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ANION INFLUENCE ON THE BINDING OF DIVALENT CATIONS TO PHOSPHATIDYLCHOLINE

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We have used the osmotic pressure technique of Rand, Parsegian and co-workers (Nature 259 (1976) 601–603) to investigate the effect of anion species on the binding of M^{2+} to dipalmitoylphosphatidylcholine bilayers. Calcium and magnesium salts show a complex behavior which is consistent with both anion binding and screening. We observe virtually no change, within the accuracy of our experiment, in the decay of repulsive pressure with inter-bilayer separation for the acetate and nitrate salts of magnesium and calcium; however, the chloride salt does show a different pressure decay. At any given bilayer separation, 35 Å $< d_w <$ 75 Å, with calcium and magnesium salts present, the anions produce a decrease in the repulsive pressure in the order acetate$^-$ > Cl^- > NO_3^-.

Introduction

The presence of divalent salt solutions has been shown to modify the characteristics of the electrostatic interactions between bilayers made from dipalmitylphosphatidylcholine (DPPC). Mitsui and co-workers [1] have measured the repeat spacing of DPPC bilayers as a function of the concentration of calcium chloride and magnesium chloride. A dramatic change in the *d*-spacing was noted for molarities between 1 and 100 mM. These workers theoretically modelled the forces necessary to produce such an effect using the Debye-Huckel approximation for the electrostatic interaction [2,3]. Lis et al. [4] corroborated these findings and went on to measure the forces between phosphatidylcholine bilayers in divalent cation solutions using the osmotic pressure technique of Rand and Parsegian [5,6]. A non-linear solution to the Poisson-Boltzmann equation was derived [7] to analyze these data. The results indicated a decay in the electrostatic potential with decreasing bilayer separation.

Specifically, it was noted [7] for DPPC in 30 mM $CaCl_2$ that binding of Ca^{2+} to DPPC was increased by the addition of up to 100 mM NaCl, the net bilayer repulsion was also decreased, and the association affinity for Ca^{2+} decreases as opposing bilayers approach, with a concomitant lowering of the surface electrostatic potential. In addition, the binding preferences among six alkaline earth cations as well as among five different acyl-chain derivatives of phosphatidylcholine were reported [8].

An increase in the binding of divalent cations to PC bilayers in the presence of NaCl was predicted on theoretical grounds by Gillespie [9]. Experimental indications for the influence of specific anions on the binding of divalent and trivalent

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Abbreviations: DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine.

cations to PCs were derived from differential scanning calorimetry [10], Raman spectroscopy [11,12], electrophoretic mobility [13,14] and NMR [16–18] studies. The latter studies indicate a synergism between cation and anion binding to PC bilayers promoted by a surface potential effect [17]. The order of enhancement of lanthanide binding by specific anions was found to be [18]: $Cl^- < Br^- < NO_3^- < SCN^- < I^- < ClO_4^-$.

In this report we investigate the role of the anion species in the modification of the electrostatic interactions between DPPC bilayers. Salts of calcium and magnesium are used with anion species varied similarly for each metal ion. The subsequent change in anion size and configuration would alter the effective charge density on the bilayer surface if the anions do in fact bind. Otherwise, their presence near the bilayer surface would contribute to the overall screening of the charge, leaving desorption of the cations on the bilayers surface as the principal cause of the observed decay in electrostatic potential. Osmotic pressure is used to exert the necessary counterforce to the electrostatic repulsion of the charged surfaces, and X-ray diffraction is used to infer inter-bilayer separations. Our findings indicate that the decay of the electrostatic force for DPPC bilayers charged by Ca^{2+} and Mg^{2+} adsorption is different for the chloride salt compared with the nitrate and acetate salts. The magnitude of the net repulsive force for any given bilayer separation, $35\ Å < d_w < 75\ Å$, does vary with anion species in the order acetate$^-$ $> Cl^- > NO_3^-$. Since cation binding could be predicted to decrease in a similar manner with respect to anion species, we infer that this effect is indicative of anion screening. The chloride anions, however, may be actually binding to the bilayer surfaces because of the observed change in electrostatic repulsive decay with bilayer separation.

Materials and Methods

DPPC was obtained from Sigma Chemical Co. and used without further purification. Water was double-distilled and salts were reagent grade. Dextran was obtained from Pharmacia Chemical Co. and mixed in known concentrations with salt solutions prior to contact with the phospholipids.

