EFFECTS OF MONOVALENT ION BINDING AND SCREENING ON MEASURED ELECTROSTATIC FORCES BETWEEN CHARGED PHOSPHOLIPID BILAYERS

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ABSTRACT In an effort to determine the role that monovalent ions play in the modification of intermembrane forces, we have measured these forces between charged phospholipid bilayers in monovalent ionic solutions. The osmotic stress technique allowed the net electrostatic pressure between the bilayers to be measured while their separation was concurrently determined by x-ray diffraction. Taken together, these measurements yielded electrostatic pressure as a function of bilayer separation. We have related measured pressures to the bilayer surface charge density and surface potential through an exact solution of the full nonlinear Poisson-Boltzmann equation for this system. Quantitative differences in bilayer separation amongst monovalent alkali metal cations indicated differential binding of these to phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidic acid (PA); binding affinity series were determined for Li^+, Na^+, K^+, Cs^+, and TMA^+ ions to these lipids. The anions Cl^-, Br^-, I^-, and CH_3COO^- were found to have no differential effect on the repulsive forces between PS bilayers. Debye lengths for the electric double layer estimated from the slopes of the experimental pressure curves were consistently longer than predicted on the basis of classic Gouy-Chapman theory. Estimates of the van der Waals Hamaker coefficient between bilayers of PS and PG in salt solution were found to be weaker than between phosphatidylcholine bilayers in pure water, a difference possibly due to electromagnetic retardation and ionic screening.

INTRODUCTION

Cell contact phenomena such as cell adhesion and aggregation, fusion at the synaptic junctions, endocytosis, and pinocytosis will depend upon the interaction energies of the membranes in question (McLaughlin, 1977). These interactions involve both long-range forces (electrostatic and van der Waals) acting across intervening aqueous space and short-range specific interactions involving molecular contact. The long-range interactions are thought to be important in influencing the initial approach of interacting membranes (Parsegian, 1973). In fact, recent experimental work has shown that sufficiently strong electrostatic repulsive forces can prevent cell-surface adhesion, although when repulsive forces are reduced, cells adhere because of attractive physical forces (Gingell and Fornes, 1975; Gingell and Todd, 1975; Gingell and Fornes, 1976).

Strong repulsive "hydration" forces that probably govern the establishment of short-range interactions have been measured between phospholipid bilayer, colloid, and mica surfaces at separations of <30 Å (Barclay and Ottewill, 1970; Barclay et al., 1972; LeNeveu et al., 1977; Cowley et al., 1978; Israelachvili and Adams, 1978; Pashley, 1981 *a* and *b*). Long-range electrostatic and van der Waals forces extend many tens of Angstroms from the membrane surfaces. We have used lipid bilayers to study these long-range nonspecific forces experimentally, as they allow the use of a much simpler system than biological membranes, while acting as good models of them.

Although much theoretical work on the electrostatic forces existing between charged planes has been done (Verwey and Overbeek, 1948; Ninham and Parsegian, 1971; Parsegian and Gingell, 1972; McLaughlin, 1977; Radic and Marcelja, 1978, Gruen and Marcelja, 1982), relatively little has been done on the measurement of electrostatic forces in biological systems. Cowley et al. (1978) found that electrostatic repulsive forces between phospholipid bilayers of low surface charge density in water gave results consistent with theory. However, no direct measurements of the electrostatic forces between charged phospholipid bilayers have been made in ionic

solutions, although estimates of the surface potentials of individual lipid vesicles have been made by McLaughlin and co-workers (McLaughlin, 1977; Eisenberg et al., 1979). Concurrent with the present study were measurements of electrostatic forces in muscle and tobacco mosaic virus gels (Millman and Irving, 1980; Millman and Nickel, 1980; Millman, Irving, Nickel, and Loosley-Millman, manuscript in preparation), between various phospholipid bilayers in divalent ionic solutions (Lis et al., 1981), and between mica sheets (Pashley, 1981 *a* and *b*). Although all of the experiments show qualitative agreement with theory, in only some cases is the agreement quantitative.

McLaughlin (1977) gives a thorough review of many different types of experiments that have shown good qualitative agreement with Gouy-Chapman theory. In this theory, the electrostatic field decays with distance from an isolated charged surface with a screening or Debye length proportional to the square root of the ionic strength of the bathing medium (Verwey and Overbeek, 1948). All of these experimental systems have dealt with isolated charged surfaces. Our approach is to use a multibilayer system in which adjacent parallel electric double layers are interacting. We may thus compare the behavior of isolated and interacting double layers.

In this study we have examined electrostatic and van der Waals interactions. Electrostatic forces were measured for bilayers of different charged lipids in a range of monovalent ionic solutions. The Debye length was consistently found to be longer (i.e., the decay of the electrostatic force with distance was slower) than that theoretically predicted by standard double-layer theory. The van der Waals attractive force was estimated for two charged bilayer systems in NaCl solution and the Hamaker coefficient was found to be lower than that previously determined by Parsegian et al. (1979) for a neutral lipid system in pure water at much smaller separations than observed here.

It is our belief that knowledge of the quantitative behavior of these forces, particularly with regard to the effects of the ionic environment on the electrostatic force, will be important in the understanding of membrane interactions.

MATERIALS AND METHODS

Materials

Egg phosphatidylglycerol (PG) was obtained from Lipid Products (North Surrey, England) and Sigma Chemical Co. (St. Louis, MO). Phosphatidylserine (PS), extracted from bovine brain, was purchased from Sigma and from Serdary Research Chemicals (London, Ontario). Phosphatidic acid (PA), isolated from hen eggs, was obtained from Sigma as was dipalmitoylphosphatidic acid (DPPA). All of these lipids were converted to sodium salts using a chloroform:methanol:aqueous solution two-phase system. The purity of all converted lipids as tested by thin layer chromatography was >99%.

Dextran was purchased from the Pharmacia Chemical Company (Piscataway, NJ) (2,000,000 mol wt) and British Drug Houses Ltd. (Poole, Dorset, England) (200,000–270,000 mol wt). Tetramethylammoniumchloride (TMACl) was obtained from the Eastman Kodak Co. (Rochester, NY) and doubly recrystallized from an ethanol:acetone solution. The various salts used were of analytical grade and were obtained from the Fisher Scientific Co. (Waltham, MA). Water used for all samples and solutions was deionized and distilled in glass. Several representative experiments using 0.2 mM EDTA gave results identical to those using pure water.

Preparation of X-ray Samples

Pure lipids were dried down from chloroform solutions, first by a rotary evaporator, and then by vacuum for several hours. They were then stored under N_2. To make a gravimetric sample, 3–15 mg of lipid were weighed into a small glass vial, and the appropriate weight of water added from a fine-tipped pipette. Samples were sealed in the vial, allowed to equilibrate for 3–5 d at 4°C and then transferred to x-ray chambers where they were sealed between mica sheets 1 mm apart.

