5°C, 4.33 Å at 23°C, and 4.25 Å at 50°C. The broad wide-angle reflection remained centered at a constant spacing of ~4.11 Å over this temperature range. These patterns are consistent with bilayers in the tilted gel ($L\beta'$) phase (Tardieu et al., 1973). For all specimens of EPC the diffraction patterns contained four or five orders of lamellar repeat period and a very broad wide-angle band centered at ~4.5 Å, typical for bilayers in the liquid crystalline ($L\alpha$) phase (Tardieu et al., 1973).

For both DBPC and EPC bilayers osmotic stress experiments were performed at three temperatures, 5, 23, and 50°C. Fig. 1, *A* and *B*, shows plots of the common logarithm of applied pressure (log P) versus the lamellar repeat period (d) for DBPC and EPC, respectively. In excess buffer, with no applied pressure, the repeat periods for DBPC were 73.7, 74.1, and 75.6 Å at 5, 23, and 50°C, respectively (Fig. 1 *A*, *arrows*) and for EPC were 64.3, 63.2, and 67.2 Å at 5, 23, and 50°C, respectively (Fig. 1 *B*, *arrows*). For both DBPC and EPC bilayers the repeat period decreased monotonically

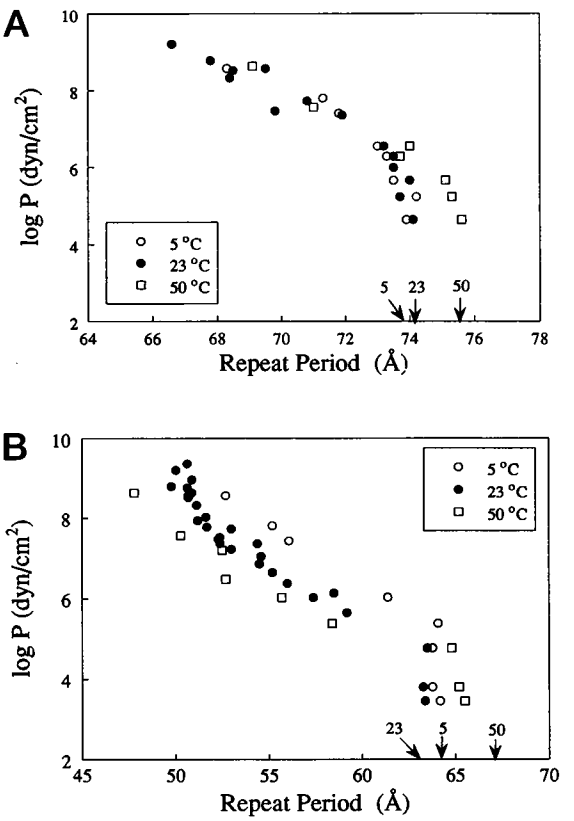

FIGURE 1 Logarithm of applied pressure (log P) plotted versus the lamellar repeat period (d) at 5°, 23°, and 50°C for (*A*) DBPC and (*B*) EPC bilayers. The arrows note the repeat periods for each of these temperatures for liposomes in excess buffer with no applied pressure. The data points for log $P < 7.5$ were obtained for unoriented samples in dextran or PEG solutions, whereas data at higher pressures were obtained from oriented samples in controlled humidity atmospheres. Data for EPC at 23°C were taken from McIntosh and Simon (1986b) and McIntosh et al. (1987).

with increasing applied pressure for each temperature. The log P versus d relations had a larger dependence on temperature for EPC than for DBPC bilayers.

The lamellar repeat period (d) represents the total thickness of the unit cell, which is the sum of the bilayer thickness (d_b) and the fluid layer thickness (d_f) between apposing bilayers. To obtain information on both d_b and d_f for the data in Fig. 1, A and B, we performed a Fourier analysis of the diffraction data. Fig. 2, A and B, shows the structure factors for the lamellar diffraction data from the osmotic stress experiments with DBPC and EPC, respectively. Phase angles for dipalmitoylphosphatidylcholine (DPPC) in the gel phase and EPC in the liquid crystalline phase have previously been determined (McIntosh and Simon, 1986b) and are used in these figures. In the case of DBPC (Fig. 2 A) the structure factors were nearly independent of temperature. In the case of EPC (Fig. 2 B) there was a small temperature effect on the structure factors. For example, for reciprocal spacings of 0.04–0.07 $Å^{-1}$, the structure factors for EPC at 5°C were smaller in magnitude than those at higher temperatures (Fig. 2 B).

