Adsorption of Divalent Cations to a Variety of Phosphatidylcholine Bilayers†

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ABSTRACT: We have determined the degree of binding of divalent cations to several kinds of phosphatidylcholine (PC) bilayers. This has been done by measuring the electrostatic interbilayer repulsive force that results when multilamellar lattices are exposed to $Me^{2+}Cl_2$ solutions. Divalent cations bind to dipalmitoylphosphatidylcholine in the sequence $Ca^{2+} \simeq Cd^{2+} \simeq Mn^{2+} > Ca^{2+} \simeq Mg^{2+} > Ba^{2+}$. Among the different synthetic lipids, preference for Ca^{2+} is in the sequence DOPC < DLPC < DMPC ≃ DPPC ≃ DSPC. The density of bound charge is proportional to the density of polar groups on the bilayer surface. Phosphatidylcholines with mixed hydrocarbon chains, such as egg PC or 1:1 mixtures of synthetic PC's, form two distinct lamellar phases in $CaCl_2$ solutions. In all cases the electrostatic force between bilayers decays exponentially with their separation but more slowly than expected from ionic double-layer theory. We suggest that the electric fields from opposing surfaces perturb the zwitterionic charge-binding polar groups and continuously modify their ion binding affinities as the bilayers approach.

The effects of divalent cations[1] on the structure and function of cells and cellular organelles are many and varied. [For recent reviews, see *Symp. Soc. Exp. Biol.* (1976), Scarpa & Carafoli (1978).] Divalent cations appear to be critical in maintaining membrane structural integrity. Extracellular and intracellular concentrations of Ca^{2+}, which are tightly regulated, affect many membrane-associated functions from transport properties and the electrical stability of excitable membranes to the communication between cells in contact and the fusion of membranes in processes such as exocytosis. Mg^{2+} often acts as an antagonist to Ca^{2+}. The varied effects could result from (a) conformational changes mediated by divalent cation binding to specific membrane components, (b) surface potential changes resulting from divalent cation binding, and (c) surface potential changes mediated by screening of surface charges by the divalent cations. Obvious candidates for binding are specific membrane proteins and acidic phospholipids. Divalent cation binding to the latter is strong and can induce some large conformational changes in the structure of the lipid aggregates. For example, crystallization of the hydrocarbon chains is induced in phosphatidylserine by Ca^{2+} but not by Mg^{2+} (Portis et al., 1979); Ca^{2+} induces a more radical structural transition, in cardiolipin, from bilayer to hexagonal structures (Rand & SenGupta, 1972). UO_2^{2+} even at millimolar concentrations rotates the frozen hydrocarbon chains of diplamitoylphosphatidylcholine to be perpendicular to the bilayer plane (Parsegian et al., 1980).

Less obvious candidates for ion binding are the neutral phospholipids. So it was at first surprising (Bangham & Dawson, 1962; Inoko et al., 1975) but is now expected (McLaughlin et al., 1978; Hauser et al., 1975, 1977; Hutton et al., 1977; Grasdalen et al., 1977; Lau et al., 1980; Lis et al., 1980, 1981) that divalent alkaline earth cations will adsorb to bilayers of zwitterionic phosphatidylcholine. The charge so imparted causes bilayers in a multilayer array to separate (Inoko et al., 1975; Ohshima & Mitsui, 1978; Lis et al., 1980, 1981) and allows vesicular bilayers to move in an applied external electric field (McLaughlin et al., 1978; Lau et al., 1980). Both effects have been used to estimate the extent of ion binding. These studies have revealed differences in binding of Ca^{2+} vs. Mg^{2+} and differences in binding to phosphatidylcholines bearing frozen or melted hydrocarbon chains (Lau et al., 1980; Lis et al., 1980).

We now report binding preferences among six alkaline earth cations as well as those among five different phosphatidylcholines. (1) For lipids in 30 mM $Me^{2+}Cl_2^-$ solutions, the extent of binding to dipalmitoylphosphatidylcholine (DPPC) increases in the sequence $Ba^{2+} < Mg^{2+} \simeq Co^{2+} < Ca^{2+} \simeq Cd^{2+} \simeq Mn^{2+}$ and to dioleoylphosphatidylcholine as $Mg^{2+} < Co^{2+} \simeq Ca^{2+}$. (2) At any particular bilayer separation the fraction of phosphaditylcholine molecules to which Ca^{2+} binds appears to be independent of the hydrocarbon chain identity or state. Different densities of adsorbed ion are in proportion to the surface density of phosphatidylcholine in the bilayer. At room temperature those densities go as DOPC < DLPC < DMPC < DPPC < DSPC. (3) As should be expected, the density of bound divalent cation increases with increasing $Me^{2+}Cl_2^-$ concentration. The surface potential varies only slightly with $MgCl_2$ concentration (at 10 and 30 mM) but changes drastically with $CaCl_2$ concentration (between 1 and 100 mM). (4) Egg phosphatidylcholine, which contains a mixture of hydrocarbon chains, separates into two distinct lamellar phases when exposed to 30 mM $CaCl_2$ solution as do 1:1 mixutres of DMPC/DLPC, DMPC/DOPC, and DLPC/DOPC. (5) All phosphatidylcholine bilayers in $Me^{2+}Cl_2^-$ solution show an exponentially decaying electrostatic repulsion at large separations and a much shorter range and dominating hydration force (LeNeveu et al., 1976, 1977) at separations < 20 Å (Lis et al., 1981; Cowley et al., 1978). Decay distances of the electrostatic contribution are consistently larger than expected from the theory of salt screening.

