Interactions between charged, uncharged, and zwitterionic bilayers containing phosphatidylglycerol

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ABSTRACT Pressure vs. distance relationships have been obtained for phosphatidylglycerol bilayers, in both charged and uncharged states. Water was removed from the lipid multilayers by the application of osmotic pressures in the range of 0–2.7 × 10^9 dyn/cm^2, and the distance between adjacent bilayers was obtained from Fourier analysis of lamellar x-ray diffraction data. For phosphatidylglycerol bilayers made electrically neutral either by lowering the pH or by adding equimolar concentrations of the positively charged lipid stearylamine, the pressure-distance data could be fit with a single exponential. The measured decay lengths were 1.1 Å at low pH and 1.5 Å with stearylamine, which are similar to decay lengths of the hydration pressure found for gel phases of other neutral bilayers. In addition, the magnitude of this repulsive pressure was proportional to the square of the Volta potential (equivalent to the dipole potential for electrically neutral bilayers) measured in monolayers in equilibrium with bilayers, in agreement with results previously found for the hydration pressure between phosphatidylcholine bilayers. For charged phosphatidylglycerol bilayers, the pressure-distance relation had two distinct regions. For bilayer separations >10 Å, the pressure-distance data had an exponential decay length (11 Å) and a magnitude consistent with that expected for electrostatic repulsion from double-layer theory. For bilayer separations of 2–10 Å, the pressure decayed much more rapidly with increasing bilayer separation (decay length <1 Å). We interpret these data at low bilayer separations in terms of a combination of hydration repulsion and steric hindrance between the lipid head groups and the sodium ions trapped between apposing bilayers.

INTRODUCTION

Two nonspecific short-range repulsive interactions have been observed for uncharged lipid membranes, such as phosphatidylcholine bilayers. For bilayer separations of 5–20 Å, the dominant repulsive interaction is the hydration pressure (LeNeveu et. al., 1977; Parsegian et al., 1979; Marra and Israelachvili, 1985; McIntosh and Simon, 1986; McIntosh et al., 1989*a*), which is thought to arise from the polarization of water molecules by the zwitterionic lipid head groups (Marcelja and Radic, 1976; LeNeveu et al., 1977; Israelachvili and Pashley, 1983). For very small separations where apposing bilayers come into contact, steric hindrance between apposing lipid head groups becomes important (McIntosh et al., 1987; McIntosh et al., 1989*a*; Simon and McIntosh, 1989*a*).

For bilayers containing a net charge, such as those composed of the membrane lipids phosphatidylglycerol, phosphatidylinositol, or phosphatidylserine, electrostatics provide the dominant repulsive interaction for bilayer separations of over 30 Å (Cowley et al., 1978; Loosley-Millman et al., 1982; Marra, 1986; McDaniel and McIntosh, 1989). However, for charged bilayers at closer separations, there are fewer experimental data available concerning the relative magnitudes of hydration and electrostatic repulsive interactions. That is, the experiments of Marra (1986) could only be performed accurately down to a bilayer separation of ~20 Å, and the osmotic stress experiments of Cowley et al. (1978) were performed at applied pressures below 1 × 10^8 dyn/cm^2 that yielded a minimum bilayer separation of ~10Å. Thus, Marra could not detect hydration repulsion for phosphatidylglycerol bilayers with his direct force measurement technique. Cowley et al. (1978) observed upward deflections in their force vs. bilayer separation data beginning at bilayer separations of <20 Å, which they attributed to hydration repulsion. They showed that, for separations of 10–20 Å, their data were in qualitative agreement with observed hydration forces measured with neutral phosphatidylcholine bilayers. However, Cowley et al. (1978) did not attempt to further quantitate the hydration pressure for the charged bilayers.

In this paper, we extend the measurements of Marra (1986) and Cowley et al. (1978) to obtain magnitude and distance dependence of the short-range pressures for charged and electrically neutral phosphatidylglycerol bilayers at small bilayer separations. By the use of x-ray diffraction analysis of bilayers subjected to applied pressures from 0 to 2.6 × 10^9 dyn/cm^2, we obtain pressure-

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distance relationships for bilayer separations ranging from 80 to 2 Å. We compare measured pressure-distance relationships for charged phosphatidylglycerol bilayers and for phosphatidylglycerol membranes made electrically neutral in two ways: by the lowering of the pH of the aqueous phase and by the addition of equimolar quantities of a positively charged lipid to the bilayer. To simplify the analysis, all experiments have been performed with lipids in the gel phase. In this phase, contributions due to thermally induced fluctuations (Harbich and Helfrich, 1984; Evans and Parsegian, 1986; McIntosh et al., 1989*b*) and bilayer deformation (Simon et al., 1988; McIntosh et. al., 1989*a*) are expected to be quite small.

MATERIALS AND METHODS

The sodium salt of dipalmitoylphosphatidylglycerol (DPPG) and stearylamine (SA) were used as obtained from Avanti Polar Lipids Inc. (Birmingham, AL) and Sigma Chemical Co. (St. Louis, MO), respectively. Poly(vinylpyrrolidone) (PVP) with an average molecular weight of 40,000 was also purchased from Sigma Chemical Co. PVP solutions in the range of 0–50% wt/wt were made in either triply distilled water, 50 mM sodium phosphate buffer at pH 7.2, or 1 N HCl at pH 0.3.

Osmotic pressures were applied to unoriented lipid suspensions by an "osmotic stress" procedure (LeNeveu et al., 1976; Parsegian et al., 1979). DPPG or equimolar mixtures of DPPG and SA were rotary evaporated from chloroform and an excess amount of the appropriate PVP solution was added. Typically the lipid concentration was 10 mg/ml (14 mM), although higher lipid concentrations (250 mg/ml) were used for several experiments with DPPG:SA or DPPG at pH 0.3. X-Ray repeat periods from the two different lipid concentrations were the same. The lipid suspensions were vortexed, covered with nitrogen, and heated two or three times for several minutes to 60°C, above the lipid's main phase transition temperature, which is ~53°C at pH 0.3 and ~40°C at pH 7.2 (Watts et al., 1978). Because PVP is too large to enter between the lipid multilayers, it competes for water with the lipid and compresses the lamellar lattice (Parsegian et al., 1979). Osmotic pressures of the PVP solutions were calculated from the virial coefficients obtained by Vink (1971). These extrapolated values are in excellent agreement with measured pressures (Parsegian et al., 1986; McIntosh et al., 1989*c*). The lipid-PVP suspensions were concentrated by centrifugation with an Eppendorf desk top centrifuge. The pellets were sealed in x-ray capillary tubes and mounted in a point collimation x-ray camera.