Fig. 3 shows electron density profiles for DBPC at 23°C

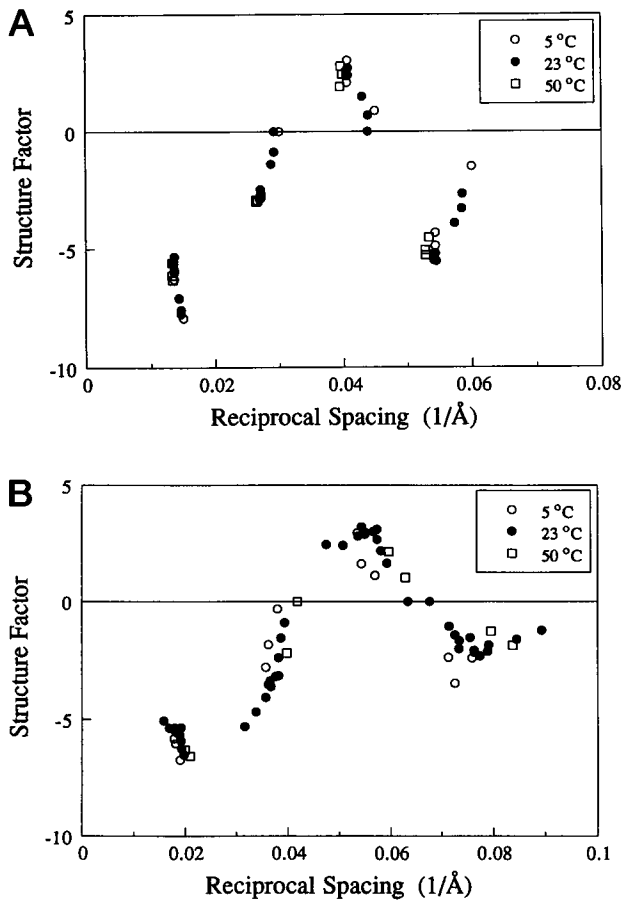

FIGURE 2 Plots of structure factors versus reciprocal space coordinates for x-ray data from osmotic stress experiments of (A) DBPC and (B) EPC bilayers. Data for EPC at 23°C were taken from McIntosh and Simon (1986b).

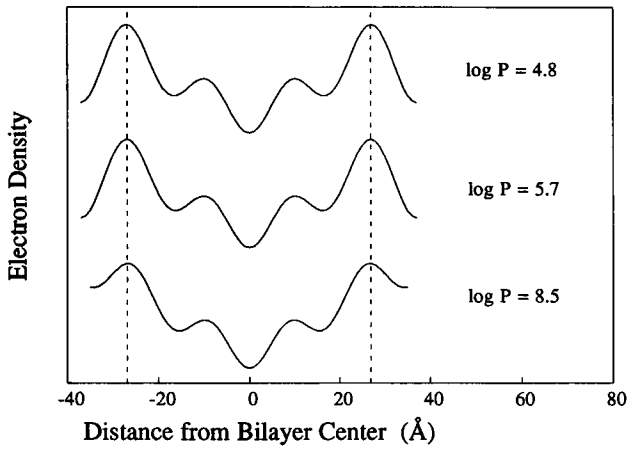

FIGURE 3 Electron density profiles of DBPC bilayers at 23°C at three applied pressures.

at three applied pressures. For each profile, the center of the bilayer is located at 0 Å. The two electron density peaks, near ±25 Å, correspond to the phospholipid headgroups and the medium density regions at the outer edges of each profile correspond to the fluid spaces between adjacent bilayers. The density dip in the geometric center of the profile corresponds to the localization of the terminal methyl groups in the center of the bilayer, and the medium density regions between the terminal methyl dip and the headgroup peaks correspond to the lipid hydrocarbon chains. Note that the width of the fluid space between bilayers decreased with increasing applied pressure. Dotted lines have been drawn through the middle of the headgroup peaks to indicate that the headgroup peak separation across the bilayer was nearly independent of applied pressure, as previously found for DPPC and EPC for a similar range of applied pressures (McIntosh and Simon, 1986b). Electron density profiles for DBPC as a function of temperature at a constant applied pressure are shown in Fig. 4. The profiles were very similar over this temperature range, although the distance between headgroup peaks increased slightly as the temperature was raised. The distance between headgroups peaks across the bilayer (d_{pp}) was 53.7 Å ± 0.1 Å (mean ± SD, N = 3 experiments) at 5°C, 53.7 ± 0.1 Å (N = 4) at 23°C, and 54.7 Å ± 0.6 Å (N = 4) at 50°C.