To obtain these results, we have used X-ray diffraction to measure the separation of bilayers in multilayers that have been swollen in alkaline earth chloride ($Me^{2+}Cl_2$) solutions (Inoko et al., 1975; Lis et al., 1980, 1981). We osmotically shrink the multilayer lattice by equilibrating it with external solutions of $Me^{2+}Cl_2^-$ and high molecular weight

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DLPC, dilauroylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; Ca^{2+}, calcium ion.

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dextran. From the known osmotic pressure of the dextran solution and the X-ray determination of bilayer separation, we extract force vs. distance curves for the electrostatic repulsion caused by ion adsorption. The stronger the repulsion at a given separation in a medium of a given ionic strength, the greater the degree of ion adsorption.

In an earlier publication (Lis et al., 1981), we showed how force and ion adsorption can be more formally connected by solving for the electrostatic potential between bilayers. We use that method here to convert force measurements into surface charge densities and surface potentials.

As in the earlier studies in 30 mM $CaCl_2$, the anomalously slow force vs. distance curves have been interpreted as showing that divalent ions bound to the bilayer surface appear to desorb when bilayers are pushed together and the potential at the plane of ion binding decreases at the same time. A possible explanation of this is that double-layer electric fields between bilayer surfaces variably perturb the conformation or arrangement of the polar groups and thus the binding characteristics of those groups as the surfaces are pushed together. Another interpretation of the data, albeit unlikely, is that the bilayers maintain nearly constant surface charge density and potential but that the 30 mM $Me^{2+}Cl_2^-$ solution creates a screening length ~30% larger than predicted by electrostatic double-layer theory.

Experimental Procedures

All phospholipids used here were from Sigma Chemical Co. Other sources for DPPC, used in the experiments involving $CaCl_2$, were reported previously (Lis et al., 1981). All lipid lots showed <1% impurity by thin-layer chromatography and gave results independent of source. Water was doubly distilled and salts were reagent grade. Dextran was obtained from Pharmacia Chemicals and mixed in known concentrations with salt solutions prior to contact with the phospholipids.

Samples were made by adding lipid directly to salt solutions containing dextran. Mixtures were allowed to equilibrate for 48 h and then mounted between mica windows. We have previously established the sufficiency of this procedure for obtaining equilibrium (Lis et al., 1981). All experiments were carried out at room temperature (25 °C).

X-ray diffraction and force measurements have been previously described (Lis et al., 1981; LeNeveu et al., 1977). Lamellar phases produce a series of X-ray reflections whose spacings are integral orders of the bilayer repeat spacing, d, which can be divided into a bilayer thickness, d_l, and a water layer thickness, d_w. Specific bilayers forced apart by electrostatic repulsion maintain the same thickness as the same bilayer in excess water (LeNeveu et al., 1977). The method for determining the net repulsive force between membranes from the osmotic pressure of the dextran solutions has been previously described (LeNeveu et al., 1977).

Simultaneous measurement of d and the osmotic pressure provides the relationship between bilayer separation and repulsive force. The measurement of this repulsion is a determination of the electrical potential midway between bilayers. The nonlinear Poisson–Boltzmann differential equation governing this potential has been integrated from the midpoint to the bilayer surface (Lis et al., 1981). One can thus infer a surface potential directly from this procedure and a surface charge density from the boundary conditions on this equation at the bilayer surface.

We assume that all the divalent cations bind at the same plane with respect to the lipid bilayer. As in earlier computations, we take the binding plane to be 3.5 Å. away from a hypothetical interface that separates water from lipid bilayers.

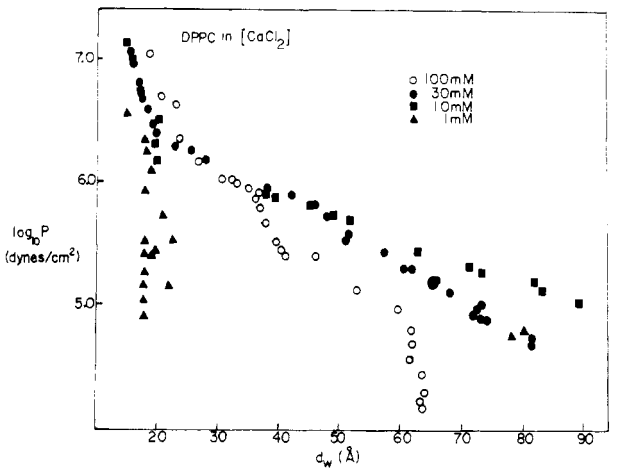

FIGURE 1: Comparison of force vs. separation relations for DPPC in four $CaCl_2$ solutions.

In none of the preparations described below did we observe perturbation of the hydrocarbon chain packing to be induced by the presence of divalent cations.

