Blackburn, M. N., Chirgwin, J. M., James, G. T., Kempe, T. D., Parsons, T. F., Register, A. M., Schnackerz, K. D., & Noltmann, E. A. (1972) *J. Biol. Chem. 247*, 1170–1179.
Bray, G. A. (1960) *Anal. Biochem. 1*, 279–285.
Bruch, P., Schnackerz, K. D., & Gracy, R. W. (1976) *Eur. J. Biochem. 68*, 153–158.
Burton, P. M., & Waley, S. G. (1966) *Biochem. J. 100*, 702–710.
Chatterjee, G. C., & Noltmann, E. A. (1967) *Eur. J. Biochem. 2*, 9–18.
Chirgwin, J. M. (1974) Ph.D. Dissertation, University of California, Riverside.
Chirgwin, J. M., & Noltmann, E. A. (1975) *J. Biol. Chem. 250*, 7272–7276.
Coulson, A. F. W., Knowles, J. R., Priddle, J. D., & Offord, R. E. (1970) *Nature (London) 227*, 180–181.
Dyson, J. E. D., & Noltmann, E. A. (1968) *J. Biol. Chem. 243*, 1401–1414.
Fischer, H. O. L. (1960) *Neuere Methoden der organischen Chemie*, Vol. 2, pp 173–207, Verlag Chemie, Weinheim.
Gibson, D. R., Talent, J. M., & Gracy, R. W. (1977) *Biochem. Biophys. Res. Commun. 78*, 1241–1246.
Hanes, C. S., & Isherwood, F. A. (1949) *Nature (London) 164*, 1107–1112.
Hartman, F. C. (1970a) *Biochemistry 9*, 1776–1782.
Hartman, F. C. (1970b) *J. Am. Chem. Soc. 92*, 2170–2172.
Hartman, F. C., Suli, B., Welch, M. H., & Barker, R. (1973) *J. Biol. Chem. 248*, 8233–8239.
Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.
Lartillot, S., & Baron, C. (1964) *Bull. Soc. Chim. Fr.* 783–786.
Mehlitz, A., Gierschner, K., & Minas, T. (1963) *Chem.-Ztg. 87*, 573–576.
Meloche, H. P. (1967) *Biochemistry 6*, 2273–2280.
Micovic, V. M., & Mihailovic, M. L. J. (1953) *J. Org. Chem. 18*, 1190–1200.
Moore, J. A., & Reed, D. E. (1961) *Org. Synth. 41*, 16–20.
Noltmann, E. A. (1966) *Methods Enzymol. 9*, 557–565.
Noltmann, E. A. (1972) *Enzymes, 3rd Ed. 6*, 271–354.
O'Connell, E. L., & Rose, I. A. (1973) *J. Biol. Chem. 248*, 2225–2231.
Phillips, T. L., & Gracy, R. W. (1976) *Biochim. Biophys. Acta 429*, 624–628.
Pon, N. G., Schnackerz, K. D., Blackburn, M. N., Chatterjee, G. C., & Noltmann, E. A. (1970) *Biochemistry 9*, 1506–1514.
Rose, I. A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 491–517.
Schnackerz, K. D., & Noltmann, E. A. (1970) *J. Biol. Chem. 245*, 6417–6423.
Schnackerz, K. D., & Noltmann, E. A. (1971) *Biochemistry 10*, 4837–4843.
Schnackerz, K. D., Chirgwin, J. M., & Noltmann, E. A. (1977) *Z. Physiol. Chem. 358*, 1276.
Shaw, E. (1970) *Enzymes, 3rd Ed. 1*, 91–146.
Shaw, P. J., & Muirhead, H. (1976) *FEBS Lett. 65*, 50–55.
Visser, L., Sigman, D. S., & Blout, E. R. (1971) *Biochemistry 10*, 735–742.

Binding of Divalent Cations to Dipalmitoylphosphatidylcholine Bilayers and Its Effect on Bilayer Interaction†

L. J. Lis,‡ V. A. Parsegian,* and R. P. Rand

ABSTRACT: We have confirmed that $CaCl_2$ swells the multilayer lattice formed by dipalmitoylphosphatidylcholine (DPPC) in an aqueous solution. Specifically, at room temperature 1 mM $CaCl_2$ causes these lipid bilayers to increase their separation, d_w, from 19 Å in pure water to >90 Å. $CaCl_2$ concentrations >40 mM cause less swelling. We have measured the net repulsive force between the bilayers in 30 mM $CaCl_2$ at T = 25 °C (below the acyl chain freezing temperature). For interbilayer separations between 30 and 90 Å, the dominant repulsion between bilayers is probably electrostatic; Ca^{2+} binds to DPPC lecithin bilayers, imparting a charge to them. The addition of NaCl to $CaCl_2$ solutions decreases this repulsion. For d_w < 20 Å, the bilayer repulsion appears to be dominated by the "hydration forces" observed previously between both neutral and charged phospholipids. From the electrostatic repulsive force, we estimate the extent of Ca^{2+} binding to the bilayer surface. The desorption of bound Ca^{2+}, apparent when bilayers are pushed together, is more rapid than one would expect if an association constant governed Ca^{2+} binding. The association affinity does not appear to be a fixed quantity but rather a sensitive function of ionic strength and bilayer separation.

There is a great deal of curiosity about the extent and kind of interaction between mobile ions and the components of biological membranes. Among the more accessible of these interactions is the affinity of Ca^{2+} and related alkaline earth ions for the zwitterionic phospholipid lecithin.[1] Electrophoretic studies by Bangham & Dawson (1962) and McLaughlin et al. (1978) have shown that the presence of Ca^{2+} in the bathing medium induces a positive charge on lecithin vesicles. Several NMR studies indicate Ca^{2+} binding to egg lecithin (McLaughlin et al., 1978; Hauser et al., 1975, 1977; Hutton et al., 1977; Grasdalen et al., 1977). Structural consequences of alkaline earth ion binding to lecithins were detected by Inoko

† From Brock University, St. Catharines, Ontario, Canada L2S 3A1. *Received October 17, 1979.* This work was supported in part by Grant No. A4920 to R.P.R. from the Natural Sciences and Engineering Research Council of Canada. L.J.L. gratefully acknowledges the grant of a postdoctoral fellowship from Muscular Dystrophy of Canada.

* Address correspondence to this author at the Physical Sciences Laboratory, Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD 20205.
‡ Present address: Illinois Institute of Technology, Chicago, IL 60616.

[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; Ca^{2+}, calcium ion; T_m, gel to liquid crystal transition temperature.

0006-2960/81/0420-1761$01.25/0

et al. (1975), who found that DPPC bilayers immersed in a 1 mM $CaCl_2$ solution would separate indefinitely rather than remain at the limited separation that these bilayers maintain in an excess amount of pure water. Higher concentrations of $CaCl_2$ caused a progressive decrease in bilayer separation; this decrease was attributed (Inoko et al., 1975; Ohshima & Mitsui, 1978) to the ionic screening of electrostatic repulsion between bilayers charged by the adsorption of divalent cations.

In this work we have repeated these observations on bilayer separation and then gone on to measure directly the repulsive forces between dipalmitoylphosphatidylcholine (DPPC) bilayers. From the repulsive force and bilayer separation, we have inferred the density of charges adhering to the bilayer surface as well as the electrostatic potential conferred by that charge. The main features of Ca^{2+} binding by lecithins in 30 mM $CaCl_2$ solutions stand out clearly: (1) Binding is such that in the presence of 100 mM NaCl and 30 mM $CaCl_2$ as many as one in twenty phospholipids bears Ca^{2+} ion. (2) The association of Ca^{2+} with lecithin cannot be described either in terms of an "association constant" or a characteristic surface potential. (3) The association affinity for Ca^{2+} goes down so rapidly as two bilayer surfaces approach that the electrostatic potential actually decreases at the assumed position of Ca^{2+} binding. (4) The presence of NaCl increases apparent Ca^{2+} binding affinity while it decreases the net bilayer repulsion. (5) Surface potentials inferred from bilayer repulsion in 100 mM NaCl and 30 mM $CaCl_2$ are consistent with those inferred from ζ potential measurements. (6) Ca^{2+} has no effect on the hydration forces that maintain lecithin bilayer separation in pure water. The use of ion association constants must therefore always include a very strict delineation of the ionic conditions under which the determination was made.