To check for hydrolysis of DPPG caused by incubation at pH 0.3, thin layer chromotography was performed using a solvent system of chloroform/methanol/water 65:25:4 (vol/vol/vol). A single spot was observed for DPPG as obtained from Avanti Polar Lipids, Inc. However, after incubation for 24 h at pH 0.3, two additional spots were observed, corresponding to lysoPG and fatty acid. Evidence for break-down products was also seen in diffraction patterns recorded from DPPG after 2 or 3 d incubation at pH 0.3. In addition to reflections from DPPG (see below), these patterns also contained reflections at 47 and 37 Å. The experiments at pH 0.3 reported in this paper, which were all completed in <24 h, contained only lamellar low-angle diffraction from DPPG (see below) and did not contain the 47 and 37 Å reflections. Even though separate phases did not appear in these patterns recorded with <24 h at pH 0.3, some break-down products were undoubtedly present in these specimens as evidenced by the chromotography results. However, neither lysoPG nor fatty acid would contribute fixed charge to the DPPG bilayer at this low pH value.

Osmotic pressures were applied through the gas phase to oriented DPPG and equimolar DPPG:SA multilayers by published procedures (Parsegian et al., 1979; McIntosh et al., 1987). In brief, oriented multilayers were formed by placing a small drop of lipid/chloroform solution on a flat strip of aluminum foil and evaporating the chloroform. The foil substrate was given a convex curvature and mounted in a controlled humidity chamber on a line-focused single-mirror x-ray camera so that the x-ray beam was oriented at a grazing angle relative to the multilayers. Relative humidity was controlled in the chamber by means of a cup of a saturated salt solution. To speed equilibration, a gentle stream of nitrogen gas was passed through a flask of the saturated salt solution and then through the chamber. The relative vapor pressures (p/p_0) of various salt solutions used in these experiments have been measured (Weast, 1984) and are indicated in parentheses: $CuSO_4$ (0.98). Na_2SO_4 (0.93), KCl (0.87), NH_4Cl (0.80), $NaNO_2$ (0.66), $CaCl_2$ (0.32), and LiCl (0.15). The vapor pressure applied to the specimen is given by

$$P = -(RT/V_w) \ln (p/p_0), \quad (1)$$

where R is the molar gas constant, T is the temperature (293 ± 1° K in these experiments), and V_w is the molar volume of water.

For both unoriented lipid suspensions and oriented multilayers, x-ray diffraction patterns were recorded on a stack of four sheets of Kodak DEF 5 x-ray film. All patterns were recorded at 20°C. Films were processed by standard techniques and densitometered with a microdensitometer (model MKIIIC; Joyce-Loebl). For films from the unoriented specimens, the densitometer traces were taken in radial direction from the center of the films. For films from the oriented specimens, the traces were taken through the center of each reflection. 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 $\{h^2I(h)\}^{1/2}$ (Herbette et al., 1977). For the oriented, line-focused patterns, there was no detectable arcing of the reflections, which were of uniform height. For these patterns, the structure factor was set equal to $F(h) = \{hI(h)\}^{1/2}$ (Blaurock and Worthington, 1966; Herbette et al., 1977). That is, the intensities were corrected by a single factor of h (the Lorentz correction factor) due to the cylindrical curvature of the multilayers (Blaurock and Worthington, 1966; Herbette et al., 1977). The validity of this correction factor has been demonstrated experimentally (McIntosh et al., 1987; McIntosh et al., 1989*a*). One-dimensional electron density profiles, $\rho(x)$, across the bilayers wre calculated by

$$\rho(x) = (2/d) \Sigma \exp [i\phi(h)] \cdot F(h) \cos (2\pi hxd), \quad (2)$$

where d is the lamellar repeat period and $\phi(h)$ is the phase angle, either 0 or π for each order h. The resolution of the profiles was $d/2h_{max} \sim 7$–8 Å. Phase angles were calculated by the use of the sampling theorem as previously described (McIntosh et al., 1987; McIntosh et al., 1989*a*). The value of the structure factor at the origin was estimated by the procedure of King and Worthington (1971).

Volta potentials (Aveyard and Haydon, 1973) were measured as described previously (McIntosh et al., 1989*a*). In brief, monolayers were formed by spreading 10 μL of the lipid/chloroform solution (25 mg/mL) onto the appropriate subphase in a Teflon trough with a surface area of 30 cm^2. Under these conditions 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 subphase was vacuum aspirated immediately before each monolayer was spread to ensure that the surface was free of surface-active impurities. The Volta potential was measured between a Ag/AgCl electrode in the subphase and a polonium electrode in the air that were connected to an

electrometer (model 602; Keithley Instruments, Inc., Cleveland, OH). The reported values of Volta potential represent the difference in the potential of the subphase surface in the presence and absence of the monolayer.

RESULTS

The diffraction pattern for DPPG in excess buffer at pH 7.2 consisted of broad bands centered at ~48 and 24 Å, plus two wide-angle bands, a sharp reflection at 4.21 Å and a broad band centered at ~4.1 Å. This is similar to patterns recorded previously from DPPG at pH 7.4 (Wilkinson et al., 1987). The presence of the broad low-angle bands, which are related to the square of the continuous Fourier transform of the bilayer, indicates that the multilayer structure swells in aqueous media so that the fluid spaces between adjacent bilayers become large and irregular in width (Wilkins et al., 1971; Wilkinson et al., 1987). Similar wide-angle patterns have previously been recorded from DPPG liposomes in water, and indicate that the lipid hydrocarbon chains are tilted relative to the bilayer normal (Watts et al., 1981). For all other specimens examined in this study, both oriented and unoriented, the x-ray diffraction patterns contained a series of sharp low-angle reflections which indexed as orders of a lamellar repeat period. For DPPG in excess 1 N HCl at pH 0.3, the lamellar repeat period was 69.7 Å and the wide-angle contained a single sharp reflection at 4.10 Å. Similar patterns for DPPG at low pH have been recorded previously (Watts et al., 1981). For 1:1 DPPG:SA in excess water with no applied pressure, the lamellar repeat period was 62.9 Å and the wide-angle pattern contained a sharp reflection at 4.10 Å. The sharpness of the single reflection for both DPPG at pH 0.3 and 1:1 DPPG:SA indicates that in these bilayers the lipid hydrocarbon chains are oriented approximately perpendicular to the plane of the bilayer (Tardieu et al., 1973; McIntosh, 1980; Watts et al., 1981), and the bilayers are in the Lβ gel phase.