Fig. 5 shows electron density profiles for EPC as a function of temperature at a constant applied pressure. The profiles were similar to those of DBPC, except that the distance between headgroup peaks was smaller and the terminal methyl dip in the middle of the profile was less pronounced, particularly at 50°C, indicating that there was more disorder in the hydrocarbon packing in the liquid crystalline phase of EPC than in the gel phase of DBPC. Moreover, in contrast to the near invariance with temperature of the bilayer profile for DBPC (Fig. 4), for EPC there was a systematic decrease with increasing temperature in the distance between headgroup peaks. In Fig. 5 the dotted lines have been drawn through the middle of the headgroup

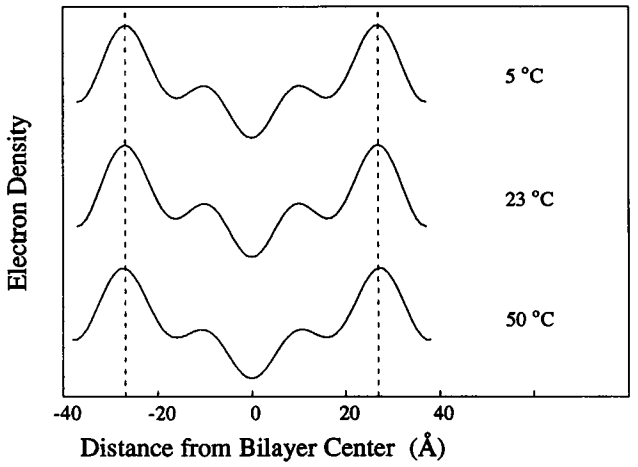

FIGURE 4 Electron density profiles of DBPC bilayers as a function of temperature at a constant applied pressure of 5×10^5 dyn/cm^2 (log P = 5.7).

peaks for the profiles at 5°C, and it can be seen that at 23 and 50°C the headgroup peaks were shifted inward from the position of this dotted line. The headgroup peak separation across the EPC bilayer was d_{pp} = 40.1 Å ± 0.2 Å (N = 3) at 5°C, 37.8 Å ± 0.8 Å at 23°C (N = 10) (McIntosh and Simon, 1986b), and 36.3 Å ± 0.6 Å (N = 2) at 50°C.

These electron density profiles can be used to estimate the width of the fluid space between adjacent bilayers for each applied pressure. As noted previously (McIntosh and Simon, 1986b, 1993; McIntosh et al., 1992), the definition of the lipid/water interface is somewhat arbitrary, because the bilayer surface is not smooth and water penetrates into the headgroup region of the bilayer (Griffith et al., 1974; Worcester and Franks, 1976; Simon and McIntosh, 1986; Wiener et al., 1991). We operationally define the bilayer width as the total thickness of the bilayer assuming that the conformation of the phosphorylcholine headgroup in DBPC and EPC bilayers is the same as it is in single crystals of PC (Pearson and Pascher, 1979). In that case the high density headgroup peak would be located between the phosphate group and the glycerol 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 ~5 Å outward from the center of the high density peaks in the electron density profiles (McIntosh et al., 1987; McIntosh and Simon, 1986b, 1993). Therefore, for each osmotic pressure we calculate the bilayer thickness (d_b) from $d_b = d_{pp} + 10$ Å. The distance between bilayer surfaces (d_f) is calculated from $d_f = d - d_b$ (McIntosh and Simon, 1986b, 1993; McIntosh et al., 1992).

Using this definition of the lipid/water interface, we plot in Fig. 6, *A* and *B*, log P versus d_f for DBPC and EPC, respectively. In each figure, the distance between bilayers in excess buffer (no applied pressure) is shown by arrows. For DBPC, d_f in excess buffer was only ~1 Å larger at 50°C than at 5°C, and the pressure-distance relations were nearly independent of temperature for 2 Å < d_f < 11 Å (Fig. 6 *A*).

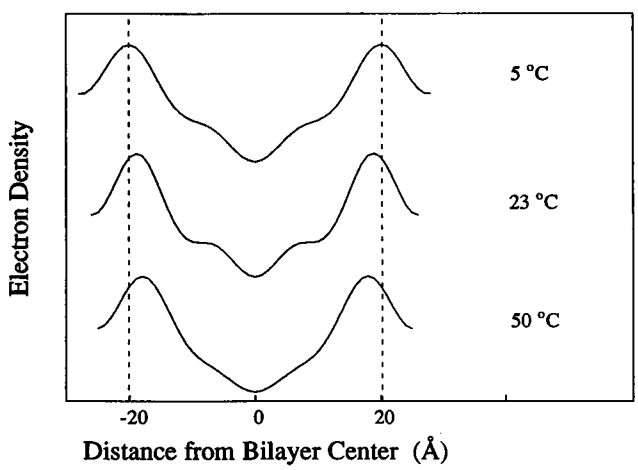

FIGURE 5 Electron density profiles of EPC bilayers as a function of temperature at a constant applied pressure of 5×10^7 dyn/cm^2 (log P = 7.7).