For lipid samples that were to be equilibrated with excess solution, dry lipid was first slightly hydrated to make a buttery paste, which aided in handling the lipid. A few milligrams of the lipid were then placed either directly in contact with or (whenever necessary) separated by a dialysis membrane from a large excess (at least 100 times) of salt solution, either with or without Dextran, and allowed to equilibrate for 2–5 d at 4°C. For samples equilibrating against a Dextran solution, the refractive index of the solution was determined with a sugar refractometer (Fisher Scientific) after equilibration. The refractive index of all solutions was corrected to compensate for the contribution of salt, to give absolute sugar concentration. The final osmotic pressure of the solution was then determined as described previously (LeNeveu et al., 1976, 1977). Lipid and some solution were transferred to the x-ray sample chamber and sealed between mica windows ~2 mm apart. The pH of all solutions was adjusted to ~7.2, and equilibration with the lipid did not alter this value.

X-ray Diffraction

The lamellar repeat distance of these phospholipid systems was measured at room temperature (21–25°C) by x-ray diffraction using mirror-monochromator, Franks' (single mirror), or toroid cameras. The type of radiation used in all cases was CuK_{α} radiation, λ = 1.540 Å. All lipids except DPPA were above their hydrocarbon chain phase transition temperatures. DPPA was also observed above its transition at 65°C.

The x-ray diffraction reflections give the dimensions of the repeat distance d, of the lamellar phase. For those samples whose composition is known (gravimetrically prepared samples) one can divide this repeat distance into a lipid layer of thickness $d_l = \phi d$ (where ϕ is the volume fraction of lipid equivalent to weight fraction assuming a specific density of 1: such an assumption gives maximum uncertainties in d_l and d_w of 1 or 2 Å [LeNeveu et al., 1977; Parsegian et al., 1979]) and a water layer of thickness $d_w = d - d_l$ (Small, 1967). Thus the bilayer thickness (d_l) and the separation between the bilayers (d_w) can be determined at all concentrations of lipid in water. Once the bilayer becomes fully hydrated, its thickness remains constant (Small, 1967; LeNeveu et al., 1977; Cowley et al., 1978; Loosley-Millman, 1980; Lis et al., 1981). For nongravimetrically prepared samples the bilayer thickness and separation can be equated to those of gravimetric samples of the same dimension (LeNeveu et al., 1977; Parsegian et al., 1979). On the strength of previous experience (Cowley et al., 1978; Lis et al., 1981), we know that the thickness of the lipid bilayer (d_l) does not change significantly when the multilamellar phases are under the weak (osmotic) pressures at which electrostatic forces are observed.

Electrostatic Repulsion and Interbilayer Separation

Dextran in solutions equilibrating against the lipid lamellar phases is prevented from entering the multilamellar array either by the presence of a dialysis membrane or, when in direct contact with the lipid, by virtue of its large size (Loosley-Millman, 1980). When equilibration across a

dialysis membrane is used, great care has been taken to ensure that no hydrostatic pressure builds up across the membrane. Even small hydrostatic pressures can significantly affect the bilayer separation.

The lipid lamellar structure must take up water against the osmotic pressure (P_π) of the Dextran solution. At equilibrium, the chemical potential of the water between the bilayers must be equal to that of the water in the Dextran solution (each being lower than that of pure water), and the net pressure between the bilayers equal to the externally applied osmotic pressure. The net pressure between the bilayers will be the resultant of the attractive van der Waals pressure (P_A) and the repulsive hydration (P_H) and electrostatic pressures (P_{ES}) (Cowley et al., 1978; Loosley-Millman, 1980). At interbilayer separations of 25 Å or more, the hydration pressures in these phospholipid systems are negligible compared with the electrostatic pressure. The van der Waals pressures are also insignificant, except at very large interbilayer separations (Loosley-Millman, 1980). In these experiments at interbilayer separations >25 Å, the osmotic pressure is balanced by the electrostatic pressure alone.

In summary, by combining osmotic stress with x-ray diffraction, the variation of interbilayer repulsive forces with interbilayer separation and ionic environment can be determined. We have called such variation "pressure curves."

THEORETICAL TREATMENT

One of our major objectives was to compare theoretically calculated electrostatic pressure and Debye lengths with our experimentally measured values. Our theoretical calculations of the electrostatic pressure are based on an exact solution of the Poisson-Boltzmann equation which can be found in the appendix. This solution allowed us to generate theoretical curves of electrostatic pressure vs. interlamellar separation for various bilayer surface charge densities and ionic strengths.

RESULTS

Lipid Bilayer and Water Layer Thickness of Lipid Lamellar Phases

X-ray diffraction of the various lipid mixtures gave several sharp reflections, all integral orders of the single repeat distance, d, of the one-dimensional crystal. This verified that the structures of these lipid-water systems were single lamellar phases. Such a structure has been shown to be one of alternating lipid bilayers and water layers (Reiss-Husson, 1967; Small, 1967; Luzzati, 1968; Tardieu et al., 1973). For such phases of PS the variation with lipid concentration of the repeat spacing, d, calculated bilayer thickness, d_l, and interbilayer separation d_w are shown in Fig. 1.

The bilayer thicknesses for the lamellar phases of hydrated PA and PG were determined in the same manner, at lipid concentrations where the bilayer thickness is invariant (51% by weight or less). Bilayer thicknesses were determined to be 38.7 ± 0.5 Å for PS, 33.9 ± 1.1 Å for PG, and 32.6 ± 0.6 Å for PA.

Effects of Ionic Strength on the Electrostatic Repulsive Forces Acting Between Bilayers of PS

Fig. 2 shows bilayer separations for PS multibilayers under constant osmotic stress in NaCl solutions of different ionic strength. Interbilayer separation increases as the ionic strength of NaCl is reduced. Only slight changes in the interbilayer separation are seen in the range of 1.0–0.5 M, whereas decreasing the salt concentration from 0.5 to 0.4 M produces a dramatic increase in separation.

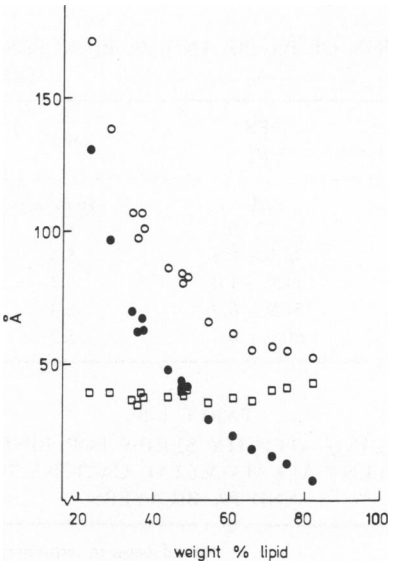

FIGURE 1 Phase diagram for PS prepared in pure water. Lattice repeat distance d (○), bilayer thickness d_l (□), and bilayer separation d_w (●), are plotted as a function of lipid weight fraction in the sample. The weight fraction of the lipid (ϕ) is used to compute the bilayer thickness (d_l) from the measured repeat spacing (d); $d_l = \phi d$. The bilayer separation (d_w) is determined as $d_w = d - d_l$.