For specimens subjected to applied pressures, the repeat period depended on the type of sample and the applied pressure. Plots of the logarithm of applied pressure (log P) vs. repeat period (d) for DPPG multilayers are shown in Fig. 1 *A*. For oriented DPPG specimens, the repeat period increased from 55.1 Å at a relative vapor pressure of 0.15 (log P = 9.4) to 59.0 Å at a relative vapor pressure of 0.98 (log P = 7.4). Unoriented DPPG liposomes at pH 7.2 gave repeat periods that ranged from 58.3 Å in 50% PVP (log P = 7.5) to 78.5 Å in 20% PVP (log P = 6.4). Fig. 1 *B* shows plots of log P vs. d for oriented and unoriented equimolar DPPG:SA multilayers and unoriented DPPG liposomes in PVP solutions at pH 0.3. For the DPPG:SA experiments, the repeat period

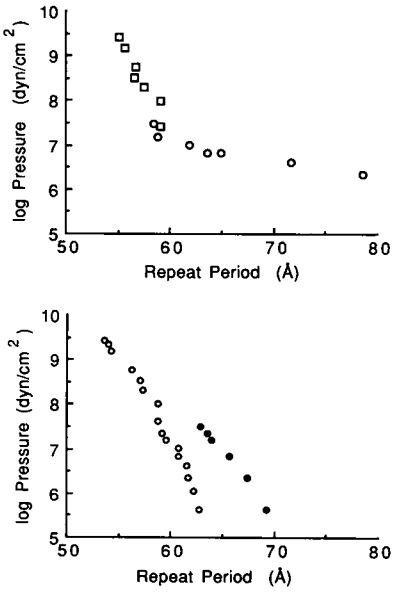

FIGURE 1 Logarithm of applied pressure vs. lamellar repeat period for (*A*) DPPG at pH 7.2 (*open circles*) and DPPG in relative humidity atmospheres (*open squares*) and (*B*) 1:1 DPPG:SA (*open circles*) and DPPG at pH 0.3 (*closed circles*).

decreased monotonically with increasing pressure, ranging from d = 53.6 Å at a relative vapor pressure of 0.15 (log P = 9.4) to 62.8 Å in 10% PVP solution (log P = 5.6). For DPPG liposomes at pH 0.3, d ranged from 62.9 Å at 50% PVP (log P = 7.5) to 69.3 Å at 10% PVP (log P = 5.6).

Wide-angle diffraction patterns were also recorded from the unoriented specimens in PVP solutions. For DPPG liposomes in PVP solutions at pH 7.2, the wide-angle pattern consisted of a sharp reflection at 4.21 Å and a broad band centered at ~4.1 Å, whereas for DPPG at pH 0.3 and equimolar DPPG:SA bilayers in PVP the wide-angle pattern contained a sharp reflection at 4.11 Å. With the assumption of no chain tilt and hexagonal close packing of the lipid chains for DPPG at pH 0.3 Å, this wide-angle spacing corresponds to an area per chain of 19.5 Å^2, or an area per DPPG molecule of 39.0 Å^2. For all three types of liposomes 1:1 DPPG:SA, DPPG at pH 0.3, and DPPG at pH 7.2, the spacings of the wide-angle reflections remained constant (to within 0.02 Å) for each PVP concentration used.

Plots of structure factors for the lamellar diffraction vs. reciprocal space coordinate are shown in Fig. 2 *A* for oriented DPPG multibilayers and DPPG liposomes at pH 7.2, in Fig. 2 *B* for DPPG liposomes at pH 0.3, and in Fig. 2 *C* for oriented and unoriented equimolar DPPG:SA multibilayers. In each figure the solid line corresponds to the continuous Fourier transform calcu-

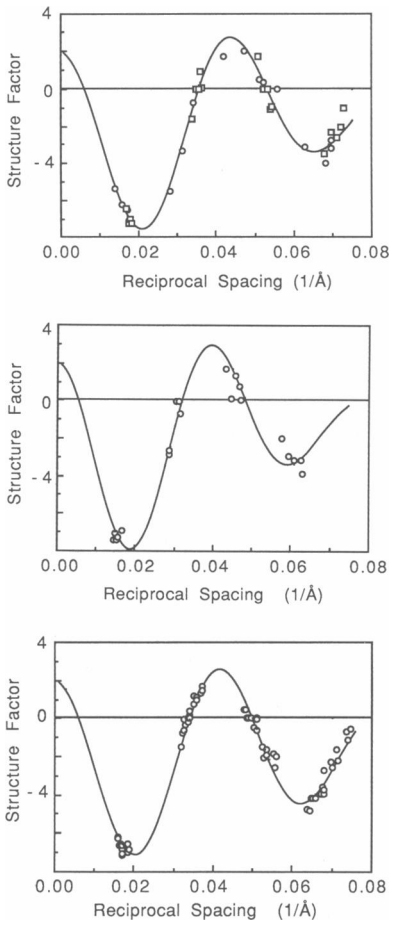

FIGURE 2 Observed structure factor plotted vs. reciprocal space coordinate for (*A*) DPPG at pH 7.2 (*circles*) and DPPG in humidity atmospheres (*squares*), (*B*) DPPG at pH 0.3 (*circles*), and (*C*) 1:1 DPPG:SA (*circles*). The lines correspond to continuous Fourier transforms of the bilayer calculated using the sampling theorem.

lated using the sampling theorem for one data set. Note, in Fig. 2 *A*, that all the structure factors for both oriented DPPG mulitlayers and DPPG liposomes (pH 7.2) fall quite near this continuous transform. This indicates that, at this resolution, the structures of all of these bilayers are similar. However, the transform for DPPG liposomes at pH 0.3 (Fig. 2 *B*) is different, in that the nodes of this transform are located at smaller values of reciprocal space than are the nodes of the transform in Fig. 2 *A*. Thus, the continuous transforms and the wide-angle diffraction patterns both indicate that the structure of DPPG bilayers differs at pH values of 0.3 and 7.2.