Results

Influence of Ca^{2+} or Mg^{2+} Concentration on Its Binding to DPPC. Figure 1 shows the relationship between net repulsive force and bilayer separation for DPPC bilayers in solutions of various $CaCl_2$ concentrations (1, 10, 30, and 100 mM). There is a sharp break between the regions of separation where hydration (d_w < 20 Å) and electrostatic repulsive forces (d_w > 30 Å) dominate the net repulsive force for 1 and 10 mM $CaCl_2$. In 30 mM $CaCl_2$ the repulsive forces for DPPC bilayers change from hydration to electrostatic repulsion more gradually. In 100 mM $CaCl_2$ solutions, DPPC bilayers swell to a finite separation with no discernible distinction between hydration and electrostatic repulsive forces. Figure 2 describes the variation in surface potential ψ_b and area per bound divalent cation S, respectively, as a function of separation between DPPC bilayers. The amount of Ca^{2+} bound to DPPC bilayers increases as the $CaCl_2$ solution concentration increases and decreases as the bilayers are forced together.

Figure 3 gives the relationship between net repulsive force and bilayer separation for DPPC bilayers in 10 and 30 mM $MgCl_2$. DPPC bilayers do not swell in 1 mM $MgCl_2$ (Lis et al., 1980). Calculated ψ_b and S are given in Figure 4. Mg^{2+} binding to DPPC bilayers increases with $MgCl_2$ concentration while the binding of Mg^{2+} to DPPC is less than that of Ca^{2+} at corresponding concentrations (Lau et al., 1980; Lis et al., 1980).

Binding of Various Me^{2+} Species to DPPC and DOPC. The strength of the net repulsive force vs. separation between DPPC bilayers in 30 mM solutions decreases in the order $CaCl_2 \simeq CdCl_2 \simeq MnCl_2 > CoCl_2 \simeq MgCl_2 > BaCl_2$ (Figure 5). DPPC bilayers in 30 mM $BaCl_2$ and in water show no detectable difference indicating little Ba^{2+} binding. Surface potential ψ_b and divalent ion area S, calculated from the data in Figure 5, are given in Figure 6. The binding of Me^{2+} to DPPC bilayers increases as $Ba^{2+} < Mg^{2+} \simeq Co^{2+} < Ca^{2+} \simeq Cd^{2+} \simeq Mn^{2+}$. This sequence is in good accord with the observations of McLaughlin et al. (1978) on egg PC and Inoko et al. (1975) on DPPC.

Similar measurements and estimates are given in Figures 7 and 8 for DOPC bilayers examined in 30 mM $CaCl_2$, $CoCl_2$, and $MgCl_2$. The binding of Me^{2+} to DOPC bilayers increases in the sequence $Mg^{2+} < Co^{2+} \simeq Ca^{2+}$. Addition of NaCl at 100 mM concentration increased Co^{2+} binding.

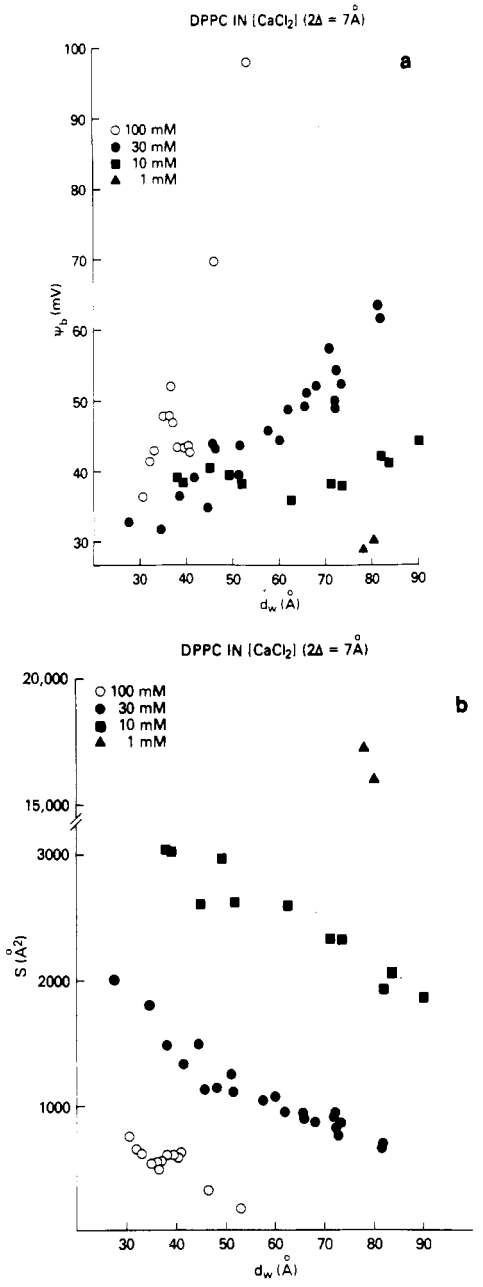

FIGURE 2: Surface electrostatic potential ψ_b (a) and area S per Ca^{2+} bound (b) vs. separation d_w, from data in Figure 1. Divalent ions are assumed to bind in a plane at distance $\Delta = 3.5$ Å from the plane dividing lipid from aqueous regions (Lis et al., 1981).