To control more finely the PS bilayer separation as the ionic strength is reduced, the lamellar phase was made to swell against an externally applied osmotic pressure P_π, held approximately constant at log P_π(dyn/cm^2) = 5.3 (Dextran concentration of ~10% by weight). Under these conditions the interbilayer repulsive pressure is constant. Fig. 2 depicts the variation of interbilayer separation as the ionic strength of NaCl is decreased. Between 1.0 and 0.5 M NaCl the bilayers separate very slowly upon reduction of the salt concentration. Below 0.5 M the bilayers increase

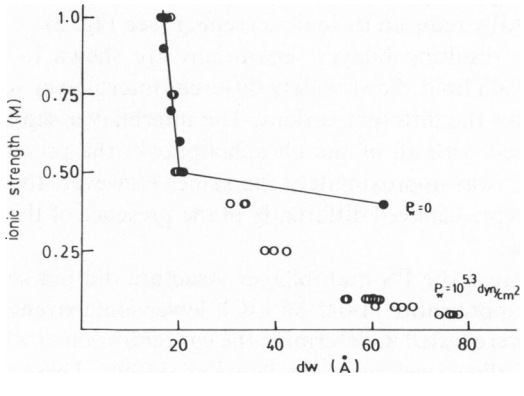

FIGURE 2 Variation in separation of PS bilayers with ionic strength of NaCl at a constant osmotic pressure, log P_π (dyn/cm^2) ~ 5.3 (○), and at zero osmotic pressure (●). (Interbilayer separations for lamellar phases in 0.01 M NaCl under no osmotic pressure were indefinitely large.)

TABLE I A

SEPARATION OF PS, PG AND PA BILAYERS IN 0.2 M CHLORIDE SOLUTIONS OF Li^+, Na^+, K^+, Cs^+, AND TMA^+ AT CONSTANT OSMOTIC PRESSURE

Ion	PS d_w	log P_π	PG d_w	log P_π	PA d_w	log P
(0.2 M)	*(Å)*	*(dyn/cm²)*	*(Å)*	*(dyn/cm²)*	*(Å)*	*(dyn/cm²)*
Li^+	23.5 ± 0.3	5.3	45.5 ± 0.6	5.4	51.8 ± 1.6	5.2
Na^+	46.9 ± 0.5	5.4	46.4 ± 0.7	5.4	51.7 ± 1.5	5.3
K^+	51.0 ± 1.0	5.3	52.3 ± 0.3	5.3	58.3 ± 1.7	5.2
Cs^+	54.9 ± 0.9	5.3	53.6 ± 2.0	5.3	58.4 ± 1.4	5.3
TMA^+	60.6 ± 0.8	5.3	58.6 ± 0.4	5.3	61.2 ± 0.8	5.2

TABLE I B

ION BINDING AFFINITY SERIES FOR BINDING OF MONOVALENT ALKALI METAL CATIONS TO PS, PG, AND PA BILAYERS

Lipid	Ion adsorption sequence
PS	$Li^+ \gg Na^+ > K^+ > Cs^+ > TMA^+$
PG	$Li^+ \simeq Na^+ > K^+ \simeq Cs^+ > TMA^+$
PA	$Li^+ \simeq Na^+ > K^+ \simeq Cs^+ \geq TMA^+$

their separation considerably as the ionic strength is reduced. These qualitative effects of ionic strength are clearly the same whether or not osmotic pressure is applied (Fig. 2). (It may be worth noting that sonicated PS vesicles in NaCl solution are reported to aggregate only above 0.5 M concentrations [Day et al., 1980].)

Modification of Interbilayer Forces by Alkali Metal Cations and TMA^+

The effects of Li^+, Na^+, K^+, Cs^+, and TMA^+ ions in 0.2 M chloride solutions on the forces between bilayers of PS, PG, and PA were investigated by allowing these multilamellar systems to swell against an externally applied osmotic pressure maintained at a value in the range of log P_π(dyn/cm²) = 5.2 - 5.4. Use of 0.2 M solutions at this osmotic pressure allowed observation of the effects of the ions on the bilayer separations where the electrostatic forces are not totally reduced by ionic screening (see Fig. 2).

The resulting bilayer separations are shown in Table I *A*. Each lipid shows widely different interbilayer separations for the different cations. The interbilayer separation observed with all of the phospholipids in the presence of TMA^+ was approximately the same. However, the three lipid types behaved differently in the presence of the other cations.

Because the PS multibilayer structure did not seem to swell appreciably in 0.2 M LiCl, lower ionic strengths of LiCl were tested to determine the concentration at which it would allow swelling of the lamellar system. These results are summarized in Table II. As the concentration of LiCl was reduced from 200 mM, the repeat spacing remained constant, but the sharpness of the x-ray reflections became increasingly diffuse. At ~10 mM LiCl, the lamellar system dispersed completely in the sample chambers. This behavior is qualitatively different from that produced by NaCl on the lamellar phases of this lipid (see Fig. 2).

Effects of Anions on Ionic Screening and Binding

Table III presents the separations between PS bilayers that swelled against a constant osmotic pressure [log P_π(dyn/cm²) = 5.3] in 0.2 M Na^+ solutions of Cl^-, Br^-, I^-, and CH_3COO^-. The bilayers were always separated (within the experimental error) to the same extent regardless of the specific anion present, indicating that there is no detectable difference in the electrostatic repulsive forces between these bilayers.

Interbilayer Pressure as a Function of Bilayer Separation

Pressure curves were obtained for PS bilayers in monovalent chloride solutions of 1.0, 0.4, 0.1, and 0.01 M Na^+ (Fig. 3), and 0.4 M Li^+, Cs^+, and TMA^+ (Fig. 4). Pressure

TABLE II

EFFECTS OF Li^+ AT VARIOUS CONCENTRATIONS On PS MULTIBILAYERS.

Ionic strength (mM)	*d*
	(Å)
200	62.2 ± 0.3 (*s*)
100	61.9 ± 0.5 (*b*)
50	61.2 ± 0.4 (*b*)
40	62.0 ± 0.6 (*b*)
30	63.7 ± 1.1 (*b*)
20	61.8 ± 0.6 (*b*)
10	66.9/dispersed (*d*)
5	all dispersed
1	all dispersed

Reflections are characterized as *s*, sharp; *b*, broad; *d*, very diffuse. All samples were under an approximately constant osmotic pressure [log P_π (dyn/cm²) ≃ 5.25]. The lamellar repeat is expressed in terms of *d* because Li^+ binding may cause a structural change in the bilayer and affect d_1 (Parsegian et al., 1981). To confirm tha the preparation temperature had no effect, some samples in 200 mM Li^+ were equilabrated with PS at 20°C as well as at the 4°C normally used to minimize sample degradation. No difference in *d* was observed.

TABLE III

BILAYER SEPARATIONS FOR PS IN 0.2 M NA^+ SOLUTIONS OF Cl^-, Br^-, I^-, AND CH_3COO^- AT CONSTANT OSMOTIC PRESSURE

Ionic Na^+ solution	d_w
(0.2 M)	*(Å)*
Cl^-	46.9 ± 0.5
Br^-	46.0 ± 0.4
I^-	46.3 ± 0.5
CH_3COO^-	44.4 ± 1.4

log P_π (dyn/cm^2) ≃ 5.4.