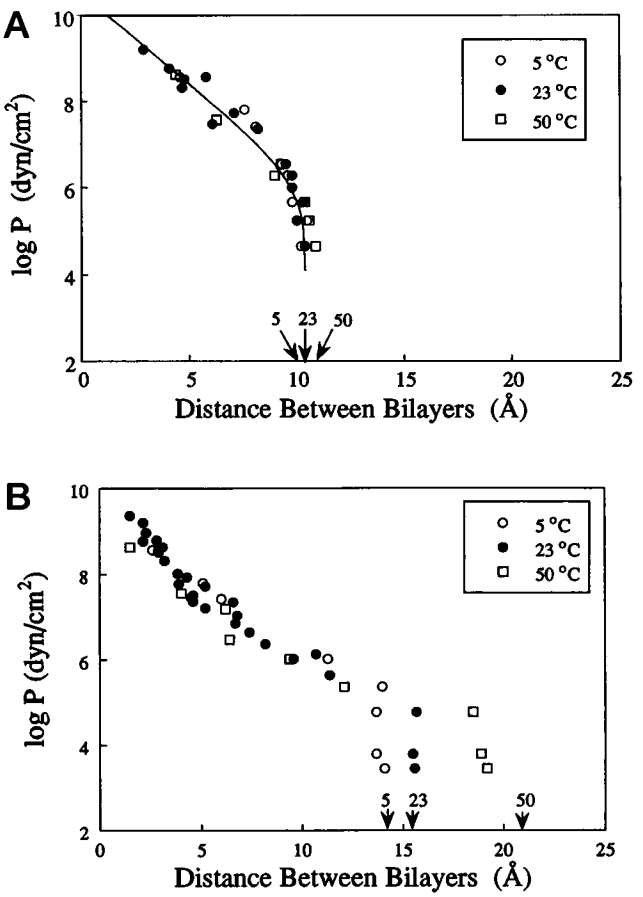

FIGURE 6 Logarithm of applied pressure (log P) plotted versus the distance between bilayers (d_f) at 5°, 23°, and 50°C for (*A*) DBPC and (*B*) EPC bilayers. The arrows note the values of d_f for each of these temperatures for liposomes in excess buffer with no applied pressure. Data for EPC at 23°C were taken from McIntosh and Simon (1986b) and McIntosh et al. (1987). In (*A*) the solid line is the fit to the DBPC data at 23°C (parameters given in Table 1).

Likewise, for EPC the pressure-distance relations were nearly independent of temperature for 2 Å $< d_f <$ 11 Å (Fig. 6 *B*). However, for EPC the pressure-distance relationship depended strongly on temperature for $d_f > 11$ Å (Fig. 6 *B*). In particular, d_f in excess buffer was significantly larger at 50°C (20.9 Å) than at 5°C (14.2 Å).

Dipole potential measurements

For DBPC monolayers, the dipole potentials were 560 ± 12 mV ($N = 3$) at 5°C, 532 ± 11 mV ($N = 4$) at 23°C and 467 ± 9 mV ($N = 2$) at 50°C. For EPC, the dipole potentials were 457 ± 11 mV ($N = 2$) at 5°C, 422 ± 8 mV ($N = 3$) at 23°C and 377 ± 12 mV ($N = 3$) at 50°C.

DISCUSSION

The data presented above provide information on the temperature dependence of both the bilayer structure and interbilayer interactions for gel and liquid crystalline bilayers.

Bilayer structure

The electron density profiles (Figs. 4 and 5) show that in the gel phase the width of the bilayer increased ~1 Å over the temperature range of 5–50°C, probably due to a small decrease in the hydrocarbon chain tilt that is known to occur in saturated lecithins (Tardieu et al., 1973; McIntosh, 1980; Tristram-Nagle et al., 1992). A change in chain tilt is consistent with the observed change in the wide-angle diffraction pattern. In contrast, in the liquid crystalline phase the bilayer thickness, as determined by the distance between headgroups in the electron density profiles, decreased 3.8 Å, going from 40.1 Å at 5°C to 36.3 Å at 50°C. Because the width of the EPC headgroup was ~10 Å (McIntosh and Simon, 1986b; McIntosh et al., 1987), this implies that the width of the bilayer hydrocarbon region decreased ~13%, going from 30.1 Å at 5°C to 26.3 Å at 50°C. This observed decrease in thickness with increasing temperature is undoubtedly due to an increase in disorder in the hydrocarbon interior, as evidenced by the lack of a pronounced terminal methyl dip in the middle of the profile at 50°C (Fig. 5). Consistent with this interpretation of increasing disorder is the previous result that the orientational order parameters along the acyl chains of liquid crystalline PC bilayers decrease monotonically from 0 to 70°C (Bloom et al., 1991). Since the change in volume with respect to temperature is equal to the sum of the changes in thickness and area, this observed decrease in bilayer thickness can be compared with that predicted from changes in volume and area per molecule for liquid crystalline bilayers measured by other physical techniques. The measured fractional increase in specific volume is $\sim 5 \times 10^{-4}$/°C (Nagle and Wilkinson, 1978) and the measured area dilation is 3.28×10^{-3}/°C (Evans and Needham, 1987; Bloom et al., 1991). Thus, a 45°C increase in temperature would be expected to produce a fractional increase in volume of ~2% and a fractional increase in area per molecule of ~15%, giving a fractional decrease in hydrocarbon thickness of ~13%. This predicted change in hydrocarbon thickness is the same as our measured change for 5–50°C for EPC bilayers.