Since the influence of Ca^{2+} on bilayer forces is best seen at 30 mM $CaCl_2$, we used that concentration in the measurements reported here. DPPC exhibits qualitatively similar but quantitatively different behavior at other Ca^{2+} concentrations and when exposed to other divalent cations. In the next paper (Lis et al., 1981) we report similar behavior for a different set of divalent cations, phosphatidylcholines, and salt concentrations.

Materials and Methods

Experimental

DPPC was obtained from Serdary Research Chemicals (London, Ontario, Canada), Sigma Chemical Co. (St. Louis, MO), and Dr. D. Papahadjopoulos (Roswell Park Memorial Institute). Some lipid obtained from Serdary was further purified by silicic acid column chromatography. DPPC was also synthesized in this laboratory from glycerophosphorylcholine, isolated from egg lecithin (Sigma) by using the method of Chadha (1970), with palmitic acid (Fluka), added by the method of Cubero-Robles & van den Berg (1969). All five sources provided lipid which was chromatographically pure; the DPPC synthesized in our laboratory had <1% impurity. All lipids gave identical results. Dextran of approximate molecular weight 2 000 000 was obtained from Pharmacia (Sweden). Water was doubly distilled and $CaCl_2$ was of reagent grade.

To extract force vs. distance curves, we follow the method of earlier related measurements (Rand & Luzzati, 1968; LeNeveu et al., 1976, 1977; Parsegian et al., 1978) to obtain two sets of X-ray data. In the first we determine the multilayer lattice repeat spacing as a function of lipid concentration and in the second the lattice repeat of the lipid in equilibrium with dextran solutions. The repeat spacing d and lipid concentration provide (1) the cross-sectional area per lipid molecule A from

$$Ad = 2(v_l + v_w)$$

where $2(v_l + \delta_w)$ is the volume filled by two phospholipid molecules plus the volume of water apportioned to these two phospholipids, (2) the lipid bilayer thickness

$$d_l = 2v_l/A$$

where v_l is the volume of a single phospholipid molecule, and (3) the bilayer separation

$$d_w = d - d_l$$

Determination of A requires only the repeat spacing, weight composition, and mean density for the entire multilayer lattice. The lipid molecular volume v_l used in finding bilayer thickness d_l may include the lipid molecule alone or, also, some water molecules considered to be incorporated into the bilayer. For the present we have neglected small (5%) differences (Tardieu et al., 1973) in specific volume of the total phosphatidylcholine and the aqueous solution.

X-ray samples of DPPC in various $CaCl_2$ solutions were prepared by adding gravimetrically known amounts of solution to the dry lipid in small weighing bottles and sealing them for 2 days before mounting for X-ray measurements. Because of the high surface to volume ratio of the sealed sample holders, we were concerned lest ions from mounted samples bind to the large mica windows. The $CaCl_2$ concentration in a known volume of solution was found not to change after a 24-h exposure to mica sheets of sufficient number to give the same surface to volume ratio as that of the sample in the sealed holder.

An osmotic stress technique which gives the repulsive pressure between the bilayers has been described in great detail (LeNeveu et al., 1976, 1977; Parsegian et al., 1979). Briefly, the multilamellar structure is equilibrated with an external dextran–$CaCl_2$ solution where the dextran molecules are too large to enter the interbilayer space. The bilayers separate by imbibing $CaCl_2$ solution against the osmotic pressure of the dextran solution ($\pi_{dextran}$) and, at equilibrium, experience mutual repulsion equal to that pressure. X-ray diffraction of these equilibrated samples yields, with reference to the gravimetrically prepared samples, the structural paramaters d, d_l, d_w, and A.

In this report of measuring electrostatic forces, the lipid molecular cross-sectional area A and bilayer thickness d_l are found to be constant as a function of water content. Under these conditions removal of water causes only a decrease in bilayer separation d_w. Methods are developed elsewhere (Parsegian et al., 1979) for analyzing systems where A and d_w both change with solvent removal.

We have ascertained that the osmotic pressure of dextran mixed in ionic solutions does not differ from that of dextran in pure water. DPPC placed in excess amounts of solution containing various concentrations of dextran, up to 40 wt %, 30 mM $CaCl_2$, and either 0, 25, 50, 75, or 100 mM NaCl was examined, and the equilibrium d spacings of the lamellar phase were obtained. These dextran-exposed samples also were allowed to equilibrate for 48 h. Neither increasing the time for equilibration nor increasing the temperature of the sample to 50 °C during equilibration affected the final measurements. The volume of this phase was always at least 10 times that of the multilayer to ensure that the dextran-containing phase acted as a reservoir for water, $CaCl_2$, and NaCl at the predetermined chemical potentials. An increase to 100 times made no difference in the observed spacings. Equilibration of lipid samples with dextran solutions that had themselves

been dialyzed for several days against large volumes of 30 mM $CaCl_2$ gave results identical with those with dextrans that were mixed directly with $CaCl_2$ solution. The addition of the calcium ionophore A23187 (courtesy of the Eli Lilly Co.) to some preparations made no difference to the attainment of equilibrium.

The only structure observed is the lamellar phase, which is identified by a series of reflections whose spacings are integral orders of a single repeat distance d. All data reported below were collected at 25 °C. Particular attention was paid to wide-angle X-ray scattering as it reflects hydrocarbon chain tilt. No changes in hydrocarbon chain tilt were observed upon addition of calcium chloride. [In strong contrast, even millimolar levels of uranyl acetate cause a large change in chain tilt (Parsegian et al., 1981).]

Theoretical

The pressure $\pi_{dextran}$ is the net repulsive pressure between bilayers. When this repulsion is dominated by electrostatic forces, it may be equated to the osmotic pressure (π_{mid}) of the mobile ions midway between the bilayers minus the osmotic pressure (π_{res}) of those ions in the reservoir (dextran plus electrolyte) phase (Langmuir, 1938; Verwey & Overbeek, 1948)

$$\pi_{dextran} = P = \pi_{mid} - \pi_{res} \tag{1}$$

We use the van't Hoff expression

$$\pi_{res} = k_T \sum_{\{n_i^0\}} n_i^0 \tag{2}$$

where k is Boltzmann's constant, T absolute temperature, and the set $\{n_i^0\}$ the concentrations of all ionic species of valence z_i in the reservoir; the pressure π_{mid} is defined by the Boltzmann relation

$$\pi_{mid} = kT \sum_{\{n_i^0\}} n_i^0 e^{-z_i(e\psi_m/kT)} \tag{3}$$

where e is the charge of a proton and ψ_m the electrostatic potential midway between bilayers relative to $\psi = 0$ in the reservoir.

The measurement of P, eq 1, is thereby tantamount to the measurement of the potential ψ_m. We integrate the nonlinear (Poisson–Boltzmann) differential equation for the electrostatic potential $\psi(x)$ from the midpoint $x = 0$, where $\psi = \psi_m$, in order to infer the entire potential distribution between the bilayers. By Gauss' theorem the slope $(d\psi/dx)|_b$ of this potential at the binding plane is proportional to the fixed charge density σ at that plane

$$\left.\frac{d\psi}{dx}\right|_b = \pm\frac{4\pi\sigma}{\epsilon} \tag{4}$$

(using cgs units and ϵ for the dielectric constant of the aqueous solvent). If the charge is due to binding of divalent cations, then σ is related to the area, S, per bound divalent cation by

$$\sigma = 2e/S \tag{5}$$

We now derive expressions to compute these quantities in a system whose reservoir contains univalent anions and cations plus divalent cations whose concentrations are written respectively as

$$\begin{aligned} n_-^0 &= n \\ n_+^0 &= n(1-a) \\ n_{2+}^0 &= na/2 \end{aligned} \tag{6}$$

For compactness, we consider a reduced potential

$$y(x) = (e/kT)[\psi(x) - \psi_m(x=0)] \tag{7}$$

(whose zero is taken at the midpoint), and we use the quantity

$$\xi = e^{-e\psi_m/kT} \tag{8}$$

In this notation, the density of charge σ on mobile ions is

$$\rho(x) = -ne\left(\frac{e^y}{\xi} - a\xi^2 e^{-2y} - (1-a)\xi e^{-y}\right) \tag{9}$$

The Poisson equation

$$\frac{d^2\psi}{dx^2} = -\frac{4\pi\rho(x)}{\epsilon} \tag{10}$$

becomes

$$\frac{d^2y}{dx^2} = \frac{4\pi ne^2}{\epsilon kT}\left(\frac{e^y}{\xi} - a\xi^2 e^{-2y} - (1-a)\xi e^{-y}\right) \tag{11}$$

Its integration here is similar to, but not identical with, the procedure of Ninham & Parsegian (1971).