Electron density profiles at various applied pressures P for DPPG bilayers at pH 7.2, DPPG bilayers at pH 0.3, and equimolar DPPG:SA bilayers are shown in Figs. 3 *A–C*, respectively. For each profile, the geometric center of the bilayer is at the origin (0 Å). The low-density troughs in the center of the each profile correspond to the localization of the lipid terminal methyl groups, and the highest density peaks, located at ~ +/− 22 Å in Fig. 2 *A* and at ~ +/− 24 Å in both Fig. 2, *B* and *C*, correspond to the DPPG head group. The medium-density regions between the terminal methyl trough and the head-group peaks represent the methylene chain region of the bilayer, and the medium-density regions at the outer edges of each profile correspond to the fluid spaces between adjacent bilayers. First, consider the profiles of DPPG at pH 7.2 and in relative humidity atmospheres (Fig. 3 *A*). In 25% PVP, with an applied pressure of 4.2×10^6 dyn/cm^2 ($\log P = 6.6$), there is a relatively wide fluid space between adjacent bilayers. As the applied pressure is increased ($\log P = 7.5, 8.5$), the distance between adjacent bilayers decreases, as judged by the narrowing of the medium density region at the outer edges of the profiles. Although the fluid space between adjacent bilayers decreases as applied pressure is increased, there is relatively little change in the shape of the bilayer portion of the profile. For $6.6 < \log P < 8.5$, the distance between head group peaks across the bilayer remains almost constant at d_{p-p} = 44.6 Å +/− 0.8 Å (mean +/− 1 SD, $N = 9$ experiments). This implies that the thickness of DPPG bilayers is nearly the same for unoriented liposomes at pH 7.2 and for oriented multilayers at partial vapor pressures of 0.66–0.98. At the highest applied pressure ($\log P = 9.2$, obtained at 0.32 relative vapor pressure), the fluid spaces between adjacent bilayers are so narrow that the head group peaks from adjacent bilayers have almost merged. At this highest applied pressure the value of d_{p-p} is ~2.5 Å larger than the average value of d_{p-p} obtained at lower pressures. However, this measurement of d_{p-p} at $\log P = 9.2$ is not as reliable as the measurements at lower values of P, due to the partial overlap of the head group peaks from adjacent bilayers.

The electron density profiles of both DPPG at pH 0.3 (Fig. 3 *B*) and 1:1 DPPG:SA (Fig. 3 *C*) have similar shapes to profiles of DPPG at pH 7.2 (Fig. 3 *A*), except that the distance between head group peaks across the bilayer is larger in the former two cases. That is, for DPPG at pH 0.3, d_{p-p} = 49.7 Å ± 0.7 Å ($N = 5$) and, for 1:1 DPPG:SA, d_{p-p} = 46.7 Å ± 0.5 Å ($N = 11$). For DPPG:SA, the values of d_{p-p} were measured for profiles at pressures of $5.6 < \log P < 8.8$. For pressures of $\log P >$ 8.8, the head group peaks from adjacent bilayers overlap (Fig. 3 *C*), making it difficult to estimate d_{p-p}. In fact, in the profiles of DPPG:SA at $\log P = 9.2$ (Fig. 3 *C*), the head group peaks from apposing bilayers have overlapped to the extent that the medium density fluid spaces between adjacent bilayers are not resolved. Note that this complete overlap of head group peaks is not observed for DPPG bilayers at the same applied pressure (Fig. 3 *A*).