Repulsive pressures

Gel phase bilayers

Since the undulation pressure is negligible in gel phase bilayers (McIntosh and Simon, 1993, 1994; McIntosh et al., 1995), the interactions between gel phase DBPC bilayers can be characterized by a balance between a repulsive exponentially decaying short-range pressure (P_{sr}) and an attractive van der Waals pressure (P_v) (LeNeveu et al., 1977; Parsegian et al., 1979). The total pressure (P) may then be written

$$P = P_{sr} + P_v \tag{3}$$

where $P_{sr} = P_o \cdot \exp(-d_f/\lambda)$ and $P_v = -H/6\pi d_f^3$, where H is the Hamaker constant. The data sets at 5, 23, and 50°C were fit to Eq. 3 with the constraints that their energy minima fell between 10 and 11 Å (Fig. 6 *A*) and were about -0.05 erg/cm^2 (Evans and Needham, 1987; McIntosh and Simon, 1986a). The fit for the 23°C data set is shown in Fig. 6 *A* and the values of P_o, λ, and H for all three data sets are given in Table 1. From the data (Fig. 6 *A*) and the fits to the data (Table 1) it can be seen that the magnitude and range of P_{sr} do not markedly change from 5 to 50°C.

As described by Parsegian et al. (1979), the work to dehydrate the bilayer (ΔG) has two main components, work arising from changes in bilayer structure (ΔG^{bs}) and the work required to remove water from between apposing bilayers (ΔG^{deh}). In the case of DBPC the electron density profiles (Fig. 3) indicate that there is little change in bilayer structure with dehydration, so that $\Delta G \approx \Delta G^{deh}$. Therefore, ΔG^{deh} can be obtained from integration of the pressure-distance curves (Parsegian et al., 1979). Because the total pressure increases exponentially with decreasing d_f (Fig. 6), the work to remove water between DBPC bilayers increases exponentially as the fluid spacing decreases, as previously found for other lipid bilayers (Parsegian et al., 1979; McIntosh et al., 1987; Rand and Parsegian, 1989; Marsh, 1989; Ulrich and Watts, 1994). Since the bilayer width (Fig. 3) and area per DBPC molecule (A) are approximately constant for this range of dehydration, the change in interbilayer spacing (Δd_f) can be converted to the number of water

TABLE 1 Parameters for the short-range repulsive pressure and van der Waals pressure between DBPC bilayers

Temperature (°C)	log P_o (dyn/cm^2)	λ (Å)	H (erg)
5	10.9 ± 0.3	0.98 ± 0.09	3.0×10^{-14}
23	10.5 ± 0.2	1.08 ± 0.07	2.9×10^{-14}
50	10.2 ± 0.2	1.13 ± 0.06	2.7×10^{-14}

molecules removed (Δn_w) with increasing osmotic stress by the relation

$$\Delta n_w = A \cdot \Delta d_f / 2V_w \quad (4)$$

where V_w is the molecular volume of water (30 Å^3) and the area per DBPC molecule is ~48 Å^2. Thus, a change in fluid space of 5 Å corresponds to the removal of about four water molecules per lipid from between the DBPC bilayers.

The free energy of dehydration can be expressed as $\Delta G^{deh} = \Delta H^{deh} - T\Delta S^{deh}$, where ΔH^{deh} and ΔS^{deh} represent the enthalpy and entropy of dehydration, respectively. Thus, the entropy can be calculated from $\Delta S^{deh} = -\partial \Delta G^{deh}/\partial T$. Since, within experimental uncertainty, the pressure-distance data for DBPC are nearly temperature-independent from 5 to 50°C (Fig. 6 *A*), it appears that $\Delta S^{deh} \approx 0$. Therefore, for the gel phase the energy of dehydration arises primarily from changes in the enthalpy of dehydration. The observation that $\Delta S^{deh} \approx 0$ implies that to compensate for the increase in entropy associated with transferring bound water from the bilayer to bulk water there must be a decrease in entropy associated with the bilayer during dehydration (McIntosh et al., 1987). The decrease in configurational entropy could arise from diminished headgroup motions (McIntosh et al., 1987; Ulrich and Watts, 1994) or out-of-plane protrusions (Israelachvili and Wennerström, 1990, 1992). An estimate of these configurational energies can be obtained if one assumes that the decrease in entropy associated with transferring bound water from the bilayer to bulk water is between 0 and 12 cal/mol·deg (Bryant, 1994; Dunitz, 1994). For the removal of a water molecule at 20°C the entropic interaction would then be between 0 and 3.5 kcal/mol (= 293 deg × 12 cal/mol·deg).