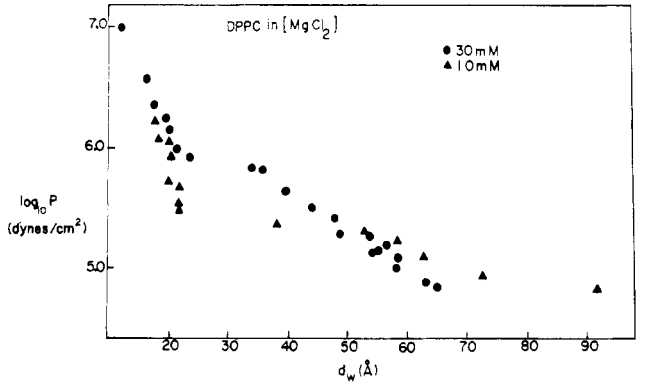

FIGURE 3: Force vs. separation of DPPC in two $MgCl_2$ solutions.

Effect of Acyl Chains on Binding of Ca^{2+} to Phosphatidylcholines. In 30 mM $CaCl_2$, the binding of Ca^{2+} to various phosphatidylcholines increases in the sequence DOPC(m) <

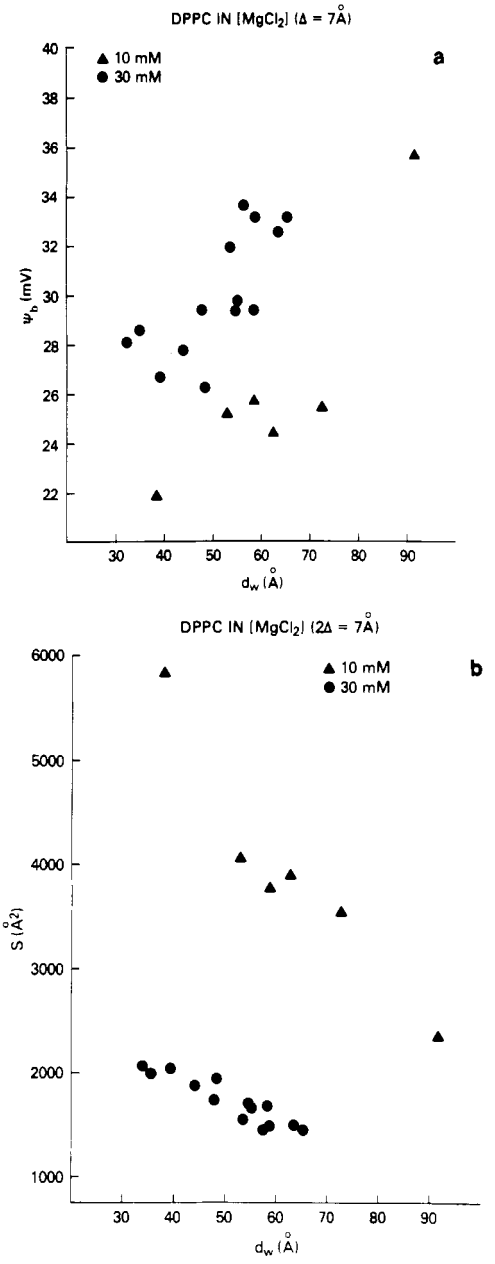

FIGURE 4: Surface electrostatic potential ψ_b and area S per Mg^{2+} bound vs. separation d_w.

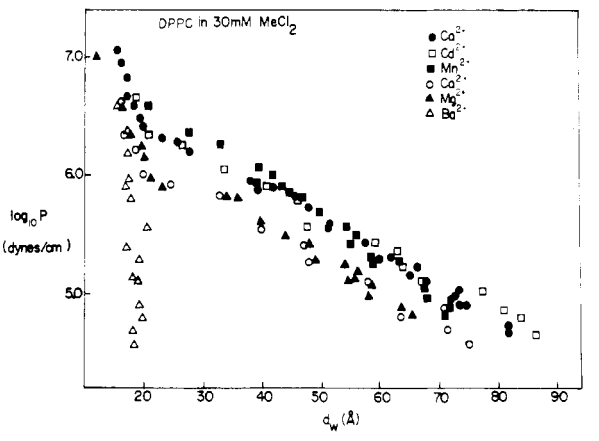

FIGURE 5: Comparison of forces conferred by addition of different divalent cations to DPPC multilayer systems. Except for $Ba^{2+}Cl_2$ to which DPPC seems indifferent, slopes (decay lengths) are similar.