The first integral is

$$\frac{dy}{dx} = \frac{e}{kT}\frac{d\psi}{dx} = \pm\kappa\left[\frac{e^y}{\xi} + \frac{a\xi^2}{2}e^{-2y} + (1-a)\xi e^{-y} - \left(\frac{1}{\xi} + \frac{a\xi^2}{2} + \xi(1-a)\right)\right]^{1/2} \tag{12a}$$

with

$$\kappa^2 = \frac{8\pi ne^2}{\epsilon kT} \tag{12b}$$

when we impose the (symmetry) condition that

$$(d\psi/dx)|_{x=0} = 0 \tag{13}$$

We define

$$\theta \equiv e^{-y} \tag{14}$$

so that the expression in the brackets in eq 12a is a polynomial in θ whose roots are 0, 1, $\theta_\pm$ where

$$\theta_\pm = \frac{-h \pm (h^2 + 8a/\xi^3)^{1/2}}{2a} \tag{15}$$

$$h = \frac{2(1-a)}{\xi} + a \tag{16}$$

The quantities $\theta_\pm$ are therefore known directly from the force measurements.

We make a standard transformation to a form appropriate for Jacobi elliptic functions (Magnus et al., 1966) and define

$$k^2 = \frac{\theta_+ - \theta_-}{\theta_+(1-\theta_-)}$$

$$z^2 = \frac{(1-\theta_-)\theta}{\theta - \theta_-}$$

so that the second integral can be written

$$\int_1^w \frac{dz}{(1-z^2)^{1/2}(1-k^2z^2)^{1/2}} = u \tag{17}$$

where

$$u = (\kappa\xi/2)\,(a/2)^{1/2}[\theta_+(1+\theta_-)]^{1/2}x \tag{18}$$

Through appropriate reduction (Magnus et al., 1966; Abramowitz & Stegun, 1964), this gives

$$w = cd(u;k) \tag{19}$$

The potential y is then

$$y = \ln\frac{1 - \theta_- - cd^2(u;k)}{-\theta_- cd^2(u;k)} \tag{20}$$

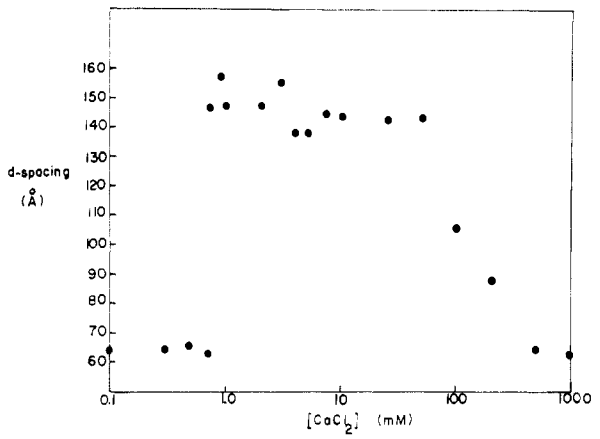

FIGURE 1: Repeat spacing of DPPC multilayer lattice in $CaCl_2$ solutions of varying concentration. All samples consisted of ~30 wt % lipid and 70% $CaCl_2$ solution. At <0.7 mM $CaCl_2$ the 62-Å repeat multilayers coexist with excess $CaCl_2$ solution. Between 0.7 and 80 mM multilayer swelling is limited by the volume of $CaCl_2$ solution available. Above 80 mM, an excess aqueous phase again occurs.

This result is like that of Ohshima & Mitsui (1978) which was for the special case (in our notation) $a = 1$.

To evaluate $cd(u;k)$, we found it convenient to use a Landen series (Abramowitz & Stegun, 1964). This method involves few programming steps and allows rapid evaluation over the full range of conditions encountered.

In order to compare our estimates of ion binding with those of others, we use two definitions of an association "constant" K_a. We imagine an association of neutral lecithin L^0 with a divalent cation C^{2+} to form L^{2+}

$$L^0 + C^{2+} \rightleftharpoons L^{2+} \tag{21}$$

and define

$$K_a = \frac{[L^{2+}]}{[L^0][C^{2+}]} \tag{22}$$

We will refer below to K_a or K^{app} as an "uncorrected" or "apparent" association constant when $[C^{2+}]$ in eq 22 is the divalent ion concentration $[C^{2+}]_r$ in the reservoir (i.e., in the dextran) phase. We will refer to K_a^{corr} as a "corrected" or "intrinsic" association constant when $[C^{2+}]$ in eq 22 is $[C^{2+}]_b$, the concentration of free ions at the plane of ion binding. The two concentrations are related by a Boltzmann expression

$$[C^{2+}]_b = [C^{2+}]_r e^{-2e\psi_b/kT} \tag{23}$$

Here, and in the text below, ψ_b is the electrostatic potential at the plane of ion binding.

Results

Experimental

We first measured the lamellar repeat spacing in preparations of 30 wt % DPPC mixed with 70 wt % $CaCl_2$ solutions of various concentrations (Figure 1). DPPC multilayers swell from 63 Å in pure water to the largest d spacings allowed by the amount of $CaCl_2$ solution (~150 Å) in 1–30 mM $CaCl_2$ solutions. Swelling is less in $CaCl_2$ solutions of >30 mM. Above 500 mM $CaCl_2$, the repeat spacing is the same as for DPPC in pure water. This behavior establishes correspondence with the findings of Mitsui and co-workers (Inoko et al., 1975; Ohshima & Mitsui, 1978).

We next determined the repeat spacing and bilayer thickness for various concentrations of DPPC at 25 °C in solutions of 30 mM $CaCl_2$ (Figure 2). Only one lamellar phase is observed for all DPPC concentrations. While DPPC in pure water stops swelling at ~30 wt % water, DPPC bilayers in 30 mM $CaCl_2$ continue to swell even after imbibing more than 70 wt % $CaCl_2$ solution. The lipid bilayer thickness decreases with decreasing lipid concentration and, between 60% and 70% lipid, reaches a limiting value of d_l = 44 Å, equal to that in excess pure water (Ladbrooke et al., 1968; Rand et al., 1975; Janiak et al., 1976; McAlister et al., 1978; McAlister, 1978).

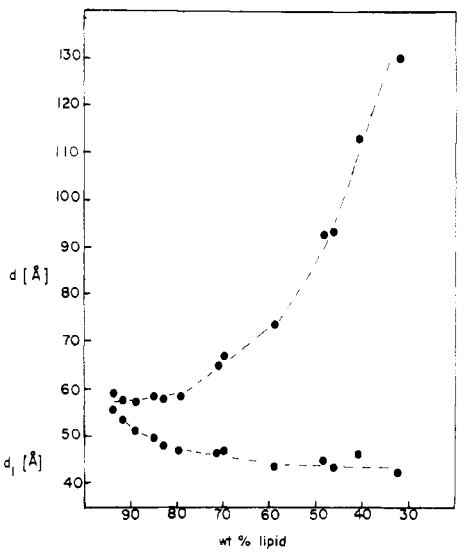

FIGURE 2: Measured repeat spacing d and computed bilayer thickness d_l of DPPC mixed with different amounts of 30 mM $CaCl_2$ solution. The volume fraction ϕ of lipid is set by the weight percent lipid (see text) and used to compute $d_l = \phi d$. The bilayer separation $d_w = d - d_l$. Note that for <60% lipid, the bilayer thickness is unchanging.