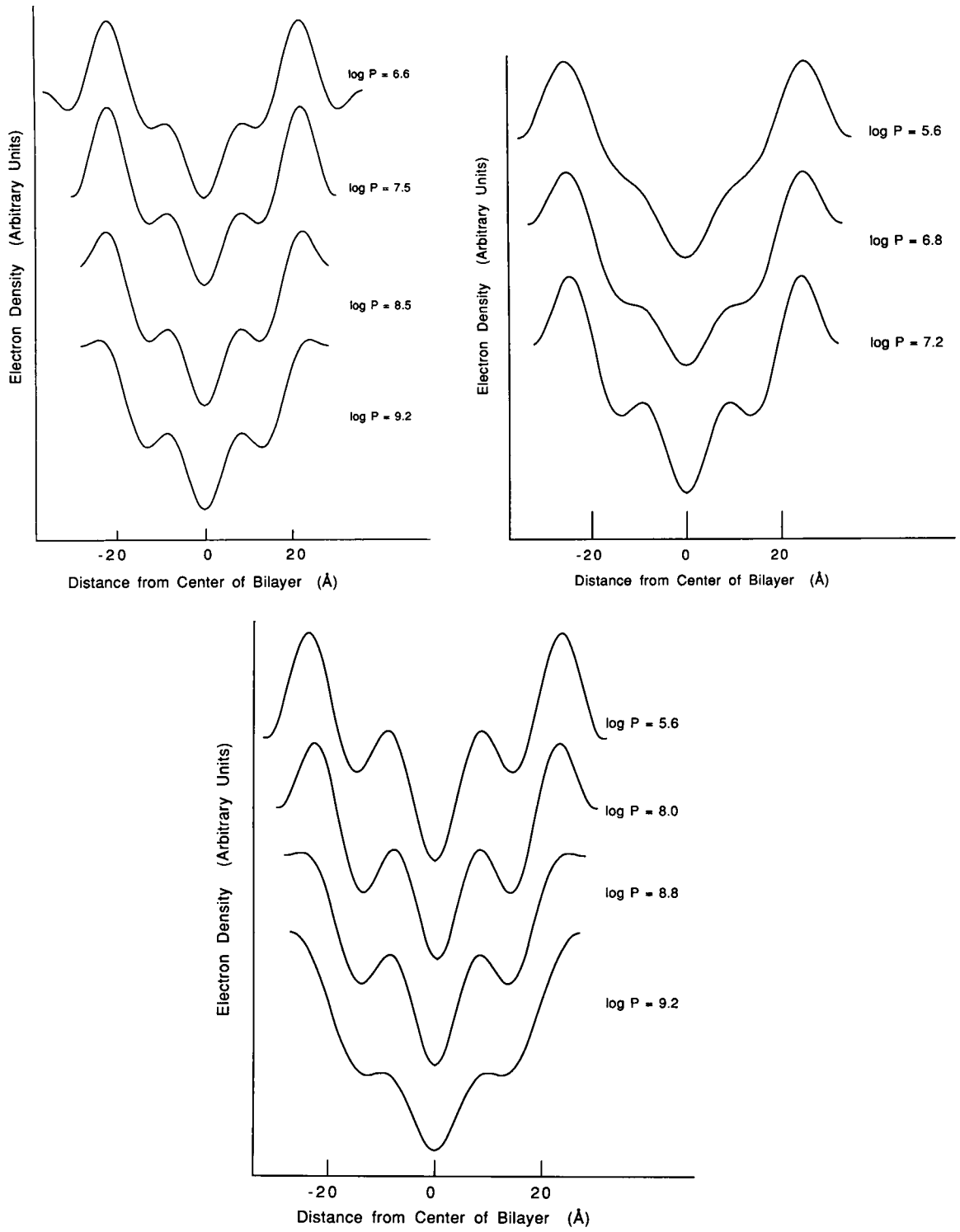

FIGURE 3 Electron density profiles for (*A*) DPPG at pH 7.2 and in relative humidity atmospheres, (*B*) DPPG at pH 0.3, and (*C*) 1:1 DPPG:SA.

The larger bilayer width for DPPG at pH 0.3 than at pH 7.2 can be completely explained by removal of the hydrocarbon chain tilt (Watts et al., 1981). The reason for the small (~3 Å) difference in the values of d_{p-p} for DPPG at pH 0.3 and DPPG:SA bilayers is not known. It seems unlikely that this difference could be completely explained by a difference in hydrocarbon chain tilt because a chain tilt of ~20 degrees for DPPG:SA bilayers would be required, and the sharpness of the wide-angle reflection indicates that there is little chain tilt in these bilayers. There are several other possible contributing factors in the difference in d_{p-p} for DPPG at pH 0.3 and DPPG:SA bilayers. First, the resolution of the x-ray data is slightly lower for DPPG at pH 0.3 ($d/2h_{max}$ ~8 Å, see Fig. 2 *B*) than for DPPG:SA bilayers ($d/2h_{max}$ ~7 Å, see Fig. 2 *C*). Second, the amine moiety in SA, which is probably located near the head group/hydrocarbon interface, might tend to shift the peak in the electron density profiles toward the middle of the bilayer, thereby reducing the value of d_{p-p}. Third, the presence of the positively charged SA could modify the orientation of the negatively charged DPPG head group. Fourth, the presence of fatty acids and lysoPG (see Materials and Methods) could change the width of the DPPG bilayer at pH 0.3.

The electron density profiles can be used to estimate the fluid space between adjacent bilayers, d_f. The definition of fluid space is somewhat arbitrary, due to the roughness of the surface of the bilayer, motion of the lipid head groups, and penetration of water into the lipid head group region. As we have done previously (McIntosh and Simon, 1986; Simon et al., 1988; McIntosh et al., 1989*a*), we define the boundary between lipid and fluid to be at the outer edge of the bilayer. The electron density profiles (Fig. 3), along with the crystal structure of phosphatidylglycerol (Pascher et al., 1987), can be used to estimate the width of the DPPG bilayer. The crystal structure of sodium dimyristoyl phosphatidylglycerol (DMPG) indicates that the phosphate groups in PG are 1.8 Å from the bilayer interface (Pascher et al., 1987). Electron density profiles of the crystalline bilayer in the subgel phase of DPPG at high resolution (Blaurock and McIntosh, 1986) can be used to estimate the location of the phosphate moiety relative to the head group peaks in electron density profiles at the lower resolution shown in Fig. 3 ($d/2h_{max}$ ~7–8 Å). In profiles of DPPG in the subgel phase at $d/2h_{max}$ = 1.3 Å, where the phosphate moiety can be clearly resolved, the distance between phosphate moieties across the bilayer is ~44 Å, whereas at $d/2h_{max}$ ~7 Å the distance between head group peaks is 41.6 Å (Blaurock and McIntosh, 1986). This indicates that the phosphate moiety is ~1.2 Å farther from the bilayer center than the high density peaks in the profiles at 7 Å resolution. That is, the distance from the head group peak in the electron density profiles in Fig. 3 *A* to the phosphate group is ~1.2 Å and the distance from the phosphate group to the edge of the bilayer is ~1.8 Å. Therefore, assuming that the structure of the head group regions is similar in crystalline DMPG, subgel phase DPPG, and the gel phases studied in this work, we add 6 Å to the average measured values of d_{p-p} to obtain the total width of the bilayer, d_b. (For the reasons discussed above, this assumption may not be strictly correct for DPPG:SA bilayers. However, the value of the decay length of the hydration pressure will be unaffected by this assumption). We then calculate the distance between bilayers from $d_f = d - d_b$.

Fig. 4 shows plots of log P vs. the distance between bilayers for DPPG at pH 7.2, DPPG at pH 0.3, and 1:1 DPPG:SA. We plot the results for DPPG in relative humidity experiments with the same symbol as DPPG at pH 7.2 because the structure factor data (Fig. 2 *A*) and the electron density profiles (Fig. 3 *A*) both indicate that the structure of the bilayer is similar at pH 7.2 and for partial vapor pressures of 0.98–0.66. The two straight lines in Fig. 4 are linear regressions to the DPPG at pH 0.3 and 1:1 DPPG:SA data. These lines fit the general expression $P = P_0 \exp(-d_f/\lambda)$, where the decay lengths were 1.1 Å and 1.5 Å for DPPG:SA and DPPG at pH 0.3, respectively. In contrast, for DPPG at pH 7.2 there is a definite break in the plot of log P vs. d_f at log $P \sim 7$. By a least-squares analysis, these data could be fit ($r^2 = 0.99$) to two exponentials with decay lengths of 0.7 and 12.9 Å. A similar relation for pressure vs. fluid spacing was found for bilayers composed of the negatively charged lipid phosphatidylinositol (McDaniel and McIntosh, 1989).

The equilibrium fluid separations (the values of d_f obtained by subtracting d_b from the repeat periods of bilayers in excess solvent with no applied pressures) are 10.2 Å and 14.0 Å for DPPG:SA and DPPG at pH 0.3, respectively.