DLPC(m) < DMPC(fr) ≃ DSPC(fr) ≃ DPPC(fr) (figures 9 and 10). Phosphatidylcholines with frozen (fr) acyl chains

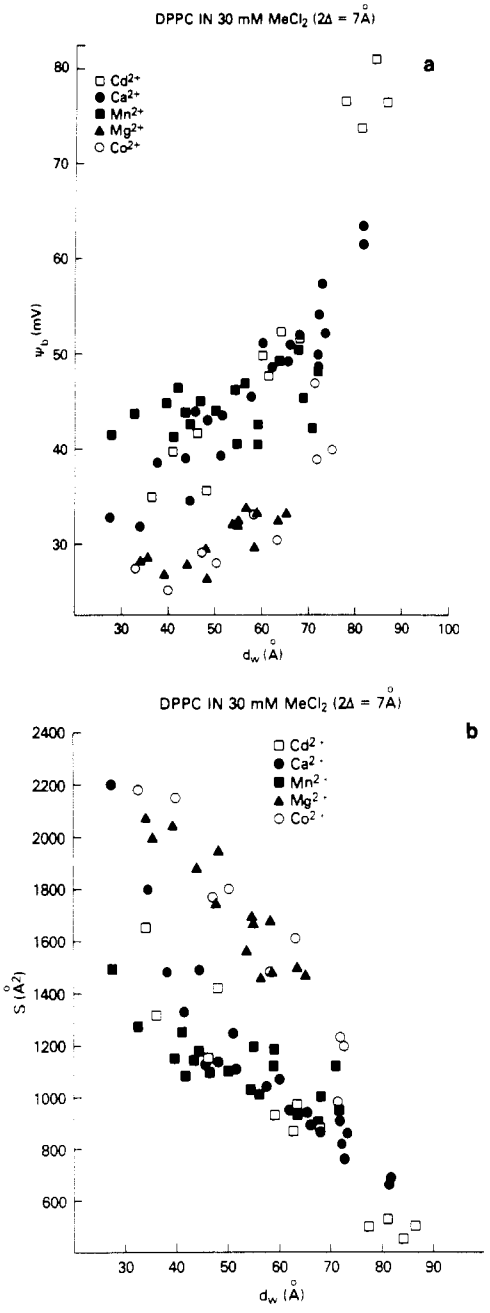

FIGURE 6: Binding plane potentials ψ_b and areas S per divalent cation bound for DPPC in various 30 mM $Me^{2+}Cl_2^-$ solutions.

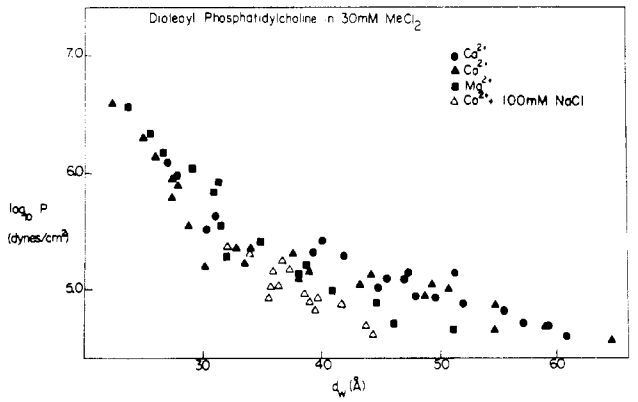

FIGURE 7: Forces created between DOPC bilayers by divalent cations in four different solutions.

apparently bind more Ca^{2+} than those with melted acyl chains (m).

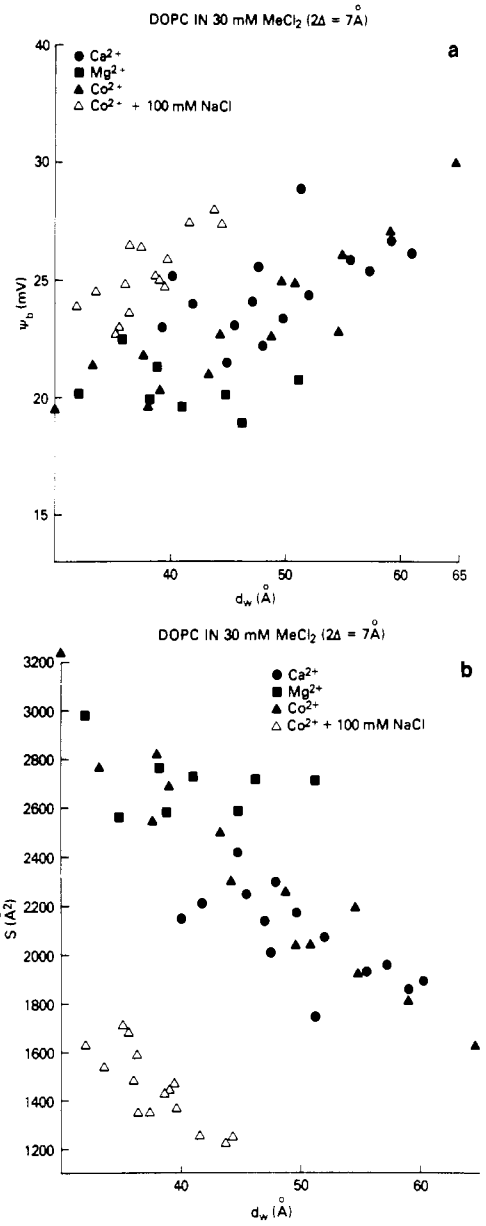

FIGURE 8: Potentials ψ_b and divalent ion areas S for DOPC.