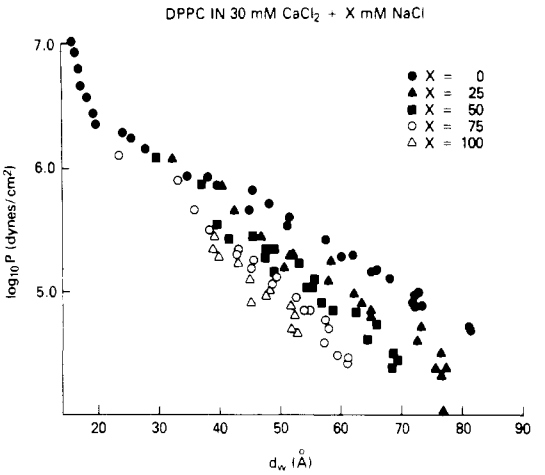

FIGURE 3: Applied osmotic pressure P vs. bilayer separation d_w for DPPC in solutions of 30 mM $CaCl_2$ plus 0, 25, 50, 75, or 100 mM NaCl. The net repulsive pressure P imparted by $CaCl_2$ alone (●) is progressively weakened by increasing concentrations of NaCl in the medium.

The net repulsive force between bilayers $\pi_{dextran}$ vs. the bilayer separation d_w in 30 mM $CaCl_2$ with various NaCl concentrations is shown in Figure 3 Also shown for comparison is the force vs. distance for DPPC in pure water (McAlister et al., 1978; McAlister, 1978).

With no NaCl, the change in repulsive force with d_w has a discontinuity in slope at d_w = 20 Å. For d_w < 20 Å the interbilayer force increases very rapidly as the bilayers approach. This is the same force as found in pure water for DPPC (McAlister et al., 1978; McAlister, 1978) and egg lecithin with (Cowley et al., 1978) or without (LeNeveu et al., 1976, 1977) added charged lipids. Even in the presence of $CaCl_2$, this "hydration force" between lecithin bilayers

Table I: DPPC and 30 mM $CaCl_2$ plus Dextran

wt % dextran	log P	d (Å)	ϕ	d_1 (Å)	d_w (Å)
40.0	7.027	62.3	75.5	46.5	15.8
38.0	6.932	62.6	75.5	46.5	16.1
36.75	6.813	63.2	74.5	46.5	16.7
33.5	6.673	63.5	74.0	46.5	17.0
31.5	6.584	64.0	73.0	46.0	18.0
29.3	6.454	64.5	72.5	45.5	19.0
27.5	6.364	64.9	72.0	45.5	19.4
25.75	6.295	68.0	69.0	45.0	23.0
24.7	6.249	69.3	67.0	44.0	25.3
23.0	6.156	71.5		44.0	27.5
18.75	5.932	78.5		44.0	34.5
18.75	5.932	82.1		44.0	38.1
17.5	5.872	85.6		44.0	41.6
13.75	5.532	95.1		44.0	51.1
13.75	5.298	105.9		44.0	61.9
9.0	5.165	109.4		44.0	65.4
7.25	4.997	116.7		44.0	72.7
6.5	4.910	115.9		44.0	72.7
5.0	4.681	127.0		44.0	83.0
3.5	4.323	131.7		44.0	87.7

appears to be the major repulsive force which must be overcome in order for lipid bilayers to approach to within a few angstroms.

For bilayer separations 30 Å < d_w < 90 Å, the force varies much more slowly with d_w; this variation is qualitatively similar to results obtained with egg lecithin bilayers containing charged lipids (Cowley et al., 1978). In this range of separations, the variation of force observed decreases monotonically with increasing NaCl concentration.

Theoretical

From earlier work (Inoko et al., 1975; Ohshima & Mitsui, 1978; Cowley et al., 1978), we expect that the extra swelling of lecithin multilayers in $CaCl_2$ solution indicates electrostatic repulsion caused by adsorption of Ca^{2+} ions to the phospholipid polar groups. One may transform the forces measured between bilayers bearing Ca^{2+} ions into estimates of the density of bound charge. To do so, we follow the prescription given under Materials and Methods and compute the electrostatic potential and area per charge, eq 20 and 5, respectively, at the hypothetical plane where Ca^{2+} is assumed to bind. These estimates, but not the direction of their change with bilayer separation, are sensitive to the assumed locus of that binding plane (Figure 4).

It is unlikely that the divalent ions bind at a hypothetical interface that separates all phospholipid from ionic solution with no overlap of lipid polar groups and the aqueous layer. The binding plane (if indeed the locus of binding positions be a plane) probably occurs some distance Δ into the aqueous region from that arbitrary lipid–water interface. The actual separation of binding planes must consequently be smaller than the d_w calculated between bilayers.

In making our estimates of surface area S per adsorbed divalent charge (Figure 5), surface potential ψ_b (Figure 6), and ion affinities K_a and K_a^{corr} (Figure 7), we have assumed Δ = 3.5 Å.[2] We know from neutron diffraction measurements

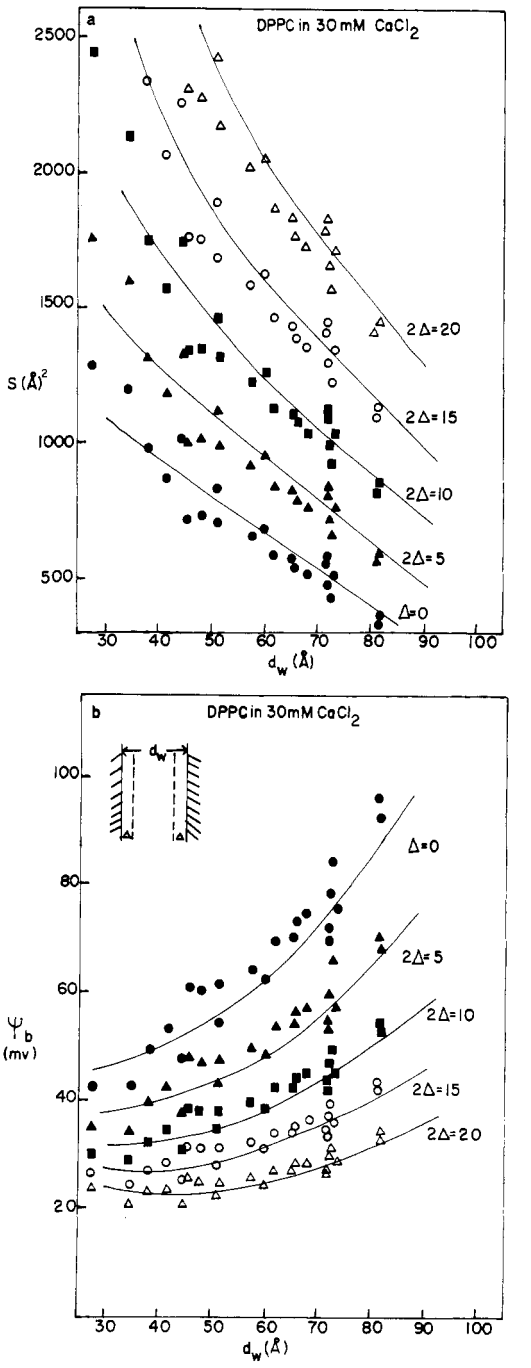

FIGURE 4: Influence of assumed position of ion binding on estimated area, S, per charge $2e$ adsorbed (a) and surface potential ψ_b (b). The difference Δ is a distance between the hypothetical binding plane and the lipid–water interface as modelled in this paper. Two qualitative features of ion binding are evident under all assumptions. The potential ψ_b *decreases* and the bound Ca^{2+} *desorbs* as bilayers are pushed together. Under no assumption of Δ shown here can these trends appear differently. Lines are values of ψ_b and S computed from smooth lines drawn through the data points of Figure 3. Only the NaCl-free case is illustrated here.

(Büldt et al., 1978) that the DPPC outer phosphate oxygens are some 24 Å from the bilayer center. Binding of Ca^{2+} to the phosphate group at that location is consistent with our choice of Δ for the binding plane. Data given in Tables I and II together with methods detailed above will allow readers to use the measurements in other ways.