Samples were made by adding lipid directly to 30 mM salt solutions containing dextran. Mixtures were allowed to equilibrate for 48 h and then mounted between mica windows. The sufficiency of this procedure has been established [7,8]. All experiments were carried out at room temperature (25°C).

The X-ray diffraction and force measurements have been previously described [5–8]. Only lamellar phases were observed by a series of X-ray reflections whose spacings were integral orders of the bilayer repeat spacing, d. The bilayer thickness d_l and water layer thickness d_w are determined from the d-spacing using a volume balance relationship. Bilayers forced apart by electrostatic repulsion maintain the same bilayer thickness as in excess water [7]. The method of determining the net repulsive force between membranes from the osmotic pressure of the dextran solution has been previously described [5,6].

Simultaneous measurements of d and the osmotic pressure provide the relationship between bilayer separation and repulsive force [7]. The measurement of this repulsion is a determination of the electrical potential midway between bilayers. The non-linear Poisson-Boltzmann equation governing this potential has been integrated from the midpoint to the bilayer surface from which a bilayer potential can be inferred [7]. The charge density can be determined from Gauss' Law at the boundary.

Bragg diffraction was used to determine the bilayer separation distance. The samples were exposed to 8 KeV X-radiations from a GE XRD-5 X-ray unit. The Cu Kα_1 line was isolated using nickel foils. The X-ray patterns were collected using a collimated slit before the sample and aligning a film cassette at the end of an evacuated plexiglass cylinder behind the sample. The cylinder was evacuated to reduce air scattering. Film, contained in light-tight cassettes, was exposed for times ranging from 2 to 12 h.

Results

Plots of applied pressure versus bilayer separation are presented in Fig. 1 for DPPC in calcium salts, and in Fig. 2 for DPPC in magnesium salts. Both data sets were subjected to least-square analysis for a linear fit of applied pressure as a func-

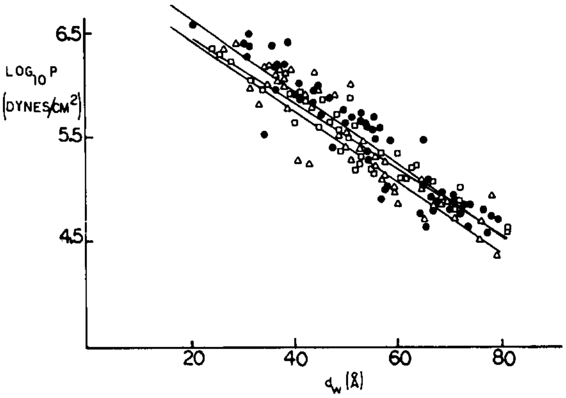

Fig. 1. Applied pressure ($\log_{10}P$) as a function of d_w for DPPC bilayers in calcium acetate (●), calcium chloride (□) and calcium nitrate (△). The solid lines represent least-squares fits of the data.

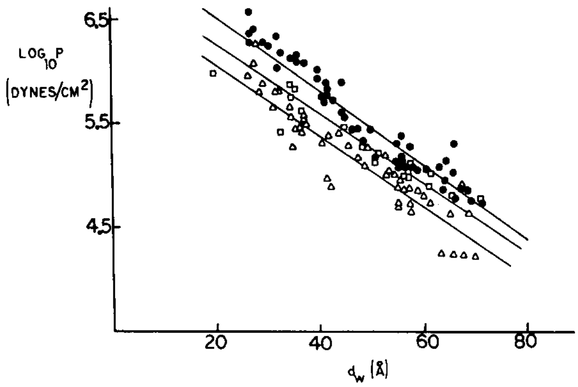

Fig. 2. Applied pressure ($\log_{10}P$) as a function of d_w for DPPC bilayers in magnesium acetate (●), magnesium chloride (□) and magnesium nitrate (△). The solid lines represent least-square fits of the data.