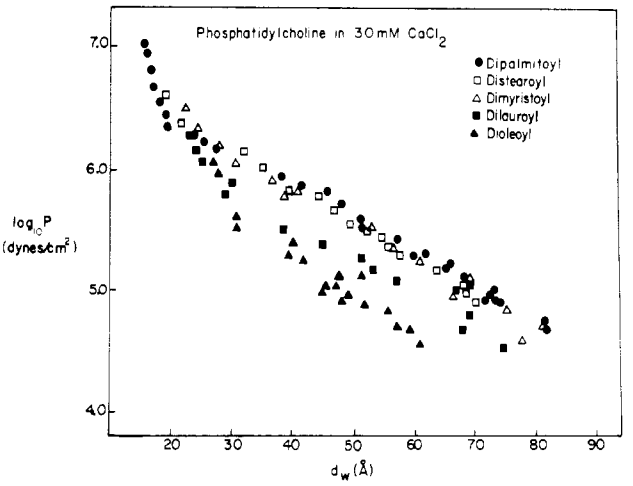

FIGURE 9: Comparison of force vs. interbilayer distance for 30 mM $CaCl_2$ acting on five different phosphatidylcholines.

Phase Separation in Mixed Phosphatidylcholine Systems. The difference in repulsive forces observed between phosphatidylcholine bilayers which differ in their hydrocarbon

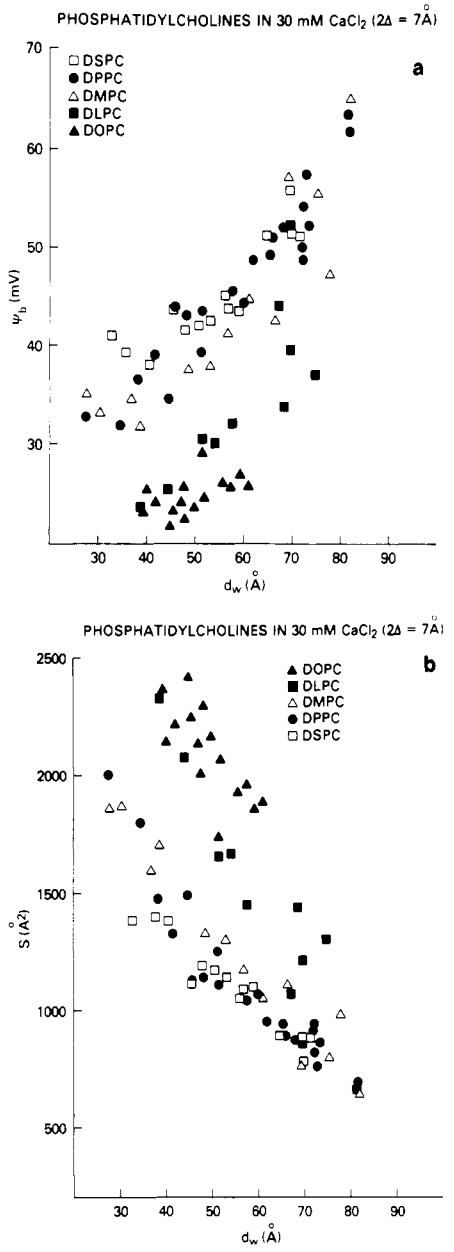

FIGURE 10: Potentials ψ_b and divalent ion areas S for data in Figure 9.

chains leads to a related phenomenon, the separation of mixed PC's into distinct populations when immersed in $Me^{2+}Cl_2^-$ solutions and subjected to osmotic stress. Egg phosphatidylcholine stressed in 30 mM $CaCl_2$ (with or without 100 mM NaCl) (Figure 11), or allowed to swell in excess solutions of varying $CaCl_2$ concentration (Figure 12), always separates into two distinct lamellar phases. Because the lipid composition of the separated phases is not yet known, one can only plot the repeat spacing rather than interbilayer distance. The lamellar separation is observed until the force rises to $10^{5.5}$ dyn/cm^2 above which all systems show only the more powerful "hydration" forces characteristic of all phosphatidylcholines (Figure 11).

To establish that the Ca^{2+}-induced separation into two lamellar phases had to do with differences in acyl chain, we stressed 1:1 mixtures of DOPC/DLPC. These mixtures displayed single lattices in pure water, but two lamellar lattices in 30 mM $CaCl_2$. Limited evidence suggests that DMPC/DLPC and DOPC/DMPC also show phase separation in 30 mM $CaCl_2$ (Figure 13 and Table I).

In all cases observed, one of the two lattices appears to swell

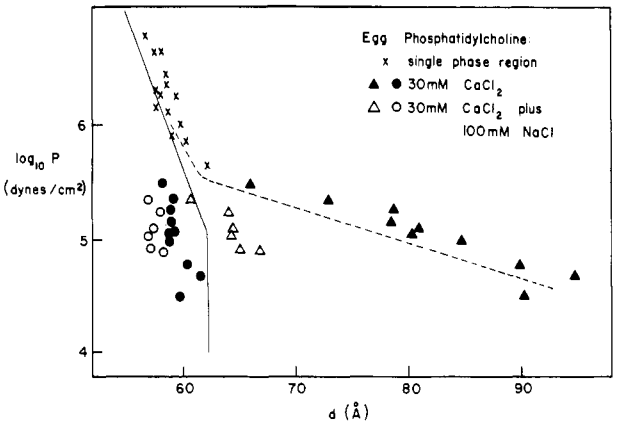

FIGURE 11: Ca^{2+}-induced separation of egg yolk phosphatidylcholine into two multilamellar phases. (This occurs with or without 100 mM NaCl.) Osmotic force P vs. repeat spacing d. (Bilayer separation d_w cannot be easily determined because bilayer thickness is not known.) (—) Trend for egg PC in water. (---) Trend for DOPC from Figure 7. The swollen phase closely resembles DOPC in 30 mM $CaCl_2$.