For phosphatidylcholine bilayers, we (Simon et al., 1988; McIntosh et al., 1989, *a–c*; Simon and McIntosh,

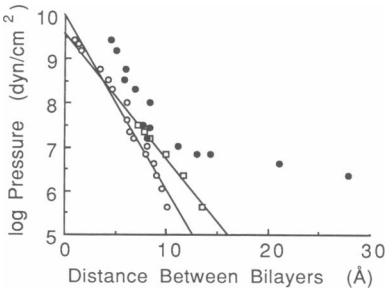

FIGURE 4 Logarithm of applied pressure vs. distance between bilayers for DPPG at pH 7.2 and in relative humidity atmospheres (*solid circles*), DPPG at pH 0.3 (*open squares*), and 1:1 DPPG:SA (*open circles*). The solid lines are linear regressions to the DPPG at pH 0.3 and 1:1 DPPG:SA data.

1989*b*) argued that the magnitude of the hydration pressure is related to the square of the Volta potential, (equivalent to the dipole potential for uncharged lipids, see below), as measured for monolayers in equilibrium with bilayers. Therefore we measured the Volta potential for DPPG and equimolar DPPG:SA monolayers. The Volta potential was 392 ± 4.3 mV (mean ± standard error, $N = 4$ measurements) for DPPG monolayers over 50 mM sodium phosphate buffer at pH 7.2, 484 ± 5.9 mV ($N = 3$) for DPPG over 1 N HCl pH 0.3, and 636 ± 19 mV for 1:1 DPPG:SA monolayers over unbuffered 10 mM NaCl at pH 7.2.

DISCUSSION

Both the low-angle and wide-angle x-ray diffraction data indicate that the bilayer width and area per lipid molecule remain nearly constant over the range of pressures applied for bilayers of DPPG at pH 7.2, DPPG at pH 0.3, and equimolar DPPG:SA. Both the reciprocal space (Fig. 2) and real space (Fig. 3) analysis of the lamellar diffraction data show that the structure of the bilayer, at ~7 Å resolution, remains nearly constant for applied pressures in the range $5.6 < \log P < 8.8$. That is, plots of structure factors for these applied pressures fall near the continuous transform of the bilayer for DPPG at pH 7.2 and in relative humidity atmospheres (Fig. 2 *A*), for DPPG liposomes at pH 0.3 (Fig. 2 *B*), and for equimolar DPPG:SA (Fig. 2 *C*) and the distance between head group peaks across the bilayer in electron density profiles remains nearly constant for DPPG at pH 7.2 and in relative humidity atmospheres (Fig. 3 *A*), for DPPG at pH 0.3 (Fig. 3 *B*), and for equimolar DPPG:SA (Fig. 3 *C*). For the higher applied pressures ($\log P > 8.8$), the bilayer width is more difficult to estimate, due to the partial overlap of the head group peaks from adjacent bilayers (Fig. 3, *A* and *C*). For $\log P > 8.8$, we have used the average value of d_{p-p} obtained from experiments at $\log P < 8.8$. This approach is rationalized by the observation that the structure amplitudes from the experiments at all applied pressures ($9.4 < \log P < 5.6$) fall near the same continuous transform (Fig. 2, *A* and *C*), implying that the bilayer width is approximately constant for these applied pressures.

The wide-angle reflections are almost invariant for each of these bilayers for all PVP concentrations (pressure range $5.6 < \log P < 7.5$). This means that the area per lipid chain and area per molecule, remains nearly constant (to within 0.2 Å^2) over this pressure range, implying that these gel-phase bilayers have very high area compressibility moduli. Thus, the fraction of the total work that goes into bilayer deformation is extremely small compared with the work of bringing apposing bilayers together (McIntosh et al., 1987). This result, that the hydration pressure is present in virtually incompressible bilayers, is not consistent with the recently proposed "osmoelastic coupling" term (Ito et al., 1989) that attributed the origin of the hydration pressure primarily to elastic deformations in the bilayer.

In this paper we have considered three systems, one with a net negative charge, DPPG at pH 7.2, and two with no net charge, DPPG at pH 0.3 and equimolar DPPG:SA. At pH 7.2, DPPG is nearly fully charged because the apparent pK of phosphatidylglycerol is 2.9 in 0.1 M NaCl (Cevc and Marsh, 1987). When the sodium salt of DPPG is neutralized, either by lowering the pH or by adding SA, Na^+ can disassociate from the bilayer surface and equilibrate with Cl^- or OH^- in the bulk solution to preserve electroneutrality. At pH 0.3 the DPPG should be ~95% neutralized by protons and ~5% by sodium ions (Lakhdar-Ghazal et al., 1983). For DPPG:SA bilayers, we tacitly assumed that the equimolar mixture should form a zwitterionic lipid in much the same way as when other anionic and cationic surfactants are cosolubilized (Kalar et al., 1989). Three lines of experimental evidence indicate that we have in fact neutralized the charge on DPPG by lowering the pH and by adding SA. First, the wide-angle patterns show that the hydrocarbon chain tilt has been removed in bilayers of DPPG at pH 0.3 and in bilayers of DPPG:SA. Watts et al. (1981) have previously shown that charged DPPG bilayers have tilted hydrocarbon chains and that electrically neutral bilayers have untilted hydrocarbon chains. Secondly, the bilayers of DPPG at pH 0.3 and DPPG:SA have equilibrium fluid spacings (d_e) in excess buffer (Table 1) that are similar to the d_e = 11.7 Å measured for neutral dipalmitoylphosphatidylcholine (DPPC) bilayers (McIntosh and Simon, 1986). In the absence of applied pressure, charged bilayers, such as DPPG at pH 7.2, swell to the extent that the sharp lamellar diffraction is not recorded and is replaced by broad bands proportional to the continuous transform of the bilayer. Third, the pressure vs. fluid spacing curves for DPPG at pH 0.3 and DPPG:SA bilayers (Fig. 4) can be fit to single exponential relations, with decay constants similar to those observed for uncharged bilayers such as DPPC (see below and Table 1).