As the estimated area S per divalent cation increases, bilayer separation decreases (Figure 5); the surface charging ions desorb as two surfaces are pushed together. The binding plane potential ψ_b *decreases* as the surfaces approach (Figures 4b

[2] It had been our original intent to use the heavy ion labeling method of Stamatoff and co-workers (Graddick et al., 1979; Stamatoff et al., 1979) to use their estimate of d_1 the distance across a bilayer between layers of UO_2^{2+} ions bound to the membrane surface. As a control, we added UO_2^{2+} acetate solutions to DPPC instead of $CaCl_2$. We learned (Parasegian et al., 1980) that UO_2^{2+}, even at 1 mM, creates a clear change in chain tilt by the criterion of wide-angle scattering introduced by Tardieu et al. (1973) recently used by McIntosh (1980).

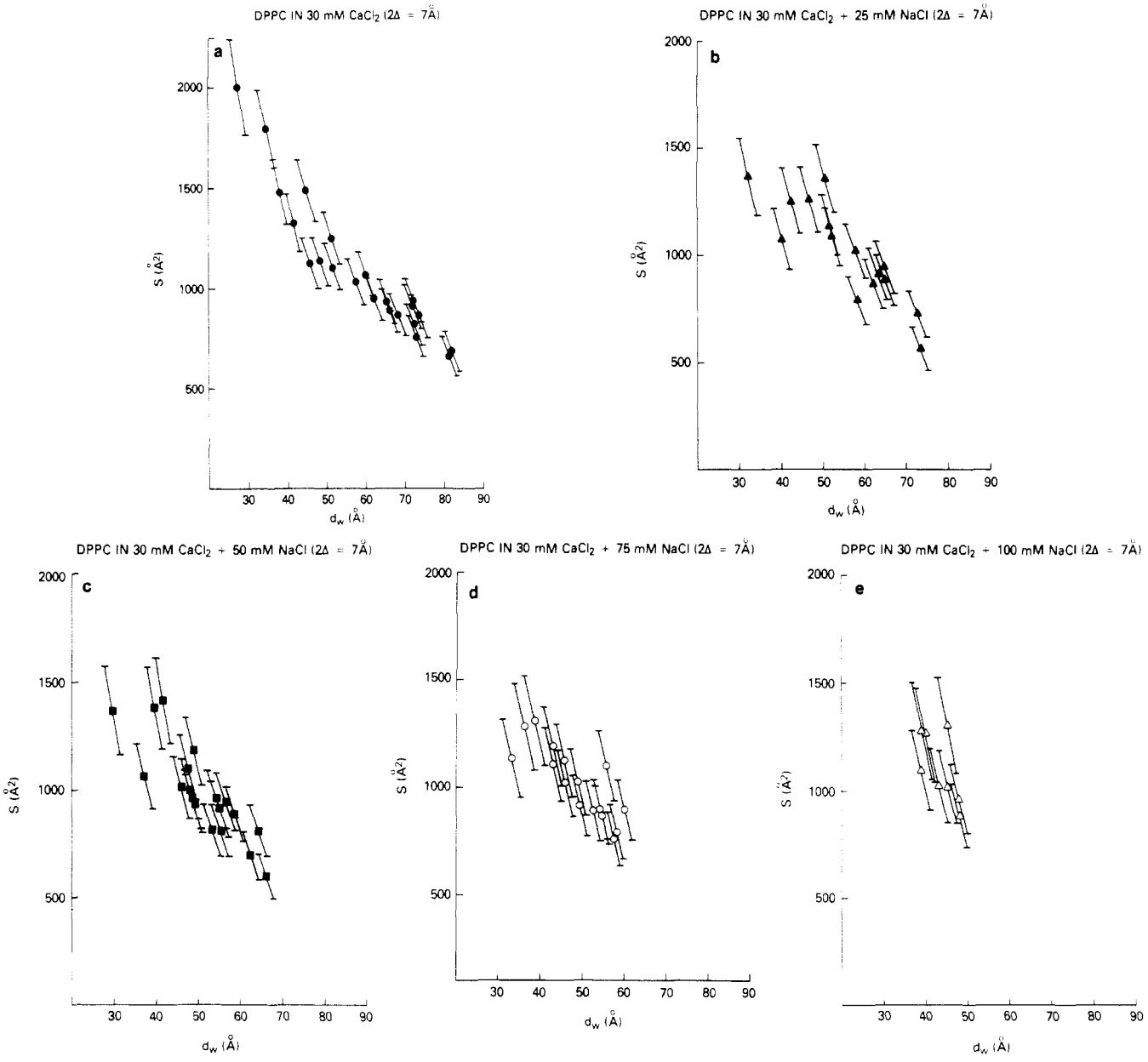

FIGURE 5: Estimated area S per divalent charge bound to DPPC bilayers vs. bilayer separation d_w. The charge density ($2e/S$) goes up with increasing levels of NaCl but always decreases as bilayers are pushed together. For these estimates we use $2\Delta = 7$ Å. Error bars indicate range of estimates from assuming ±2-Å error in d_w.

and 6). The cause of ion desorption cannot therefore be due to an increase in surface potential. Rather there is a rapid decrease in the apparent and corrected binding "constants" upon approach of two bilayers (Figure 7).

In these calculations we have corrected $\pi_{dextran}$ for contributions of van der Waals and hydration forces so that $F_{es} = \pi_{dextran} - F_{hyd} - F_{vdw}$. These forces have been measured previously (McAlister et al., 1978; McAlister, 1978) for DPPC at 25 °C in pure water. The hydration repulsion is described by $F_{hyd} = 7 \times 10^9\, e^{-d_w(\text{Å})/2.0\text{Å}}$ and the van der Waals attraction by (Ninham & Parsegian, 1970; Parsegian, 1975)

$$F_{vdw} = \frac{A_H}{6\pi}\left(\frac{1}{d_w^3} - \frac{2}{(d_w + d_1)^3} + \frac{1}{(d_w + 2d_1)^3}\right)$$

The coefficient A_H is evaluated by setting $F_{vdw} = F_{hyd}$ at the equilibrium separation $d_w = 19$ Å in pure water where $d_1 = d - d_w = 44.0$ Å. The corrections increase slightly the estimated electrostatic force but have no qualitative effect on the parameters inferred. Any image charge corrections, due to low polarizability of lipid, are expected to be negligible (Brenner et al., 1978).

Discussion

Two aspects of these measured forces between DPPC bilayers are immediately apparent. First, Ca^{2+} imparts electrostatic repulsive forces that are decreased by NaCl. Although these forces decay exponentially the decay constants are, surprisingly, ~30% lower than expected from Debye–Huckel theory. The forces do not obey expectations assuming constant surface potential, constant surface charge density, or constant Ca^{2+} surface binding affinity. Second, Ca^{2+} or $CaCl_2$ solution has no effect on the very strong hydration forces observed for separations of <20 Å between DPPC bilayers at 25 °C in water (McAlister et al., 1978; McAlister, 1978).

As the bilayers are pushed together, bound Ca^{2+} desorbs (Figure 5) in a way suggesting that there is a progressive decrease in binding affinity (Figure 7) and surface potential (Figure 6). The trends, but not the specific values, are insensitive to detailed assumptions about the locus of Ca^{2+}