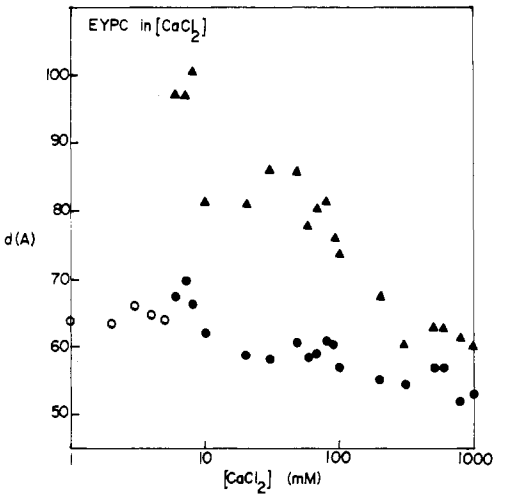

FIGURE 12: Separation of egg yolk PC into two lamellar phases when immersed in an excess volume of $CaCl_2$ solution. With increasing $CaCl_2$ concentration, the swelled phase decreases its repeat spacing while the other behaves like uncharged phospholipid. Open circles designate single-phase samples.

Table I: Lamellar Repeat Distances (d Spacings) for 1:1 Mixtures of Synthetic Phosphatidylcholines in Water and 30 mM $CaCl_2$ Taken at 25 °C[a]

	d spacing(s) (Å)	
lipid mixture	pure water	30 mM $CaCl_2$
DOPC/DLPC	58.3	59.6 72.1
DMPC/DLPC	61.7	64.8 78.1
DOPC/DMPC	61.3	59.7 80.7

[a] Bilayers were allowed to swell by competition with a 5.5 wt % dextran solution across a dialysis membrane.

negligibly while the other follows a force vs. repeat spacing decay like that of a pure PC in 30 mM $CaCl_2$. Until it is possible to separate these two phases, which must extend over several layers or else they wouldn't diffract X-rays independently, one cannot tell their composition. From figures 9 and 10, we expect a demixing to allow Ca^{2+} to bind to the species of higher surface density.

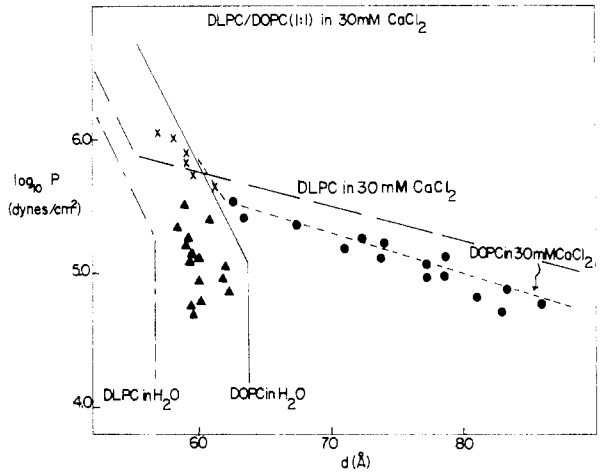

FIGURE 13: Formation of two lamellar phases by equimolar mixtures of DLPC and DOPC. The swollen phase resembles DOPC.

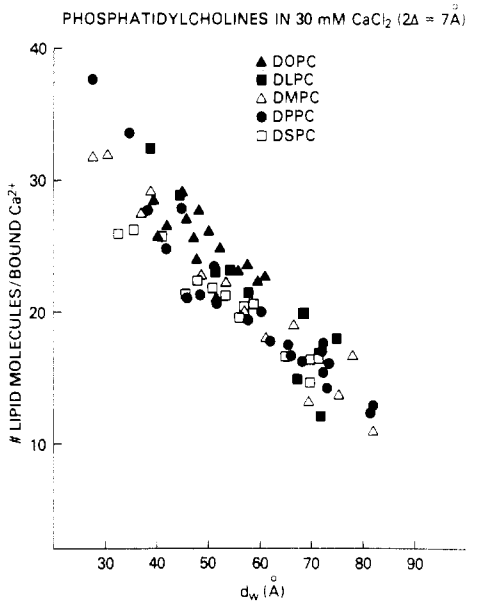

FIGURE 14: Ratios of the number of phospholipid molecules per Ca^{2+} bound. Even while it changes with separation d_w, this ratio is not detectably dependent on phospholipid acyl chain length or conformation.