We now consider the pressure-distance relationships (Fig. 4), starting with the electrically neutral bilayers of DPPG at pH 0.3 and DPPG:SA. The $\log P$ vs. d_f data for these bilayers can each be fit with single straight line, indicating that over this pressure range a single exponential repulsive pressure opposes the applied osmotic pressure. This pressure is almost certainly the hydration pressure because for neutral bilayers such as DPPC the hydration force has been shown to decay exponentially with increasing fluid separation with a magnitude (P_0)

TABLE 1 Parameters for repulsive and attractive interactions between bilayers containing phosphatidylglycerol

Lipid	P_o	λ	P_{es}	λ_{es}	H	d_c	E_a	r^2
	dyn/cm²	Å	*dyn/cm²*	Å	*ergs*	Å	*erg/cm²*	
DPPG:SA	9.8×10^9	1.1	0	—	0	10.2	—	0.98
DPPG:SA	9.7×10^9	1.1	0	—	1.4×10^{-14}	10.2	−0.06	0.99
DPPG pH 0.3	3.8×10^9	1.5	0	—	0	14.0	—	0.99
DPPG pH 0.3	2.6×10^9	1.7	0	—	1.4×10^{-14}	14.0	−0.03	0.99
DPPG pH 7.2	1.5×10^{12}	0.7	2.2×10^7	12.9	0	—	—	0.99
DPPG pH 7.2	8.9×10^{11}	0.8	2.7×10^7	11.3	1.4×10^{-14}	—	—	0.99
DPPG pH 7.2	7.9×10^{11}	0.8	3.3×10^7	10.5	6.0×10^{-14}	—	—	0.99

and decay length (λ) similar to that observed in Fig. 4 (McIntosh and Simon, 1986; Simon et al. 1988; Simon and McIntosh, 1989*b*). That is, P_0 and λ of the hydration pressure measured for DPPC are 4.7×10^9 dyn/cm² and 1.3 Å, respectively (McIntosh and Simon, 1986), which are very similar to the values found in the present study (Table 1).

The pressure-distance data and the values of the equilibrium fluid separation (d_e) in excess solvent (Fig. 4) can be used to estimate the energy of adhesion (E_a) for the DPPG at pH 0.3 and DPPG:SA bilayers. For these electrically neutral systems, the total pressure, P_t, consists of the difference between the repulsive hydration pressure, P_h, and the attractive van der Waals pressure, P_v, which has the form $H/6\pi d_f^3$, where H is the Hamaker constant. As noted by Evans and Parsegian (1986) the planes of origin for P_h and P_v may differ and there is no clear definition for either of these planes. To simplify the analysis, we assumed that the two pressures have the same plane of origin as defined in Results, so that $P_t = P_0 \cdot \exp(-d_f/\lambda) - H/6\pi d_f^3$. The energy of adhesion can be calculated by integrating P_t and evaluating the energy at the equilibrium fluid separation. Using a least squares fit to the pressure-distance data (Fig. 4) and the value of the equilibrium fluid separations, we calculated H and E_a for DPPG at pH 0.3 and DPPG:SA bilayers (Table 1). For both systems a Hamaker constant of 1.4×10^{-14} ergs was obtained. This value is similar to other predicted and measured values of H (Requena et al., 1977; Marra and Israelachvili, 1985; Marra, 1986). The energy of adhesion was estimated to be −0.03 and −0.06 erg/cm² for DPPG at pH 0.3 and DPPG:SA, respectively. These are quite reasonable values because the adhesion energy between electrically neutral gel and liquid-crystalline bilayers is −0.035 erg/cm² (Evans and Needham, 1986) and the adhesion energy between two electrically neutral gel phase bilayers would be expected to be somewhat larger (Dr. David Needham, personal communication). Thus, although several assumptions are involved in these calculations, including the location of the planes of origin of the hydration and van der Waals pressures, our values of H and the energy of adhesion are in close agreement with values obtained by other methods. It should also be noted that the inclusion of the attractive van der Waals pressure changes only slightly the values of the magnitude (P_0) and decay length (λ) of the hydration pressure (Table 1). That is, for both DPPG at pH 0.3 and DPPG:SA bilayers the values of P_0 and λ are only slightly modified when the van der Waals pressure is included in the analysis (in Table 1, compare line 1 with line 2 and line 3 with line 4).

Measurements of Volta potential (V) provide a further test for the idea that the observed pressure-distance relations for DPPG at pH 0.3 and DPPG:SA correspond primarily to the hydration pressure. That is, Cevc and Marsh (1985) have developed a theory for the magnitude of the hydration pressure which predicts that $P_0 = 2\chi(\Psi_h/\lambda)^2$, where χ is the orientational susceptibility and Ψ_h is the "hydration potential." In previous studies with electrically neutral bilayers of phosphatidylcholine (Simon et al., 1988), phosphatidylcholine/cholesterol (McIntosh et al., 1989*a*, *c*), and monoglycerides (McIntosh et al., 1989*b*), we found empirically that the measured Volta potential (which is equivalent to the dipole potential for electrically neutral bilayers) provides a good estimate for Ψ_h. Thus, according to this analysis we would expect that $P_0 = 2\chi(V/\lambda)^2$, where we have substituted V for the hydration potential. Using this formula and our measured values of V and λ, we obtain predicted values of $P_0 = 6.0 \times 10^9$ dyn/cm² and 1.8×10^9 dyn/cm² for DPPG:SA and DPPG at pH 0.3, respectively, which are within a factor of 2 of our measured values (Table 1). We consider this agreement between theoretical and experimental values to be quite satisfactory, considering that these measured values of P_0 are estimated from extrapolation of an exponential function with a relatively small decay length. A change of ~1 Å in the definition of the bilayer/fluid boundary would put the experimental and theoretical values of P_0 in complete agreement. Thus, the same relationship which has been used to predict the magnitude of the hydration pressure for other neutral bilayers (Simon et al., 1988; McIntosh et al., 1989, *a–c*; Simon and McIntosh, 1989*b*), also closely predicts the

magnitude of the observed repulsive pressure between bilayers of DPPG:SA and DPPG at pH 0.3.

Hydration pressures which decay exponentially with increasing distance between bilayers surfaces have now been found for three types of electrically neutral gel bilayer surfaces: zwitterionic lipids such as DPPC (Lis et al., 1982; McIntosh and Simon, 1986), bilayers containing equimolar positive and negative lipids such as DPPG:SA, and bilayers containing uncharged lipids such as DPPG at pH 0.3 and monoglycerides (McIntosh et al., 1989*b*). Although these systems have quite different symmetries and chemical compositions in the head group region of the bilayer, they all have similar decay lengths (1.1–1.7 Å). For these systems we find no compelling evidence for the presence of an attractive hydration pressure, as has recently been proposed by Rand et al. (1988) for phosphatidylethanolamine bilayers.