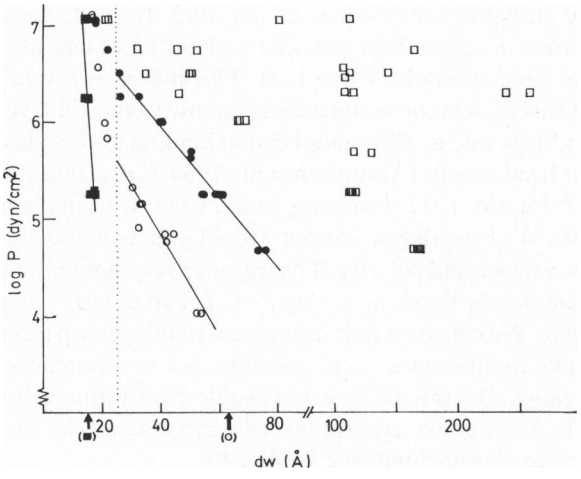

FIGURE 3 Pressure curves for PS bilayers in 1.0 (■), 0.4 (○), 0.1 (●), and 0.01 (□) M NaCl. The arrows represent the separations for PS bilayers in 1.0 and 0.4 M NaCl at $P_\pi = 0$. The solid lines are the hydration pressure curve as determined from a least-squares fit to the 1.0 M data, and least-squares fits to the experimental pressure curves in the electrostatic region. The vertical dotted line represents a boundary: for data at greater d_w the pressure is almost totally electrostatic. Note that the data for 0.01 M NaCl to the right of the break appears on a different scale.

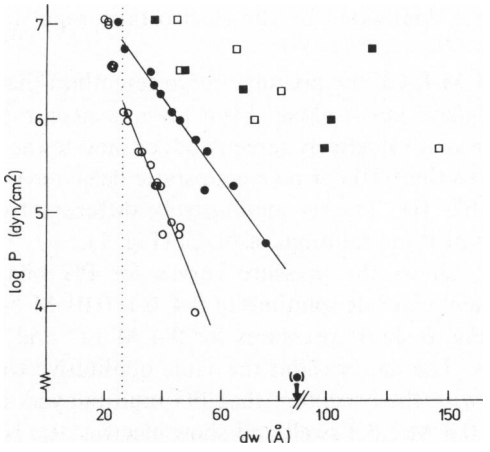

FIGURE 5 Pressure curves for PG bilayers in 0.4 (○), 0.1 (●), 0.01 (□) M NaCl, and in water (■). Solid and dotted lines as in Fig. 3. The arrow represents the separation for PG bilayers in 0.1 M NaCl at $P_\pi = 0$. Note that some of the data for 0.01 M NaCl and water appear on a different scale.

curves with the Cs^+ ion were difficult to obtain because Cs^+ is an efficient x-ray absorber, and only faint diffraction patterns were obtained. In 10 mM NaCl, the lamellar system swells to enormous proportions (Fig. 3). Because of the extremely shallow slope of the pressure curve at this ionic strength, small pressure fluctuations cause large changes in d_w. Hence these results show a good deal of scatter. The lowest ionic strength where consistent results could be obtained with NaCl was 0.1 M.

At bilayer separations of <20 Å, all of the pressure curves are dominated by a sharply exponentially decaying hydration pressure. At lower ionic strengths and bilayer separations >25 Å, the pressure curves decay much more slowly because the hydration pressure is negligible and the

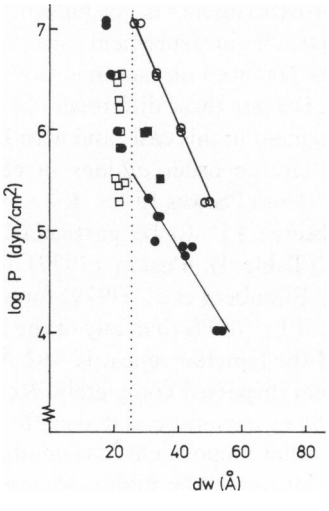

FIGURE 4 Pressure curves for PS bilayers in chloride solutions of 0.4 M Li^+ (□), Cs^+ (■), Na^+ (●), and TMA^+ (○). Solid and dotted lines as in Fig. 3.

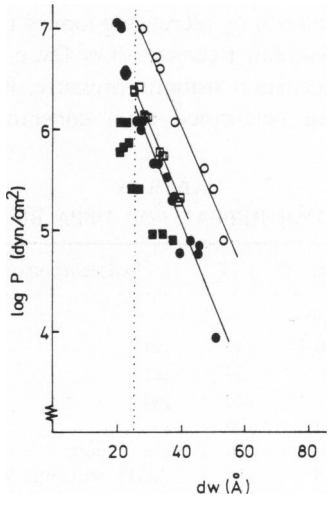

FIGURE 6 Pressure curves for bilayers of PG in 0.4 M chloride solutions of Li^+ (□), Na^+ (●), and TMA^+ (○); and PA bilayers in 0.4 M NaCl (■). Solid and dotted lines as in Fig. 3.

curves are dominated by the electrostatic repulsive pressure.

In 0.4 M LiCl, the pressure curve resembles the hydration repulsive curve alone. Even lower concentrations of LiCl can very effectively screen and/or bind to the bilayer charges so that little or no electrostatic behavior is apparent (Table II). This is qualitatively different from the behavior of PS in solutions of NaCl (Fig. 1).

Fig. 5 shows the pressure curves for PG bilayers in monovalent chloride solutions of 0.4, 0.1, 0.01 M Na^+ and water; Fig. 6 shows pressures for 0.4 M Li^+ and TMA^+ solutions. The data exhibit the same qualitative trends as for PS, with the exception that PG multibilayers in solutions of 0.4 M LiCl swell and show electrostatic behavior typical of PG bilayers in other 0.4 M ionic solutions (Fig. 6).

Fig. 6 also presents results for egg PA in 0.4 M NaCl, which shows swelling like that of PG. DPPA, however, did not show such swelling. Table IV shows typical data indicating that bilayers of DPPA exhibit the swelling behavior of electrostatic repulsion only when their hydrocarbon chains are not in the gel state, as indicated by the high angle x-ray reflection at 4.2 Å. At room temperature, in 0.4 M NaCl and various levels of Dextran, or in water, the multilayers were in an unswelled state. Above their transition temperature the lipids swelled to large dimensions in water and collapsed reversibly again below their transition. This surprising behavior is unlike other gel-state lipids (DPPC and DSPC) that do show electrostatic repulsion when charged by the weak adsorption of divalent ions (Lis et al., 1981).

INTERPRETATION AND DISCUSSION

Ionic Strength Effects

The results shown in Fig. 2 are in qualitative agreement with electric double-layer theory; charged interfaces are increasingly screened by increasing ionic strengths of their aqueous environment. Because all of these measurements were made at constant osmotic pressure, which is tantamount to being performed at a constant electrostatic pressure, reduced screening of the electrostatic field should cause an increase in interbilayer separation. This is exactly what was observed.

TABLE IV
LAMELLAR REPEAT FOR DPPA BILAYERS

log P_π	[NaCl]	T	hydrocarbon chains		d
(dyn/cm²)	*(M)*	*(°C)*			*(Å)*
7.1	0.4	20	gel		58
6.6	0.4	20	gel		59
6.1	0.4	20	gel		57
0	0.4	20	gel		57*
0	0	20	gel	done	55‡
0	0	52	fluid	sequentially	101
0	0	20	gel		55

*Lipid in large excess of solution.
‡Lipid concentration of 39% by weight in water.