Discussion

The binding of divalent ions to interacting bilayers of phosphatidylcholine seems to depend on every variable we have examined: ion type, ion concentration, the presence of NaCl, lipid hydrocarbon chain, and bilayer separation. The ratios S/A, i.e., the number of phospholipid molecules per Ca^{2+} ion bound (Figure 14), follow the same function of d_w for all these synthetic phosphatidylcholines. At any given separation d_w, the amount of bound Ca^{2+} per lipid molecule appears to be independent of PC species.

Why, Then, Should Ion Binding Change with Bilayer Separation d_w? The easiest, but most disturbing, interpretation is that binding only appears to change because the intervening 30 mM $Me^{2+}Cl_2^-$ salt solution does not provide the exponential screening length expected of it from double-layer theory. For all ions, except Ba^{2+} which does not have any effect on bilayer forces, the decay of force vs. distance (Figure 3) is consistently exponential but slower than that expected from the Debye screening length.

In Table II we list apparent and expected Debye lengths for several preparations. The large deviations seen at low ionic strength are especially surprising. In this regime one expects traditional double-layer theory to be accurate.

Table II: Theoretical and Observed Decay Lengths

salt	1/K(theor) (Å)	lipid	1/K(exptl) (Å)
1 mM $CaCl_2$	55.4	DPPC	
10 mM $CaCl_2$	17.5	DPPC	24.9
10 mM $MgCl_2$		DPPC	36.5
30 mM $CaCl_2$	10.1	DLPC	18.2
		DMPC	15.5
		DPPC	15.5
		DSPC	14.1
		DOPC	12.8
30 mM $MgCl_2$		DPPC	13.5
		DOPC	9.4
30 mM $MnCl_2$		DPPC	12.3
30 mM $CdCl_2$		DPPC	17.0
30 mM $CoCl_2$		DPPC	15.5
30 mM $CoCl_2$		DOPC	21.2
30 mM $CaCl_2$ + 25 mM NaCl	8.9	DPPC	12.4
30 mM $CaCl_2$ + 50 mM NaCl	8.1	DPPC	11.4
30 mM $CaCl_2$ + 75 mM NaCl	7.5	DPPC	9.2
30 mM $CaCl_2$ + 100 mM NaCl	6.9	DPPC	9.4
100 mM $CaCl_2$	5.5	DPPC	10.4

We do not think it likely that anomalous ionic screening is the cause of the slow decay of repulsion. Measurements on charged phospholipids in water (Cowley et al., 1978) do not show this anomalous exponential decay of the repulsive force.

Is Ion Binding Perturbed by Approaching Bilayers? We expect rather that the explanation more likely involves peculiarities of ion binding to the phosphorylcholine polar group. Should ion binding change polar group orientation relative to the plane of the bilayer (Brown & Seelig, 1977), then any force which changes orientation should change binding affinity. The large dipole moment of the phosphorylcholine zwitterion might allow it to turn under the influence of an electric field from an approaching charged bilayer, although the turning energies available will be fairly weak.

Induction of Phase Separation. Quite distinct from the puzzle of anomalous screening is the dramatic appearance of two distinct lamellar phases effected only by the addition of divalent ions to multilayers of mixed PC's (Figures 11–13). Here, the lipids differ only in their hydrocarbon chains. The different force vs. distance curves shown by bilayers of these same lipids in their pure state (Figure 9) are an apparently sufficient reason for separation when mixed in excess $CaCl_2$ solution or when stressed by osmotic pressure. It is important therefore in studying mixed lipid systems, such as by electrophoretic measurements or by the spectroscopic techniques of ESR and NMR, for example, to recognize the possibility of lipid segregation and to interpret results with some caution. Because of the likelihood of phase separation, such measurements may be from only one of these phases or may be a mixed signal from more than one structure.

Modification of Bilayer Structure or Mutual Approach. In several ways, we can now recognize ways in which mutually interacting bilayers, and cell membranes, can affect each other as they approach to within tens of angstroms. We can expect lateral separation of the lipids to occur in vesicles where the more strongly repulsive molecules may be expected to evacuate the areas of closest approach (Rand et al., 1979; Rand, 1981). This possibility has also been noted for phospholipid cholesterol mixtures (Rand et al., 1980) and is now likely even for phosphatidylcholines in solutions containing divalent ions.

We can now expect also that the ion binding affinity itself changes as membranes approach. For the case of the phosphatidylcholines, which bind relatively weakly compared to anionic phospholipids, there will be strong desorption of divalent ions as surfaces come together (Figures 2, 4, 6, 8, and 10). In our present studies the amount of cationic material bound and eventually desorbed is not small; it is of the same magnitude as that which is in solution between bilayers.

Several kinds of study appear potentially instructive in bringing out qualitative features of divalent ion action on phospholipids. First, resonance experiments of the kind discussed by Brown & Seelig (1977) might be used to test the idea that polar group orientation changes, not only with ion adsorption but also with the mutual approach of bilayers. Second, analysis of physically separated lamellar phases created by addition of divalent ions would allow one to relate phase separation to the energetics of ion adsorption. Third, more theoretical study of the anomalous decay shown here might clarify the responsible molecular properties. The recent work of Copeland & Andersen (1980) is worthy of study in this connection.

Acknowledgments

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