Now let us consider the charged DPPG bilayers at pH 7.2. For charged DPPG the situation is more complex than for the neutral bilayers, due to the break in the plot of log P vs. d_f at a bilayer separation of ~10 Å (Fig. 4). Without including a term for van der Waals attraction ($H = 0$), the pressure distance curve can be fit by two exponentials, with decay lengths of 12.9 and 0.7 Å (Table 1, fifth line). The following analysis indicates that the exponential with the larger decay length corresponds primarily to electrostatic repulsion, whereas the decay with the smaller decay length probably corresponds to a combination of hydration repulsion and steric hindrance. Let us first consider the region for $d_f > 10$ Å. The decay length in this region is much higher than that normally associated with hydration repulsion, but is quite similar to that expected for electrostatic repulsion. An approximate expression for the electrostatic pressure, P_{es}, between two charged parallel plates is (Israelachvili, 1985)

$$P_{es} = -64 \times 10^3 R \cdot T \cdot C \cdot [\tan h(ze\Psi(0)/4kT)]^2 \exp(-\kappa d_f), \quad (3)$$

where T is temperature, k is the Boltzmann constant, R is the molar gas constant, C is the bulk salt concentration (which in our experiments was 50 mM Na-Phosphate and 14 mM Na-DPPG), e is the elementary charge, $\Psi(0)$ is the electrostatic double layer potential, which is −0.154 V (Marra, 1986), and $(\kappa)^{-1}$ is the Debye length = $(kT\epsilon\epsilon_0/e^2\Sigma z_i^2 c_i)^{1/2}$, where ϵ_0 is the permittivity of free space, ϵ is the dielectric constant of the aqueous phase, and z_i and c_i are the valence and concentration of ion i. For the experimental conditions used in our experiments, this formula gives $P_{es} = 8.9 \times 10^6$ dyn/cm^2 with a Debye length of 9.0 Å. These values are in quite reasonable agreement with the magnitude (P_{es}) and decay length (λ_{es}) of the observed pressure for $d_f > 10$ Å (see Table 1). Thus, there is little question that electrostatic repulsion is the dominant pressure for $d_f > 10$ Å, as previously found by Cowley et al. (1978) and McDaniel and McIntosh (1989). Least squares fits to the data points were also performed with the addition of a van der Waals term. We tried two different values of the Hamaker constant, $H = 1.4 \times 10^{-14}$ ergs, the value obtained for DPPG at pH 0.3 and for DPPG:SA bilayers, and $H = 6.0 \times 10^{-14}$ ergs, the value used by Marra (1986). Inclusion of the van der Waals term lowers the value of the decay constant of the exponential decay associated with electrostatic repulsion (see last two lines of Table 1).

For DPPG at pH 7.2 for $d_f < 10$ Å, the decay length of the repulsion is 0.7–0.8 Å, depending on the value of H selected. These values for λ are less than the 1–2 Å usually measured for hydration pressures for other lipids (Lis et al., 1982; McIntosh and Simon, 1986; Simon et al., 1988; McIntosh et al., 1989*a*, *b*). Moreover, the value of P_0 is much larger than normally obtained for hydration pressures using the plane of origin as defined here (McIntosh and Simon, 1986; Simon et al., 1988; McIntosh et al., 1989*a*). There are at least three reasons to explain the relatively high value of P_0 and low value of λ for charged DPPG compared with other lipid bilayers. First, as detailed in Results, there are uncertainties in estimations of bilayer thickness and fluid separations from the electron density profiles, particularly in the case of DPPG:SA. However, it should be noted that the standard deviations in bilayer thickness are <1 Å and the differences in fluid thickness between DPPG and DPPG:SA at the highest applied pressures ($\log P > 9$) are >3 Å. Moreover, at the highest applied pressure ($\log P = 9.2$) the electron dense head group peaks from apposing bilayers have merged for DPPG:SA (Fig. 3, *C*), but are clearly separated for DPPG (Fig. 3, *A*). Thus, although the experimental uncertainties could well explain some of the observed differences between DPPG and DPPG:SA, they are unlikely to be the only reason for the unusual values for λ and P_0 for charged DPPG. A second possible explanation is that the hydration pressure might be unusual for the charged form of DPPG compared with other lipid systems. There is no direct experimental evidence for or against this possibility. A third, and we think a likely contributing factor, is the onset of steric hindrance to close approach of apposing bilayers at small values of d_f caused by the presence of interlamellar sodium ions. That is, in three-dimensional crystals of Na-DMPG, one Na^+ is associated with every $DPPG^-$ to insure electroneutrality (Pascher et al., 1987). The same electroneutrality conditions must hold for DPPG bilayers in our controlled relative humidity atmospheres because the Na^+ associated with $DPPG^-$ cannot leave the interbilayer space and equilibrate through the vapor phase. Therefore, one cation per DPPG is trapped in the interbilayer space as the bilayers are dehydrated. The diameter

of a dehydrated Na ion is ~2 Å and the $Na^+ - O^-$ contact distance in the Na-DMPG crystal is ~1.5 Å (Pascher et al., 1987). Therefore, a fully or partially hydrated sodium ion occupies an appreciable volume which would result in steric hindrance when two charged DPPG bilayers are forced close together. This could at least partly explain why, for fluid spaces <7 Å, the log P vs. d_f relation for DPPG at pH 7.2 is shifted ~3 Å to the right of the log P vs. d_f relations for DPPG at pH 0.3 and DPPG:SA bilayers (Fig. 4). For electrically neutral DPPG at pH 0.3 or DPPG:SA multilayers, the Na^+ need not be associated with the DPPG as Na^+ and Cl^- (or OH^-) can diffuse together into the bulk aqueous phase or merely precipitate when the salt concentration reaches its saturation limit. This implies that sodium would be trapped in appreciable quantities between charged DPPG bilayers, but not between neutral DPPG at pH 0.3 or between DPPG:SA bilayers. Thus, we would expect that interlamellar sodium would cause much larger steric hindrance to close approach for charged DPPG than for neutral bilayers. We argue that for fluid spaces <7 Å in charged DPPG bilayers, the repulsive pressure could contain contributions from both steric and hydration pressures. Further attempts to separate these contributions would require unsubstantiated assumptions.

In summary, at least two repulsive interactions operate between charged DPPG bilayers. At large bilayer separations, the pressure can be described by classical double layer theory. At small bilayer separations ($d_f < 7$ Å), the pressure-distance curve has a much smaller decay length and is due to hydration pressure, possibly with an additional steric contribution from counterions trapped between apposing bilayers. For DPPG bilayers neutralized by either the addition of a cationic surfactant or by titration at low pH, the entire repulsion can be attributed to hydration pressure whose decay length is similar to that of other neutral and uncharged bilayers.

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