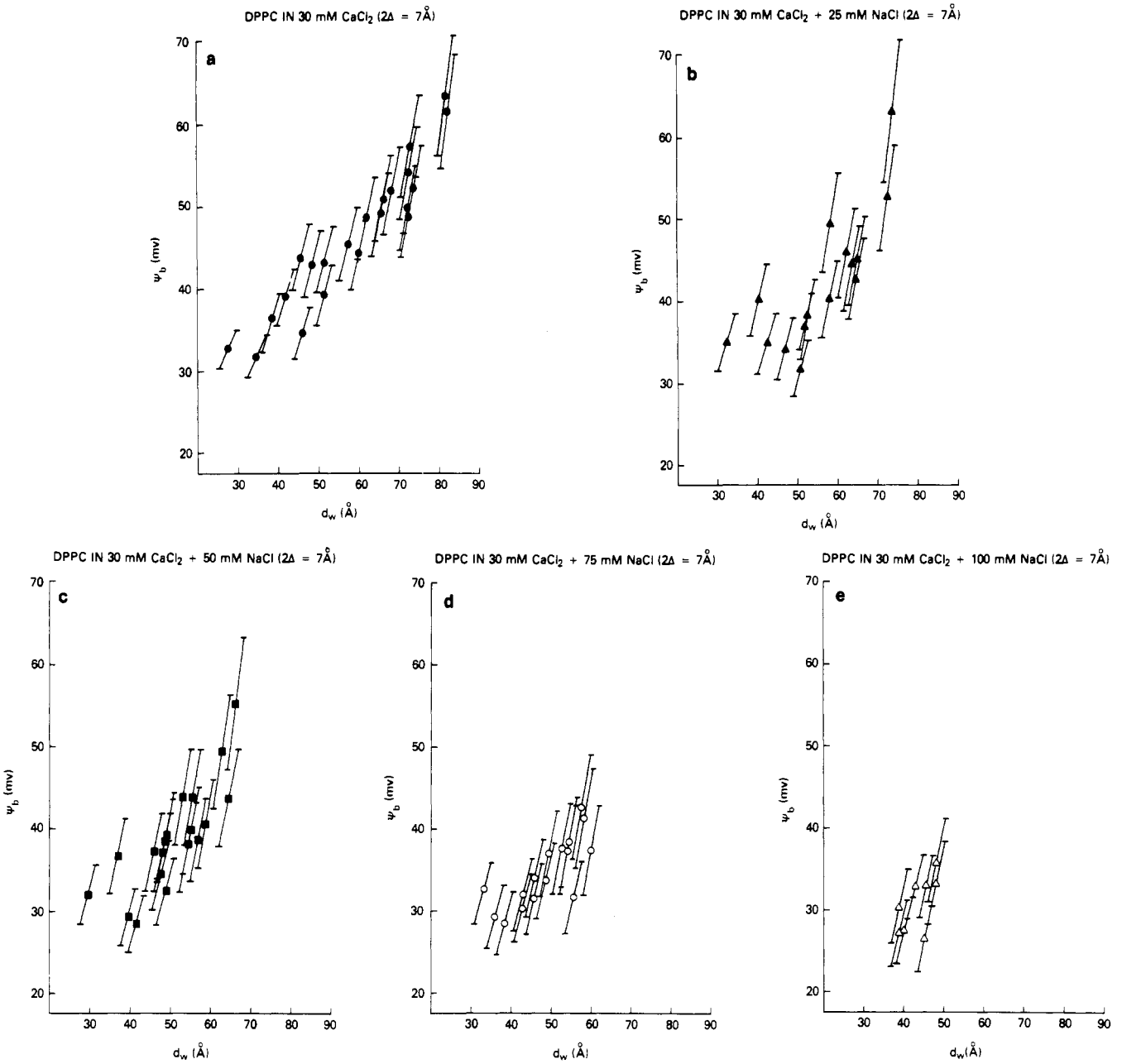

FIGURE 6: Surface electrostatic potential ψ_b vs. d_w. The potential ψ_b always decreases as the bilayers are forced together. Lines are transformations of smooth curves drawn through the data points of Figure 3. Error limits follow from assumed ±2-Å error in d_w.

binding or to systematic changes in estimated electrostatic repulsion from the contribution of other forces.

Addition of NaCl to the $CaCl_2$ medium decreases the measured net repulsion at a given separation (Figure 3) but increases both the amount of Ca^{2+} bound (Figure 5) and the surface potential (Figure 6). For a 100 mM NaCl plus 30 mM $CaCl_2$ solution where the lipid lattice is allowed to swell ad libitum, ~5% of the phospholipids are estimated to bind Ca^{2+} (Figure 6). In all the NaCl concentrations we used, the surface potential showed its surprising decrease as the lipid planes were pushed together (Figure 6).

In the limit where the lamellae in 100 mM NaCl are allowed to swell to their fullest extent, one infers apparent association coefficients and surface potentials slightly higher than those expected from ζ potential measurements on lamellar suspensions of DMPC or DPPC with frozen hydrocarbon chains (McLaughlin et al., 1978; Lau et al., 1980). We believe correspondence between that system and our measurements is adequately established within experimental error. The electrophoretic measurements have yielded surface potentials and their dependence on a variety of aqueous solutions for several different lipids. The present technique provides additional information in allowing the potentials to be probed between interacting bilayers and at various distances from the surface.

To illustrate the point of nonconstant surface parameters, we have plotted theoretically expected force vs. separation (d_w) in 30 mM $CaCl_2$ (without NaCl) by assuming constant surface charge density (Figure 8b), constant surface potential (Figure 8b), constant apparent binding affinity K_a^{app} (Figure 8a, solid line), and constant intrinsic binding affinity K_a^{corr} (Figure 8a, dashed line). It is obvious that in no way do the theoretical lines follow the experimental points. One could imagine that the Ca^{2+} binding plane shifts continuously as bilayers approach. From the example of Figure 8, it appears that this progressive shift must amount to an improbable 10 Å (2Δ = 20 Å in the notation of Figure 4).

How can one account for the unexpected variation of surface parameters? It is possible that the weak electric fields emanating from each charged bilayer can perturb neighboring

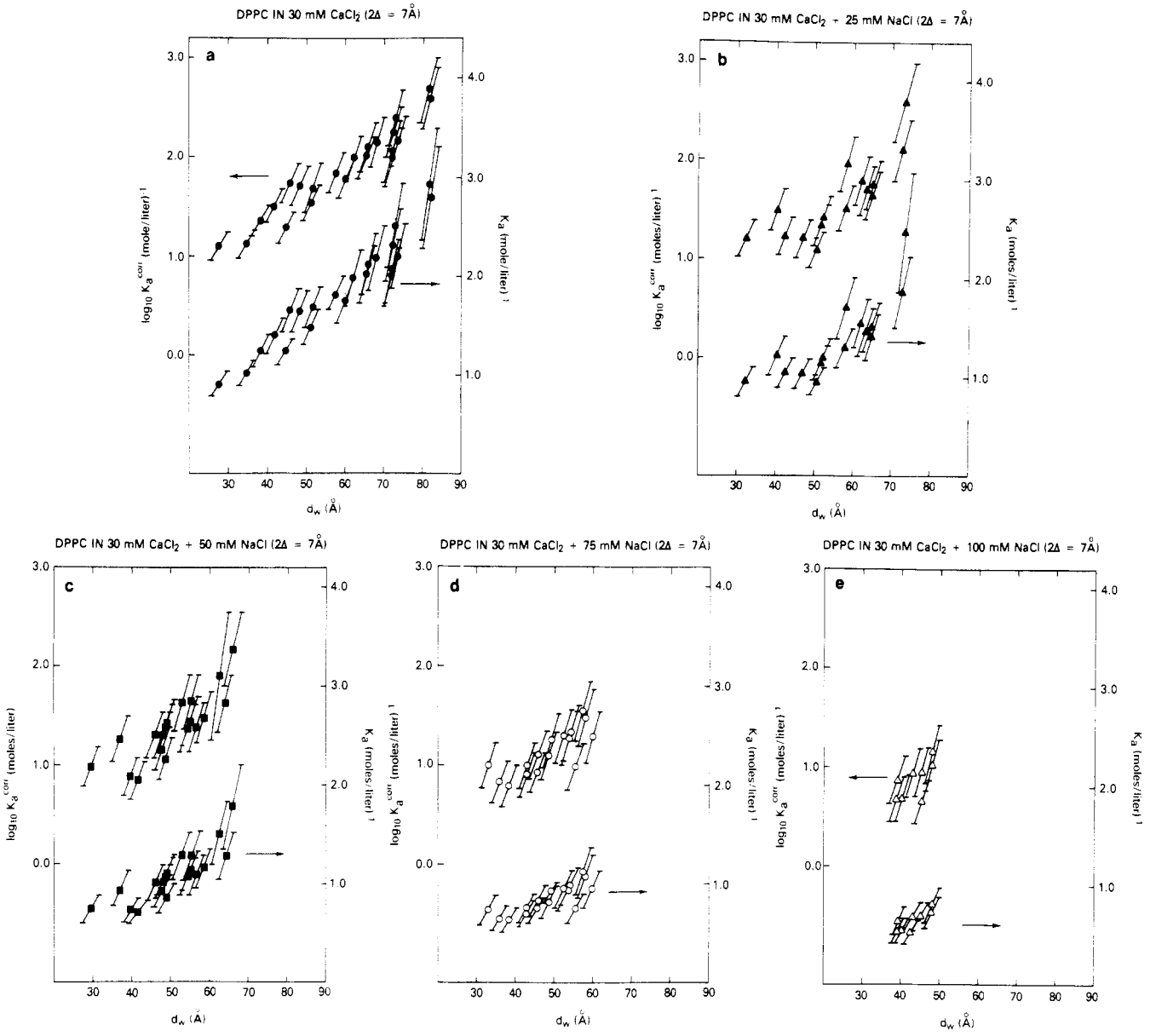

FIGURE 7: (Right axis) Apparent "uncorrected" association coefficient $K_a = [L^{2+}]/([L^0][C^{2+}]_r)$ (eq 22). (Left axis) "Corrected" association coefficient $K_a^{corr} = [L^{2+}]/([L^0][C^{2+}]_b)$ (eq 22 and 23). This parameter varies with both ionic strength and bilayer separation. Error estimates follow from ±2-Å assumed error in d_w.