We now use this information on the energetics of dehydration to compare two different hypotheses for the origin of the short-range repulsive interactions. The first is that the repulsive pressure is a hydration pressure that arises from the polarization of water molecules (Gruen and Marcelja, 1983; Cevc and Marsh, 1985; Kornyshev, 1986; Dzhavakhidze et al., 1988; Leikin and Kornyshev, 1990), and the second is that the pressure arises from out-of-plane protrusions of lipid molecules (Israelachvili and Wennerström, 1990, 1992).

In terms of the hydration pressure, a mean field theory was developed by Cevc and Marsh (1985) that extended the work of Marcelja and Radic (1976) and Gruen and Marcelja (1983). Cevc and Marsh (1985) assumed the partial charges and dipoles of the lipid are the source of the electric fields that polarizes interbilayer water molecules so that

$$P = P_o \exp(-d_f/\lambda) = 2\chi(\Psi_{hyd}/\lambda)^2 \exp(-d_f/\lambda) \quad (5)$$

where $\chi = \epsilon_0(\epsilon - 1)/\epsilon$, where ϵ is the dielectric constant, ϵ_0 is the permittivity of free space, and Ψ_{hyd} is the hydration potential. Several computer simulations of PC-water systems reveal that water is indeed polarized at the PC headgroup and that a potential arising from the partial charges of the lipid and water give rise to a potential that decays approximately exponentially a few angstroms into the fluid phase (Zheng and Vanderkooi, 1992; Marrink et al., 1993; Damodaran and Merz, 1994; Chiu et al., 1995). The Cevc and Marsh (1985) analysis (Eq. 5) has proven useful for predicting the magnitude of the short-range repulsive pressure when the theoretical hydration potential (Ψ_{hyd}) was equated to the dipole potential (V) measured in monolayers equilibrated with liposomes (Simon and McIntosh, 1989; Simon et al., 1991, 1992). Substituting our measured values of V and λ into Eq. 3, we find that $\log(P_o) = 9.72$ and 9.44 at 5 and 50°C, respectively. Thus, the water polarization model is consistent with the experimental data in that it predicts small decreases in the magnitude of the short range pressure with increasing temperature. Although the changes with respect to temperature are similar, the predicted values of P_o are smaller than the experimental values. This can be understood, since the absolute values of P_o critically depend on the choice of the ill-defined "plane of origin", where $d_f = 0$ Å.

Based solely on this polarization model, one would expect that gel phase bilayers would absorb more water than liquid crystalline phase bilayers, since the dipole potential is larger for gel bilayers (this paper and MacDonald and Simon, 1987; Simon and McIntosh, 1989). In fact, the opposite is true, as liquid crystalline bilayers adsorb more water than gel bilayers (Janiak et al., 1979; McIntosh and Simon, 1986a; Nagle and Wiener, 1988). Because of that fact, Cevc and Marsh (1985) concluded that Ψ_{hyd} must be larger in the liquid crystalline than gel phase. However, in that paper, the authors did not consider the effect of bilayer undulations on water adsorption in the liquid crystalline phase. We demonstrate below that the primary reason that lipids in the liquid crystalline phase adsorb more water than do gel phase lipids is because of the influence of the undulation pressure in the liquid crystalline phase.

In terms of the hydration model, it is interesting to compare the energetics of dehydrating ions to the energetics of dehydrating DBPC bilayers. Since for DBPC $\Delta S^{deh} \approx 0$, it follows that $\Delta G^{deh} \approx \Delta H^{deh}$. This near equivalence of energy and enthalpy of dehydration (i.e., the Born energy) is also found in measurements of removing water from ions or dipoles (Israelachvili, 1991; Marcus, 1991). The main contribution to the hydration energy of ions arises from solvent immobilization in a primary hydration shell, electrostriction, and further effects of water surrounding the shell (Marcus, 1991). The free energy to remove one water molecule from the hydration shell of a tetramethylammonium ion $\{(CH_3)N^+\}$ (which resembles choline) is 8.7 kcal/mol (Cevc and Marsh, 1988), and the energy to completely dehydrate this ion is 35.1–38.2 kcal/mol (Cevc and Marsh, 1988; Marcus, 1991). These numbers can be compared with the dehydration energy for DBPC obtained by integration of the pressure-fluid spacing curve (Fig. 6 *A*). The energy to go from $d_f = 10$ Å to $d_f = 1$ Å (or from Eq. 4, the energy to remove about seven water molecules per DBPC molecule) is ~9.3 kcal/mol. It follows that the energy of dehydration of DBPC to $d_f = 1$ Å can be accounted for by partial

dehydration of the choline moiety. It should be realized that, even at small values of d_f, complete dehydration has not taken place since water molecules are intercalated within the lipid headgroup region (McIntosh and Simon, 1986a; Nagle and Wiener, 1988).