Effect of Specific Ions on the Electrostatic Forces

According to classic Gouy-Chapman theory, ions with the same valence should be equally effective in screening the electric double layer. Thus if each of the different ions investigated were only screening the charges on the phospholipid bilayers, the different bilayer systems should equilibrate at the same interbilayer separation for a given ionic strength, independent of ion kind. Instead, a wide variation of separations was seen with different ions at the same ionic strength (Table I *A*). This indicates a significant variation in the surface charge density on the bilayers, most likely due to differential cation binding to the bilayer polar head groups (Abramson et al., 1964; Grasdalen et al., 1977; Puskin, 1977; Eisenberg et al., 1979; Kurland et al., 1979). We have shown, at least with PG, that all ions of the same valence and polarity of charge give very similar decay distances: the slopes of the log P vs. d_w curves in Fig. 6 are similar. We conclude that differences in bilayer separation are due to differences in ion binding, not to differences in screening. On this basis it is possible to determine from Table I *A* an ion adsorption selectivity series for each different phospholipid (see Table I *B*).

The ion adsorption affinity series for PS, $Li^+ >> Na^+ > K^+ > Cs^+ > TMA^+$, is in the order of decreasing hydrated radius. This adsorption series of monovalent ions to PS agrees with that obtained by Eisenberg et al. (1979) from their measurements of the zeta potentials of PS vesicles, made in 0.1 M chloride solutions of the various alkali metal cations. It is difficult to make quantitative comparisons because the relationship between the hydrodynamic plane of shear in the zeta potential measurements and the plane of charge in our experiments is not known. Also, whereas our bilayer repulsion measurements are for interacting bilayers, the zeta potential measurements are for noninteracting surfaces. Despite these differences however, there is qualitative agreement in this case, and with Puskin (1977), who reported that the order of ions in effectiveness of displacing Mn^{2+} from PS was $Li^+ > K^+ = Na^+ = Cs^+ > TMA^+$. We observe Li^+ to be particularly effective in binding to PS (Table I). Puskin (1977) found a much greater binding. Eisenberg et al. (1979) found only slightly greater binding of Li^+ to PS than any of the other ions. For $[Li^+] \geq 10$ mM the lamellar repeat is ~62 Å. For $[Li^+] \leq 5$ mM the system dispersed completely. Recently Hauser and Shipley (1981) have observed specific effects of Li^+ compared with other monovalent ions on dimyristoylphosphatidyl serine bilayers. The highly selective behavior of PS bilayers for Li^+ we observed was not seen with PG or PA bilayers (Table I *B*). This suggests a highly specific interaction between Li^+ and the PS head group.

The observation that the interbilayer separations for PS, PG, and PA bilayers are much the same in the presence of TMA^+ ions lends support to the previous working assumption (Eisenberg et al., 1979) that TMA^+ binds very weakly to these bilayers.

Both cations and anions may bind to neutral lipids (Grasdalen et al., 1977; Eisenberg et al., 1979). The data, however (Table III), clearly indicate that for PS bilayers there are no detectable differences in the interbilayer separation, and hence net surface charge, in the presence of any of the anions investigated.

Experimental Pressure Curves

The pressure curves of these charged bilayer systems are qualitatively similar to those measured previously by Cowley et al. (1978). Below separations of ~25 Å the interbilayer pressure changes exponentially with a rate characteristic of a hydration force. Complete characterization of this force requires compression of the bilayers to very close proximity, 3–5 Å, using hydrostatic and vapour pressure techniques (Parsegian et al., 1979; Rand, 1981). For separations greater than ~25 Å, repulsion falls off much more slowly and is dominated by electrostatic pressure. In this work, we have studied electrostatic repulsion and therefore confine our analysis to separations >25 Å.

The horizontal shifting of the pressure curves with respect to one another for the same ionic strength of different ions (Figs. 4, 6) is consistent with the ion binding behavior we have found (Table I) where ions with the least binding affinity (TMA^+) give the greatest interlamellar separation at any particular osmotic pressure.

The DPPA results (Table IV) were very interesting in that the lipid system clearly did not behave as though charged until the hydrocarbon chains were melted. The reason for this is as yet unclear. Data for egg PA bilayers in 0.4 M NaCl show the same general behavior seen for PS and PG bilayers (Fig. 6).

Calculation of the Hamaker Coefficient

The van der Waals attractive pressure in a multibilayer system can be described in terms of a Hamaker coefficient, A_H, as (Parsegian et al., 1979)

$$P_{\text{van der Waals}} = \frac{-A_H}{6\pi}\left[\frac{1}{d_w^3} - \frac{2}{(d_w + d_l)^3} + \frac{1}{(d_w + 2d_l)^3}\right].$$

Thus, if the van der Waals pressure is known at a particular bilayer separation, the calculation of the Hamaker coefficient is straightforward. For charged lipid bilayers at equilibrium with excess solution (where $P_\pi = 0$), the lamellae are at such great separations that the hydration force is negligible. Thus the van der Waals attractive pressure is equal to the (extrapolated) electrostatic repulsive pressure at the equilibrium separation.

Table V presents the Hamaker coefficients, A_H, determined in this way from the pressure curves of bilayers of PG in 0.1 and PS in 0.4 M NaCl (Figs. 3, 5). As the ionic strength is decreased, the equilibrium separation and its associated uncertainty increase (Loosley-Millman, 1980). Thus, the Hamaker coefficient calculated for PG bilayers in 0.1 M NaCl at 91.2 Å separation has a larger uncertainty than that calculated for PS bilayers in 0.4 M NaCl at 62.6 Å separation. The values of A_H estimated here (Table V) are lower than those reported for egg lecithin (Parsegian et al., 1979).

TABLE V
CALCULATED HAMAKER COEFFICIENTS FOR PS, PG AND PC BILAYERS.

Lipid	Ionic solution	$d_w \pm$ SD	A_H
		(Å)	*(erg)*
PS	0.4 M NaCl	62.6 ± 3.5	$2.8 - 7.1\ (\times 10^{-15})$
PG	0.1 M NaCl	91.2 ± 16.4	$1.3 - 38\ (\times 10^{-15})$
PC*	Water	27.5 ± 0.5	$51 - 69\ (\times 10^{-15})$

The range in A_H is given by one standard deviation of the data.
*from Parsegian et al., (1979).

The lower values lend some experimental support to theoretical predictions that the Hamaker coefficient decreases with both increased separation and ionic strength (Parsegian, 1975; Mahanty and Ninham, 1976). If experimental scatter were reduced, then one could determine the Hamaker coefficient both as a function of ionic strength and separation. However, given the scatter in the present data and the anomalous decay of electrostatic forces used to determine the coefficient, the A_H estimates in Table V should be taken only as a qualitative indication of weaker attraction between widely separated PS or PG bilayers in NaCl solution compared with that between closely spaced PC bilayers in pure water.