bilayers to change the ion binding characteristics of their zwitterionic polar groups. We do know that ions can induce changes in lecithin head group conformation in bilayers (Brown & Seelig, 1977). If, for example, Ca^{2+} binding causes zwitteronic polar groups to move toward a perpendicular orientation with respect to the bilayer surface with the positive $-N(CH_3)_3^+$ group outward, then any rotation of those groups away from the perpendicular can be expected to lower their affinity for Ca^{2+}. The electric field from a positive apposing bilayer might create such a perturbation, but its magnitude is likely to be too small to effect the required change in ion affinity.

Anion binding (Grasdalen et al., 1977) might be influencing the decrease in ψ_b as bilayers are moved together. However, to explain a decrease in positive surface potential ψ_b with mutual approach of bilayers, such binding would have to increase with bilayer approach even as the free anion concentration at the bilayer must decrease. Anion binding, at least as described by a single intrinsic association constant, cannot explain the behavior observed here. Perhaps the complete nonlinear Poisson–Boltzmann equation does not give a sufficiently accurate description of the electrostatic double layer in 30 mM $CaCl_2$ solutions. Judging from our measurements of the repulsion between bilayers of negatively charged phospholipids with a Na^+ counterion (Cowley et al., 1978), such inaccuracy appears unlikely. Those measurements, using the same osmotic stress method as used here, show striking agreement between the rate of change of repulsive force and the prediction via the nonlinear Poisson–Boltzmann equation.

Still, one possibility that should not be ignored is that the ionic solutions do not show the screening length expected from the usual Gouy–Chapman Debye–Huckel Poisson–Boltzmann theory. It may be that $CaCl_2$ solutions are sufficiently nonideal as to disobey the van't Hoff osmotic behavior of eq 2 and 3.

If we scale uniformly all the ionic strengths to make the theoretical screening lengths match the values apparent from Figure 3 and if we next compute potentials by using those scaled ionic strengths, we obtain apparent surface potentials and adsorbed charge densities. By the very nature of this construction, the surface parameters are constant with separation d_w. The surface potentials so obtained are near 60 mV for all NaCl concentrations.

Table II: DPPC in 30 mM $CaCl_2$ and x mM NaCl

$x = 0$		$x = 25$		$x = 50$		$x = 75$		$x = 100$	
log F	d_w	log F	d_w	log F	d_w	log F	d_w	log F	d_w
7.027	15.8	6.075	32.2	6.088	29.5	6.107	25.3	5.450	38.9
6.932	16.1	5.872	40.2	5.872	36.9	5.910	33.0	5.353	38.7
6.813	16.7	5.662	42.3	5.537	39.6	5.662	35.8	5.290	39.8
6.673	17.0	5.450	46.7	5.450	45.7	5.499	38.3	5.241	42.9
6.584	18.0	5.306	52.1	5.422	41.4	5.353	42.9	5.103	45.1
6.454	19.0	5.298	51.5	5.422	41.4	5.311	42.7	5.025	48.0
6.364	19.4	5.249	58.2	5.340	47.8	5.249	45.7	4.968	47.8
6.295	24.0	5.207	50.5	5.340	48.5	5.186	45.5	4.910	45.1
6.249	25.3	5.082	57.8	5.340	48.8	5.124	49.2	4.881	51.7
6.156	27.5	4.996	62.1	5.290	47.4	5.071	48.6	4.810	52.2
5.932	34.5	4.899	63.3	5.223	52.9	4.945	52.5	4.698	51.9
5.932	38.1	4.853	64.7	5.165	48.7	4.853	53.9	4.681	52.3
5.872	41.6	4.810	64.6	5.098	55.3	4.853	54.3	4.681	52.3
5.830	45.6[a]	4.724	73.4	5.025	54.3	4.767	57.4		
5.725	48.1	4.610	72.5	5.025	55.0	4.698	58.0		
5.662	44.6	4.502	76.3	4.910	56.6	4.581	55.5		
5.612	51.4[a]	4.384	77.2	4.853	58.5	4.490	59.7		
5.532	51.1	4.383	75.5	4.819	62.4	4.468	61.1		
5.415	57.5[a]	4.323	76.5	4.725	65.9	4.418	61.0		
5.298	61.9	4.039	76.7	4.610	64.1				
5.290	60.0[a]			4.490	68.5				
5.181	65.8[a]			4.442	69.3				
5.165	65.4			4.384	68.3				
5.108	68.0[a]								
4.997	72.7								
4.968	72.2[a]								
4.910	71.9								
4.886	73.4[a]								
4.882	72.0								
4.715	81.4[a]								
4.681	81.6								

[a] Dextran solution dialyzed against a 30 mM $CaCl_2$ solution for 24 h before being placed in contact with lipid.

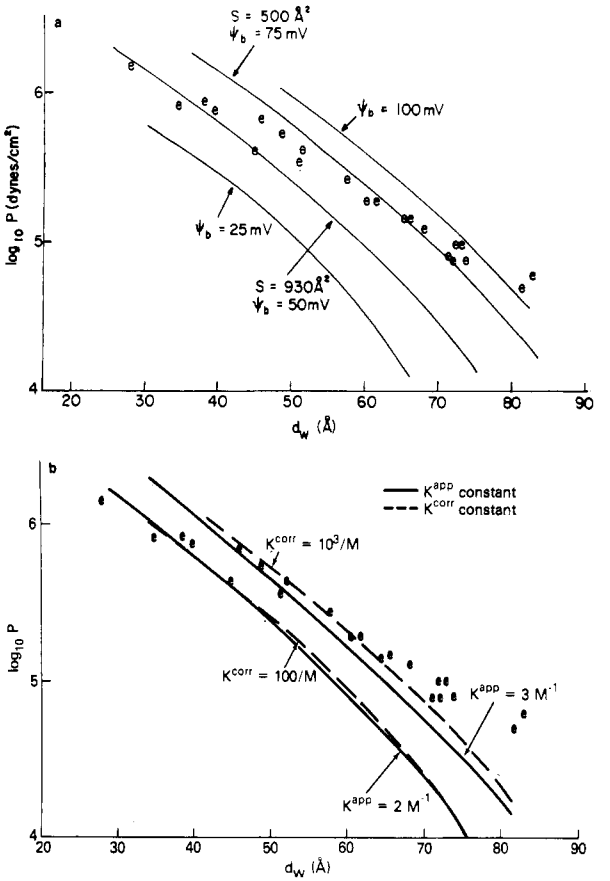

FIGURE 8: Comparison of force measurements in 30 mM $CaCl_2$ solution with theoretical curves assuming constant surface potential or surface charge density $2e/S$ (a) or constant association coefficients (eq 22) (b). In no way can the curves be shifted (e.g., by redefining Δ's as in Figure 4) to force them to behave like the experimental points. The slight deviation of theoretical lines from purely exponential variation is due mostly to corrections for van der Waals and "hydration" forces.

Or if we, for example, imagine the effective $CaCl_2$ concentration to be 23 mM, rather than 30 mM, all data points for the NaCl concentrations lead to surface potentials of 25–35 mV (when $2\Delta = 7$ Å). Whether this remarkable consistency indicates an unrecognized truth or betrays an obvious error, we have not been able to determine.