The temperature independence of the energetics of dehydration is not unique to gel phase bilayers. Virtually the same behavior was observed between DNA in 50 mM $MnCl_2$ (Leikin et al., 1991) over the identical temperature range and for a similar range of fluid spacings (3–7 Å). Thus the temperature independence of this short-range repulsive pressure is primarily enthalpic in origin and the relatively small entropy change likely reflects a decrease in configurational entropy of the macromolecule compensating for the increase in entropy of water being released from the macromolecule.

Another model for the origin of this short-range pressure is that it arises from steric interactions between apposing headgroups that may arise from a combination of headgroup rotations, that could extend from $d_f = 0 \sim 3$ Å into the fluid space (McIntosh et al., 1987), and molecular protrusions of the lipid molecule (Israelachvili and Wennerström, 1990, 1992). This possibility must be seriously considered because the range of the repulsive pressure ($d_f/2 \approx 5.5$ Å) is small and corresponds to the length of about four CH_2 groups. Molecular simulations of gel phase PC bilayers do show that the bilayer surface is rough (Heller et al., 1993), although quasi-elastic neutron scattering experiments demonstrate molecular protrusions only in the liquid crystalline phase and not in the gel phase (König et al., 1995). Israelachvili and Wennerström (1990, 1992) express the protrusion pressure as

$$P_p = P_o' \exp(-d_f/\lambda') = 2.7\pi\sigma\gamma/A \exp(-d_f/\lambda') \quad (6)$$

where σ is the lateral dimension of the protruding molecule that we estimate as $\sigma \approx (4A/\pi)^{1/2}$, γ is the effective interfacial energy, and $\lambda' = 1.15\ kT/\pi\sigma\gamma$. Eq. 6 predicts that both the preexponential factor and the decay length should change with temperature. These changes can be calculated using $\gamma = 20$ dyn/cm at 20°C (Israelachvili and Wennerström, 1990, 1992), $\partial\gamma/\partial T = -0.1$ dyn/cm/°C (Jasper, 1972), and $A = 48$ Å^2. Substitution of these numbers into Eq. 6 yields: $\log(P_o') = 9.47$ and $\lambda' = 0.83$ Å at 5°C and $\log(P_o') = 9.36$ and $\lambda' = 1.23$ Å at 50°C. Thus, the protrusion pressure theory predicts that increasing the temperature 45°C would produce a small decrease in P_o', a result in basic agreement with experiment (Table 1). The theory also predicts that the decay length should increase ~0.4 Å with an increase in temperature from 5 to 50°C. Such an increase was not observed (Table 1).

Thus, for gel phase bilayers, the hydration (water polarization) model appears to fit the total experimental pressure-distance relation somewhat better than the protrusion model. However, from these data we cannot rule out the possibility that molecular protrusions contribute to the total repulsive pressure, particularly at small interbilayer separations.

Liquid crystalline bilayers

For 2 Å $< d_f <$ 10 Å, the pressure-fluid spacing data for the liquid crystalline phase (Fig. 6 *B*) are similar to those of the gel phase (Fig. 6 *A*) in that both are nearly independent of temperature. Thus, despite the increase in headgroup motion with increasing temperature (Ulrich and Watts, 1994), over this range of fluid spacings the work to remove water is nearly independent of temperature, implying that the work of dehydration is primarily enthalpic in origin. However, the liquid crystalline phase is very different than the gel phase bilayers in that in the liquid crystalline phase the equilibrium fluid spacing increases dramatically with temperature (Fig. 6 *B*). The temperature dependence of this process suggests that near the equilibrium fluid spacing the dehydration process has a strong entropic (steric) component. We will now provide evidence that this entropic component arises from the thermally driven bending modes in the lipid bilayer that increase in a predictable manner with increasing temperature.

A self-consistent field approximation has been developed by Evans (1991) that provides a method to predict the essential features of the temperature-induced increase in fluid spacing between liquid crystalline membranes as functions of the underlying exponential repulsive pressure (P_{sr}), the attractive van der Waals pressure, and the bilayer undulation pressure. For our calculations, we use for P_{sr} the repulsive pressure that we measured for DPPC bilayers in the crystalline subgel phase, where entropic pressures are small (McIntosh and Simon, 1993). Evans (1991) assumed that the van der Waals pressure has a power law dependence and a magnitude characterized by the Hamaker constant, A_H, and that the undulation pressure is characterized by the Helfrich energy, $E_H = (kT)^2/16\pi^2\, c \cdot k_c$, where the parameter $c \approx 0.1$ and k_c is the bilayer bending modulus. Evans (1991) plotted λ/d_{eq} (where d_{eq} is the equilibrium fluid separation in excess water) versus A_H/E_H for different values of the parameter $V_r = P_o\lambda^3/E_H$. Evans (1991) used a different definition than ours for the plane of origin of the attractive and repulsive pressures, as he used the plane of origin obtained from gravimetric analysis of x-ray diffraction data (Tardieu et al., 1973; LeNeveu et al., 1977). From our previous comparisons of electron density profiles and gravimetric analysis of EPC bilayers (McIntosh et al., 1989b), we have found that the fluid spaces calculated using these different planes of origin differ by 7.9 Å. This means to use our data with the Evans (1991) plane of origin we must 1) increase the magnitude of P_o that we obtained for DPPC in the subgel phase (1.1×10^9 dyn/cm^2) by the factor (7.9 Å/1.4 Å), where 1.4 Å is λ for the subgel phase (McIntosh and Simon, 1993); and 2) add 7.9 Å to our measured value of d_{eq}. Using these modified values, we calculate $V_r \approx 3 \times 10^3$ for EPC bilayers. In subsequent