Experimental and Theoretical Electrostatic Pressure Curves Compared

In the Debye-Hückel approximation, the electrostatic potential can be written as: $\psi(x) = \psi_0 \exp(-\kappa x)$, where

TABLE VI
COMPARISON OF THEORETICAL AND EXPERIMENTAL DEBYE LENGTHS FOR PS AND PG BILAYERS IN THE PRESENCE OF DIFFERENT MONOVALENT CATIONS

Lipid	Ionic Solution	$1/\kappa_{\text{Debye}}$	$1/\kappa_{\text{experimental}}$
	(M)	*(Å)*	*(Å)*
PS	0.4 NaCl	4.8	$8.4\ (= 1.8/\kappa_D)$
PS	0.4 TMACl	4.8	$5.4\ (= 1.1/\kappa_D)$
PS	0.1 NaCl	9.7	$11.8\ (= 1.2/\kappa_D)$
PG	0.4 NaCl	4.8	$5.5\ (= 1.1/\kappa_D)$
PG	0.4 LiCl	4.8	$5.7\ (= 1.2/\kappa_D)$
PG	0.4 TMACl	4.8	$5.9\ (= 1.2/\kappa_D)$
PG	0.1 NaCl	9.7	$10.1\ (= 1.0/\kappa_D)$

$\kappa = [(8\pi ne^2)/(\epsilon kT)]^{1/2}$ (Verwey and Overbeek, 1948). We speak of $1/\kappa$, the effective thickness of the electric double layer, as the Debye length or screening length. The membranes we use are highly charged, and hence we cannot use this linear approximation. Far away from the membrane surface the decay of the electrostatic pressure will be exponential, and it is from this region ($d_w > 25$ Å) that the Debye lengths shown in Table VI were calculated from best-fit slopes to the pressure curves.

The experimentally determined Debye lengths are consistently longer than theoretically predicted. Although some theoretical values are within experimental error, interacting bilayers of PS in Na^+ solutions showed Debye lengths that were considerably greater than predicted. Debye lengths longer than those theoretically predicted have also been discovered in recent experiments with phospholipid bilayers interacting in divalent cationic solutions (Lis et al., 1981).

Longer-than-expected decay lengths were reported by Israelachvili and Adams (1978) for electrostatic forces between mica sheets in KNO_3 solutions. In contrast, they reported Debye lengths shorter than predicted between mica sheets in $BaCl_2$ and $Ca(NO_3)_2$. Pashley (1981 *a* and *b*) obtained good accuracy using double-layer theory at millimolar concentrations to describe repulsion between mica sheets immersed in millimolar alkali halide solutions. Similarly Pashley and Israelachvili (1982) found the theory to be accurate up to 1 mM concentration for mica in Mg^{++}, Ca^{++}, Sr^{++}, and Ba^{++} chloride solutions.

That the measured and theoretical screening lengths are in some cases different is not entirely surprising. Gouy-Chapman theory was developed for a maximum ionic strength of 0.01 M. Millman and Nickel (1980) have shown that even at 0.1 M ionic strength the correction to the Debye length is probably <5% (a correction that our data cannot distinguish). Higher ionic strengths, nonuniform surface charge, ion binding properties of the surface, and the energy required to establish gradients in electric polarization (Radic and Marcelja, 1978; Gruen and Marcelja, 1982) could all contribute to deviations from predicted Debye lengths.

Figs. 7 and 8 provide a comparison of the experimental pressure curves for PS, PG, and PA bilayers with the theoretical electrostatic pressure curves obtained at the constant surface charge density indicated, and as outlined in the Appendix. The area per phospholipid head group (and hence area per charge assuming complete dissociation) for the bilayers in water has been calculated (Loosley-Millman, 1980) to be ~66 Å^2 for PS, 75 Å^2 for PG, and 72 Å^2 for PA. Because the theoretical curves show little difference for charge densities of 1 e/70 Å^2 and 1 e/100 Å^2, these curves can be used to test the behavior predicted for electrostatic repulsion. To give some idea of the relative insensitivity to charge density of the pressure curves at these high surface charge densities, curves are also plotted for e/1 Å^2 and e/500 Å^2. The plot of the theoretical van der

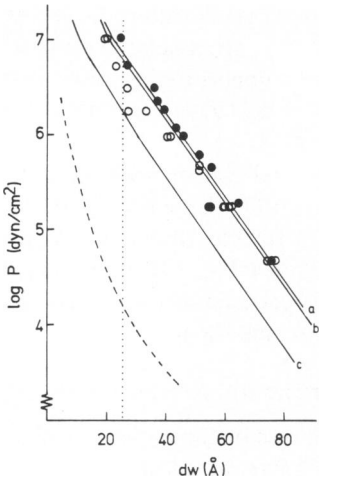

FIGURE 7 Theoretical electrostatic curves *a*, e/70 Å^2; *b*, e/100 Å^2; *c*, e/500 Å^2; and experimental net pressure points for PS (○) and PG (●) bilayers in 0.1 M NaCl. The van der Waals pressure (---) is plotted for a bilayer of thickness 33.9 Å (PG) with a Hamaker coefficient of 6×10^{-15} erg. Dotted line as in Fig. 3.

Waals pressure in Fig. 7, using the equation and Hamaker coefficient found above, shows that the van der Waals attraction is negligibly weak compared with the electrostatic force at the bilayer separations observed. Only the data for PG bilayers in 0.1 M NaCl show good agreement with the theoretical electrostatic pressure curve for a surface charge density of e/70 Å^2. The slopes shown by the rest of the data are different from the electrostatic curves, (as was previously indicated by the disagreement of experimental with theoretical $1/\kappa$), and some of the data (PS and PG in 0.4 M TMACl, Fig. 8) lie in theoretically unallowed regions of the graph. These unallowed regions arise because the nonlinear Poisson-Boltzmann equation pre-

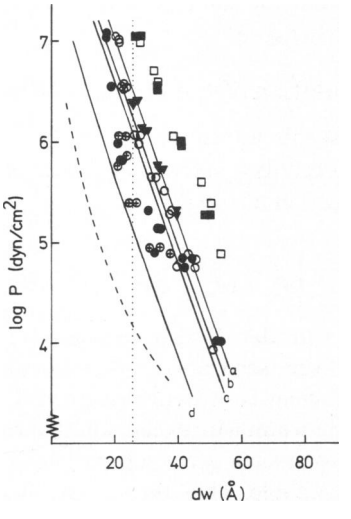

FIGURE 8 Theoretical electrostatic curves *a*, e/1 Å^2; *b*, e/70 Å^2; *c*, e/100 Å^2; *d*, e/500 Å^2; and experimental net pressure points for PS, PG, and PA bilayers in 0.4 M chloride solutions. PS (Na^+ ●, TMA^+ ■); PG (Na^+ ○, Li^+ ▼, TMA^+ □); PA (Na^+ ⊕). Dashed and dotted curves as in Fig. 7.

dicts that the pressure will reach a maximum as surface charge density $\rightarrow\infty$. Hence the pressure is quite insensitive to surface charge density in the experimental region. It is therefore of little value to use the data to calculate actual surface charge densities.

The theoretical calculations have been made assuming the surface charge lies on a hypothetical plane separating lipid and water. Clearly the lipid-water boundary is less definite as water must penetrate the polar-group layer (Büldt et al., 1979). The head group charges presumably lie somewhere in that layer, and consequently the charge plane, if it is a plane, would reside at lower bilayer separations than those given by d_w. The effect of this would be to shift all of the pressure data horizontally to lower separations. Such a shift could place all of the data points of Fig. 8 into a theoretically allowed region. It is interesting to note that the shift required to get to the boundary of the allowed region becomes progressively greater as the ions become larger. This very likely reflects the assumptions made in the theory regarding finite ion size. The maximum shift, required by TMA^+, is 12 Å, which amounts to assuming that the charge plane is 6 Å further out from the plane of the membrane surface as defined by our convention that all the lipid molecules but only the lipid molecules occupy a layer of thickness d_l.