Anomalous screening of electrostatic repulsion between mica sheets in 1 mM $CaCl_2$ solutions was reported by Israelachvili & Adams (1978) (Israelachvili, 1978). There, the screening length was *shorter* than expected from Debye–Huckel theory rather than the longer decay length seen here. If the slow decay seen here stems from special properties of ion binding to zwitterionic groups, then one need not expect similar results from the PC and mica systems.

Since much of the interest in the role of alkaline earth ions near lipids is in their influence on membrane–membrane forces [e.g., Papahadjopoulos et al. (1976, 1977), Newton et al. (1978), Morris et al. (1979), Lansman & Haynes (1975), and Haynes (1974, 1977)], it is disturbing to learn now that these forces act contrary to what was expected from indirect studies on membranes. This unexpected behavior may explain why Ohshima & Mitsui (1978) enjoy only partial success in explaining lamellar repeat spacings vs. $CaCl_2$ concentration. In the following paper we report that all these parameters—ion types, hydrocarbon chain length, and conformation—affect intermembrane forces. In fact, Ca^{2+} bathing a multilayer of mixed phosphatidylcholines will actually cause the mixture to separate into two distinct lamellar lattices. Divalent ions appear to confer stronger repulsion between phosphatidylcholine bilayers with more densely packed polar groups.

Even with the massive theoretical literature on electrostatic forces in ionic solution, there exist surprisingly few direct measurements. Other systems must now be examined in order to determine the conditions under which deviations occur between measurement and theoretical expectation.

Added in Proof

Ohshima et al. (1981) describe the swelling of DPPC multilayers in mixed $CaCl_2/MgCl_2$ solutions. Their analysis using methods from Ohshima & Mitsui (1978) yields ion binding affinities within the range found here and in the following paper (Lis et al., 1981).

Acknowledgments

We appreciate Nola Fuller's generous help in this project. We thank Stuart McLaughlin for many instructive discussions and suggestions.

References

Abramowitz, M., & Stegun, I. A. (1964) *Handbook of Mathematical Functions*, pp 569–574, National Bureau of Standards, Washington, D.C.

Bangham, A. D., & Dawson, R. M. (1962) *Biochim. Biophys. Acta 59*, 103–115.

Brenner, S. L., Parsegian, V. A., & Gingell, D. (1978) *J. Phys. Chem. 82*, 1727–1731.

Brown, M. F., & Seelig, J. (1977) *Nature (London) 269*, 721–723.

Büldt, G., Gally, H. U., Seelig, A., Seelig, J., & Zaccai, G. (1978) *Nature (London) 271*, 182–184.

Chadha, J. S. (1970) *Chem. Phys. Lipids 4*, 104–108.
Cowley, A. C., Fuller, N., Rand, R. P., & Parsegian, V. A. (1978) *Biochemistry 17*, 3163–3168.
Cubero-Robles, E., & van den Berg, D. (1969) *Biochim. Biophys. Acta 187*, 520–526.
Graddick, W. F., Stamatoff, J., Eisenberger, P., Berreman, D. W., & Spielberg, N. (1979) *Biochem. Biophys. Res. Commun. 88*, 907–912.
Grasdalen, H., Eriksson, L. E. G., Westman, J., & Ehrenberg, A. (1977) *Biochim. Biophys. Acta 469*, 151–162.
Hauser, H., Phillips, M. C., Levine, B. A., & Williams, R. J. P. (1975) *Eur. J. Biochem. 58*, 133–144.
Hauser, H., Hinckley, C. C., Krebs, J., Levine, B. A., Phillips, M. C., & Williams, R. J. P. (1977) *Biochim. Biophys. Acta 468*, 364–377.
Haynes, D. H. (1974) *J. Membr. Biol. 17*, 341–366.
Haynes, D. H. (1977) *Jerusalem Symp. Quantum Chem. Biochem., Part 1 9*, 189–212.
Hutton, W. C., Yeagle, P. L., & Martin, R. B. (1977) *Chem. Phys. Lipids 19*, 255–265.
Inoko, Y., Yamaguchi, T., Furuya, F., & Mitsui, T. (1975) *Biochim. Biophys. Acta 413*, 24–32.
Israelachvili, J. N. (1978) *Faraday Discuss. Chem. Soc. No. 65*, 20–24.
Israelachvili, J. N., & Adams, G. E. (1978) *J. Chem. Soc., Faraday Trans. 1 74*, 975–1001.
Janiak, M. J., Small, D. M., & Shipley, G. G. (1976) *Biochemistry 15*, 4575–4580.
Ladbrooke, B. D., Williams, R. M., & Chapman, D. (1968) *Biochim. Biophys. Acta 150*, 333–340.
Langmuir, I. (1938) *J. Chem. Phys. 6*, 873–896.
Lansman, J., & Haynes, D. H. (1975) *Biochim. Biophys. Acta 394*, 335–347.
Lau, A. L. Y., McLaughlin, A. C., MacDonald, R. C., & McLaughlin, S. G. A. (1980) in *Bioelectrochemistry: Ions, Surfaces, Membranes* (Blank, M., Ed.) American Chemical Society, Washington, D.C.
LeNeveu, D. M., Rand, R. P., & Parsegian, V. A. (1976) *Nature (London) 259*, 601–603.
LeNeveu, D. M., Rand, R. P., Parsegian, V. A., & Gingell, D. (1977) *Biophys. J. 18*, 209–230.
Lis, L. J., Lis, W. T., Parsegian, V. A., & Rand, R. P. (1981) *Biochemistry* (following paper in this issue).
Magnus, W., Oberhettinger, F., & Soni, R. P. (1966) *Formulas and Theorems for the Special Function of Mathematical Physics*, pp 366–369, Springer-Verlag, New York.
McAlister, M. J. (1978) M.Sc. Thesis, Brock University, St. Catharines, Ontario, Canada.
McAlister, M. J., Fuller, N., Rand, R. P., & Parsegian, V. A. (1978) *Biophys. J. 21*, 213a.
McIntosh, T. J. (1980) *Biophys. J. 29*, 237–246.
McLaughlin, A. C., Grathwohl, C., & McLaughlin, S. G. A. (1978) *Biochim. Biophys. Acta 513*, 338–357.
Morris, S. J., Chiu, V. C. K., & Haynes, D. H. (1979) *Membr. Biochem. 2*, 163–201.
Newton, C., Pangborn, W., Nir, S., & Paphadjopoulos, D. (1978) *Biochim. Biophys. Acta 506*, 281–287.
Ninham, B. W., & Parsegian, V. A. (1970) *J. Chem. Phys. 52*, 4578–4587.
Ninham, B. W., & Parsegian, V. A. (1971) *J. Theor. Biol. 31*, 415–428.
Ohshima, H., & Mitsui, T. (1978) *J. Colloid Interface Sci. 63*, 525–537.
Ohshima, H., Inoko, Y., & Mitsui, T. (1981) *J. Colloid Interface Sci.* (in press).
Papahadjopoulos, D., Vail, W. J., Pangborn, W. A., & Poste, G. (1976) *Biochim. Biophys. Acta 488*, 265–283.
Papahadjopoulos, D., Vail, W. J., Newton, C., & Nir, S. (1977) *Biochim. Biophys. Acta 465*, 579–598.
Parsegian, V. A. (1975) in *Biological Membranes* (Colbow, K., Ed.) pp 168–315, Simon Fraser University, Vancouver, B.C.
Parsegian, V. A., Fuller, N., & Rand, R. P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2750–2754.
Parsegian, V. A., Rand, R. P., & Stamatoff, J. (1981) *Biophys. J.* (in press).
Rand, R. P., & Luzzati, V. (1968) *Biophys. J. 8*, 125–139.
Rand, R. P., Chapman, D., & Larsson, K. (1975) *Biophys. J. 15*, 1117–1124.
Stamatoff, J., Bilash, T., Ching, Y., & Eisenberger, P. (1979) *Biophys. J. 28*, 413–421.
Tardieu, A., Luzzati, V., & Reman, F. C. (1973) *J. Mol. Biol. 75*, 711–733.
Verwey, E. J. W., & Overbeek, J. Th. G. (1948) *Theory of the Stability of Lyotropic Colloids*, Chapter 9, Elsevier, Amsterdam.