calculations we use the closest published curve, where $V_r = 10^3$ (Fig. 7). To predict the expected d_{eq} for EPC bilayers at different temperatures it is necessary to know the value of E_H for EPC bilayers at 23°C. This can be estimated by assuming $A_H = 3 \times 10^{-14}$ erg (Gingell and Parsegian, 1972) and selecting the value of A_H/E_H that corresponds to our value of λ/d_{eq} = 1.4 Å/23.3 Å = 0.06 (Fig. 7). This gives $A_H/E_H = 400$, so that $E_H = 7.5 \times 10^{-17}$ erg, a reasonable value (Evans, 1991). We now make the simplest assumptions and determine whether varying only one parameter, the bending modulus (k_c), is sufficient to explain the observed increase in d_{eq} with increasing temperature. In the Evans (1991) theory this modulus appears in the Helfrich energy scale such that $E_H \alpha T^2/k_c$. In turn $k_c = b K_T \delta^2$ (Needham, 1995), where b is a constant, K_T is the isothermal compressibility modulus, and δ is the acyl chain thickness. Since for SOPC bilayers it has been found that K_T is temperature-invariant from 14 to 35°C (E. Evans, personal communication), we assume that K_T is nearly constant for 5–50°C for EPC. As described above, we set $\delta = d_{pp} - 10$ Å so that δ = 30.1, 28.7, and 26.3 Å at 5, 23, and 50°C, respectively. Using these values of δ, we calculate values of E_H for each temperature and use the relation in Fig. 7 to determine the equilibrium fluid spacings at 5 and 50°C. The results (Table 2) indicate excellent agreement between theory and experiment.

Thus, at zero or small externally applied pressures the undulation pressure is the dominant repulsive pressure between electrically neutral liquid crystalline bilayers, at least from 5 to 50°C. The presence of the undulation pressure explains why liquid crystalline bilayers adsorb more water than do gel bilayers. As expected from theoretical treatments (Evans and Parsegian, 1986; Evans, 1991), we find that the undulation pressure increases with increasing temperature because of decreases in the bilayer bending modulus.

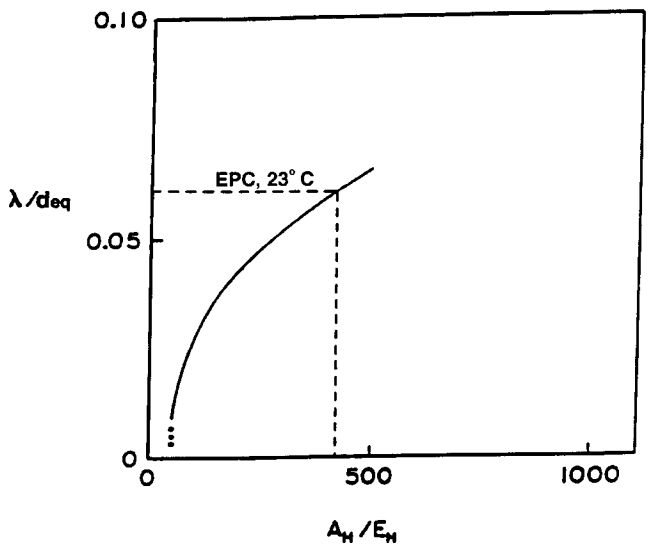

FIGURE 7 Plot of λ/d_{eq} versus A_H/E_H, where λ is the decay length of the underlying repulsive pressure, d_{eq} is the equilibrium fluid spacing in excess water, A_H is the Hamaker constant, and E_H is the Helfrich energy (see text for details). The line is redrawn from Fig. 7 of Evans (1991). The values for EPC at 23°C are shown by the dashed lines.

TABLE 2 Theoretical and experimental equilibrium fluid spacings* for EPC bilayers

Temperature (°C)	d_{eq} (experimental) (Å)	d_{eq} (undulation theory) (Å)
5	22.1	22.1
23	23.3	23.3
50	28.8	28.0

*Calculated using Evans' (1991) theory and definition for plane of origin.

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