Although shifting the data points on the d_w axis by changing the location of the charge plane can help to reconcile the experimental data with expected electrostatic pressures, it does not change the slope of the data and the derived Debye length. Such shallower slopes would be anticipated if the surface potential remained constant and surface charge decreased as the bilayers were pushed together. However, our calculations (Loosley-Millman, 1980) show that this slope is still steeper than that observed, and so this cannot account for the disparity in the Debye lengths. Again, our calculations of surface pH (Loosley-Millman, 1980) show that the minimum pH value is well above the pK of the various lipid head groups used in this study. Thus it is unlikely that this has any effect on the ionizable groups, and hence on the pressure curves.

Radic and Marcelja (1978) and Gruen and Marcelja (1982) have shown that consideration of both the effects of finite ion size and solution structure near the charged bilayer surface will lead to a renormalized $1/\kappa$, which is increased relative to the classic Debye length, $1/\kappa_D$. One can think of the finite ion size effect as deriving from short-range forces that try to keep ion density uniform, and thus oppose screening of the membrane charge near the interface. The factor that leads to the more dramatic renormalization of $1/\kappa_D$ is brought about by the strong gradients in polarization of solvent near a charged surface. The dielectric polarization in this case will reflect the nonlocal response of the solvent in a rapidly varying electric field. Its effect is thus the greatest when ionic densities and electric field gradients are high. The renormalization suggested by Marcelja and co-workers (Radic and Marcelja, 1978; and Gruen and Marcelja, 1982) does yield corrections to the Debye length of the magnitude needed here. Because their actual equations are not intended for use at our high ionic strengths (D. W. R. Gruen, personal communication), quantitative comparison is not practical between present theory and measurement. Nevertheless, it is our expectation that the above-mentioned renormalization is probably responsible for most of the observed deviations from classic double-layer theory.

Differences seen with different ionic solutions of the same concentration and valence or with different phospholipids in the same ionic solution must, of course, still be attributed to ion- or lipid-specific phenomena such as ionic binding or hydration. Such for example, must be true of TMACl vs. NaCl with PS or PS vs. PG in 0.1 M NaCl.

To go further, one may consider the data from two perspectives. One is to assume still the validity of the older theory and ascribe all deviant force decay to changes in surface charge, potential and ionic binding. The other is to take the observed exponential decays as characteristic of the intervening ionic medium and to assume that surface charge, potential and ion binding are fixed when charged membrane surfaces are brought together.

Following the first method, Fig. 9 shows the variation of the surface potential, calculated from the classic theory with d_w for PS bilayers in 0.1 and 0.4 M NaCl. It can be seen from this figure that the surface potential might actually decrease in magnitude as the bilayers are pushed together. This is just the opposite of what should happen if the surface charge density remained constant. The slow decay in electrostatic repulsion would occur because Na^+ ions are increasingly bound as the bilayers are forced

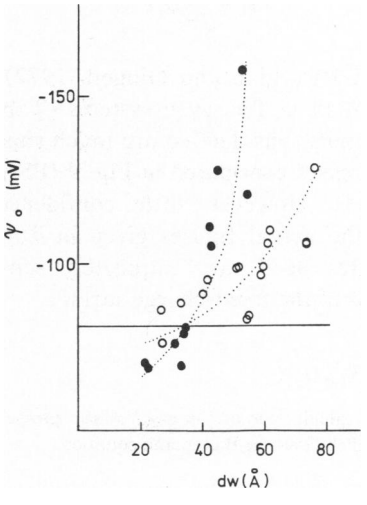

FIGURE 9 Theoretically determined surface potentials for pressure data points vs. interlamellar separation for bilayers of PS in 0.4 (●) and 0.1 (○) M NaCl. The solid line represents the surface potential for (isolated) PS bilayer surfaces in 0.1 M NaCl as determined by Eisenberg et al., (1979).

together, decreasing both surface potential and surface charge density. These results are like that of Lis et al. (1981), who reported desorption of Ca^{++} ions as positively charged bilayers of DPPC-Ca^{++} were forced together. The binding coefficient of ions to charged bilayers might increase as bilayers approach one another, possibly as a result of their electric fields perturbing the conformation of neighboring polar groups. Ions can induce changes in phosphatidylcholine head group conformation in bilayers (Brown and Seelig, 1977); conversely an induced change in conformation could alter ion binding.

Eisenberg et al. (1979) have used classic theory to interpret zeta potentials obtained for isolated charged lipid vesicles in monovalent ionic solutions. It is difficult to make direct comparisons with their results because of the relative insensitivity to surface charge density and therefore surface potential in our experimental region. Although we cannot make a good estimate of the magnitude of these potentials, they appear to be changing (Fig. 9) as the bilayers vary their separation. In systems of lower surface charge density, where surface potentials can be reliably estimated, the surface potential also appears to decrease as bilayers approach each other. (Lis et al., 1981). It might be noted that while our data cannot be used to get a good estimate of the surface charge density, it does give a very accurate measure of the minimum possible distance of the charge plane (polar head groups) from the middle of the bilayer.

Following the second scenario, we imagine that the exponential decay in Figs. 3–6 can be described by a linearized Poisson-Boltzmann equation, with the effective Debye length (or effective ionic strength) found experimentally. For separations much greater than $1/\kappa$, we write

$$P = \frac{\epsilon\kappa^2}{2\pi}\psi_s^2 e^{-\kappa d_w}$$

(Eq. 35 from Parsegian and Gingell, 1972) and extract surface potentials ψ_s for seven systems (Table VII). The surface potentials thus derived are much smaller than the ψ_0 in the two cases considered in Fig. 9 (PS in 0.1 M and 0.4 M NaCl). However, little confidence should be attached to the actual figures given in Table VII since these estimates of ψ_s are exquisitely sensitive to the assumed locus of the fixed-charge surface.

TABLE VII
SURFACE POTENTIALS INFERRED USING EFFECTIVE SCREENING LENGTHS

Lipid	Ionic solution	$1/\kappa_{experimental}$	ψ_s
	(M)		*(mV)*
PS	0.1 NaCl	11.8	49.7
PG	0.1 NaCl	10.1	85
PS	0.4 NaCl	8.4	18.8
PS	0.4 TMACl	5.4	143.5
PG	0.4 NaCl	5.5	54.9
PG	0.4 TMACl	5.9	157.
PG	0.4 LiCl	5.7	72.6

APPENDIX

Our theoretical calculations of the electrostatic pressure are based on exact solutions of the Poisson-Boltzmann equation

$$\frac{d^2\psi}{dx^2} = -\frac{4\pi/e}{\epsilon}\sum_{(n_i^0)} z_i n_i^0 e^{-z_i e\psi/kT} \qquad \text{(A1)}$$

for a region of uniform dielectric between two negatively charged bilayers of a fixed surface charge, bathed by monovalent ions. The ions are assumed to move in an average electrostatic potential ψ, but are otherwise noninteracting. $z_i e$ specifies their charge and n_i^0 their number density, in the reservoir.

These calculations are similar to those of Ninham and Parsegian (1971) and of Lis et al. (1981), but are specialized to the case of monovalent:monovalent solutions.

Using the dimensionless potential, $y = e\psi/kT$, to simplify the expression, we can obtain the first derivative:

$$\frac{dy}{dx} = \pm\left[\frac{8\pi e^2}{\epsilon kT}\left(\sum_{(n_i^0)} n_i^0 e^{-z_i y} + C\right)\right]^{1/2} \qquad \text{(A2)}$$

where C is a constant of integration. What we need to do is relate the potential to the interbilayer separation, as this is a quantity we can measure experimentally. We can redefine the potential as $y = (e\psi)/(kT) - (e\psi_{midpoint})/(kT)$, which sets $y_{midpoint} = 0$ (Fig. 10). The expression can now be written as

$$\frac{dy}{dx} = \pm\left[\frac{8\pi ne^2}{\epsilon kT}\left(\frac{e^{-y}}{z} + e^{y}z + C\right)\right]^{1/2} \qquad \text{(A3)}$$

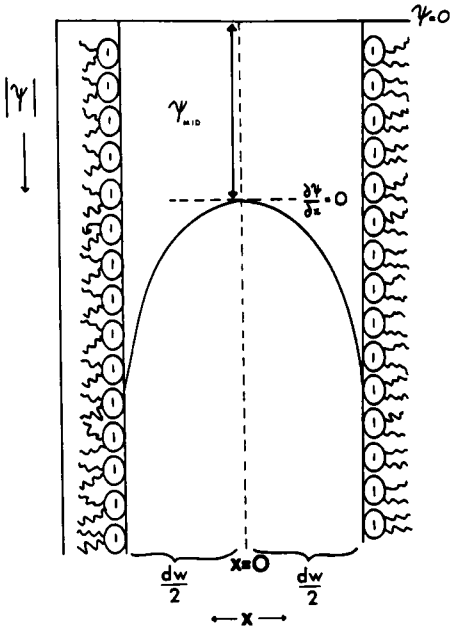

FIGURE 10 Schematic illustration of the system of two negatively charged bilayers with an electrostatic potential ψ. At $x = 0$, the midpoint, $(d\psi)/(dx) = 0$ between two parallel bilayers of equal charge. The bulk or reservoir potential is taken to be zero.

where $z = e^{(e\psi_{mid}/kT)}$, and $n_+^0 = n_-^0 = n$ by electroneutrality. Substituting $\kappa^2 = (8\pi ne^2)/(\epsilon kT)$, and finding C from $(dy/dx) = 0$ at the midpoint (Fig. 10) we have the complete expression for the first derivative of the potential:

$$\frac{dy}{dx} = \pm \kappa \left[e^y z + \frac{e^{-y}}{z} - \left(z + \frac{1}{z} \right) \right]^{1/2}. \quad \text{(A4)}$$

The expression can now be further simplified by setting $\theta = e^y$. Because y is always negative for the case of negatively charged bilayers, $0 \leq \theta \leq 1$. Hence we obtain

$$(d\theta)/(dx) = \pm \kappa \left\{ \theta \left[z\theta^2 + \frac{1}{z} - \theta \left(z + \frac{1}{z} \right) \right] \right\}^{1/2}. \quad \text{(A5)}$$

To find θ, or the potential, it is necessary to integrate the expression (A5)

$$\int_1^X \frac{d\theta}{\left\{ \theta \left[z\theta^2 + \frac{1}{z} - \theta \left(z + \frac{1}{z} \right) \right] \right\}^{1/2}} = \pm \int_0^{d_w/2} \kappa dx \quad \text{(A6)}$$

where the integration is from $\theta = 1$ at $x = 0$, the midpoint, to $\theta = X$ at $x = d_w/2$, the bilayer surface. After factoring out and choosing signs, the integral becomes

$$\int_1^X \frac{(d\theta)}{\left\{ \left[\theta (\theta - 1) \left(z\theta - \frac{1}{z} \right) \right]^{1/2} \right\}} = - \int_0^{d_w/2} \kappa dx. \quad \text{(A7)}$$

Note that $0 \leq \theta \leq 1$, so $X < 1$. Substituting $\theta = g^2$, $d\theta = 2gdg$, we can put this integral into the form of an elliptic integral of the first order (Abramowitz and Stegun, 1964):

$$\int_1^{\sqrt{X}} \frac{dg}{[(1 - g^2)(1 - z^2g^2)]^{1/2}} = - \int_0^{d_w/2} \frac{\kappa dx}{2\sqrt{z}} \quad \text{(A8)}$$

However, we now note that $\int_1^X = \int_0^X - \int_0^1$ where

$$\int_0^1 \frac{dg}{[(1 - g^2)(1 - z^2g^2)]^{1/2}} = K(z) \quad \text{(A9)}$$

by definition of an elliptic integral of the first kind (Abramowitz and Stegun, 1964).

Because our integral in g is not a complete integral, nor are its values tabulated for our region of interest, it is necessary to perform the following steps to find the value of X, and hence the potential. The value of the elliptic integral of the first kind can also be expressed as an inverse Jacobian elliptic function. That is, $u = sn^{-1}(a)$ or $a = sn(u)$ (Abramowitz and Stegun, 1964). Thus

$$u = \int_1^{\sqrt{X}} \frac{dg}{[(1 - g^2)(1 - z^2g^2)]^{1/2}} = sn^{-1}(\sqrt{X}, z). \quad \text{(A10)}$$

From the above, then,

$$\int_0^{\sqrt{X}} = \int_1^{\sqrt{X}} - \int_0^1$$

$$sn^{-1}(\sqrt{X}, z) = - \int_0^{d_w/2} \frac{\kappa dx}{2\sqrt{z}} + K(z)$$

$$\sqrt{X} = sn \left[- \frac{\kappa d_w}{4\sqrt{z}} + K(z); z \right]$$

$$sn[v + K(z); z] = cd(v; z) = \frac{cn(v; z)}{dn(v; z)}$$

so that

$$\sqrt{X} = \frac{cn\left(- \frac{\kappa d_w}{4\sqrt{z}}; z \right)}{dn\left(- \frac{\kappa d_w}{4\sqrt{z}}; z \right)} = g.$$

The parameter g can then be evaluated by using a Landen series (Abramowitz and Stegun, 1964). When g is known, the potential at the bilayer membrane surface as well as the surface charge density of the bilayer can be found.

An independent test for the values of the potential and surface charge density given by this treatment is taken from Gouy-Chapman theory (Verwey and Overbeek, 1948). In the limit of large separations, each bilayer should act like a single diffuse double layer, but as the bilayers approach one another contributions from the electric fields of both bilayers will produce larger surface potentials than seen for one diffuse double layer alone. Thus in the limit of large separations $\psi_{midpoint} \rightarrow 0$.

Tests were done to compare the potential equation we used with the overlap of two double layers derived in the limit of large separations. For separations such that $\psi_{midpoint} < 1$ mV, the agreement was always to within 1% or better.

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