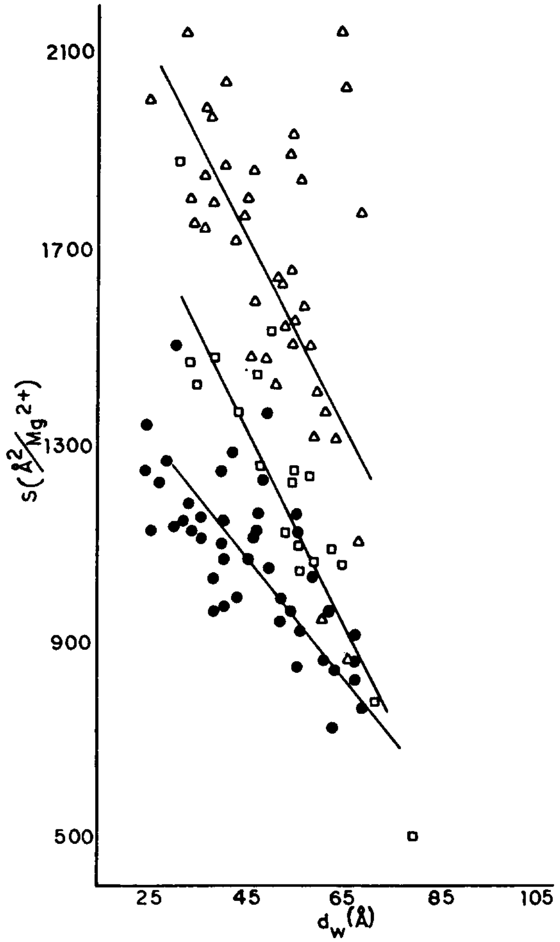

Fig. 3. Effective surface area, S, per Mg^{2+} on the bilayer surface as a function of d_w for magnesium acetate (●), magnesium chloride (□) and magnesium nitrate (△). The solid lines represent least-squares fits of the data.

TABLE I

LEAST-SQUARES SLOPES OF THE DATA IN FIGS. 1 AND 2 FITTED TO $\log_{10}P = \alpha d_w + \beta$

Also shown are the probabilities, P, that any two slopes are the same.

	Calcium		Magnesium	
Acetate	-0.034 ± 0.003		-0.034 ± 0.002	$0.6 < P < 0.7$
		$0.8 < P < 1.0$		
Nitrate	-0.034 ± 0.003		-0.034 ± 0.004	$0.9 < P < 1.0$
		$P < 0.002$		
Chloride	-0.027 ± 0.003		-0.024 ± 0.004	$0.2 < P < 0.3$

TABLE II

LEAST-SQUARES INTERCEPTS (AT $d_w = 0$) OF THE DATA IN FIGS. 1 AND 2 FITTED TO $\log_{10}P = \alpha d_w + \beta$

Also shown are the probabilities, P, that any two intercepts are the same.

	Calcium		Magnesium	
Acetate	7.34 ± 0.17		7.16 ± 0.10	$0.06 < P < 0.08$
		$P < 0.08$		
Nitrate	7.13 ± 0.16		6.74 ± 0.21	$P < 0.01$
		$P < 0.08$		
Chloride	6.92 ± 0.13		6.48 ± 0.22	$P < 0.001$

tion of bilayer separation, and an analysis of variance was performed to infer confidence levels on the slopes and intercepts. Results of these calculations are presented in Tables I and II.

Magnesium and calcium acetate and magnesium and calcium nitrate show a similar decay in electrostatic pressure with increasing bilayer separation. The strength of the electrostatic repulsion at any given bilayer separation is greater for the acetate anion than for the nitrate anion, and the calcium salts produce a greater electrostatic repulsion than the magnesium salt.

The decays of electrostatic pressure in the chloride salts of calcium and magnesium are margi-

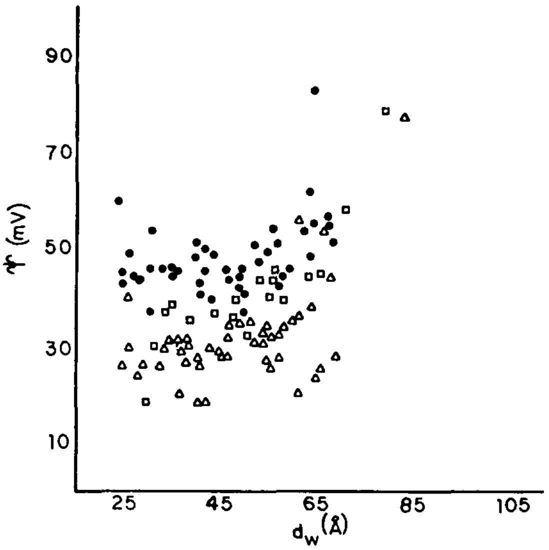

Fig. 4. Surface potential (ψ) as a function of d_w for DPPC bilayers in magnesium acetate (●), magnesium chloride (□) and magnesium nitrate (△).

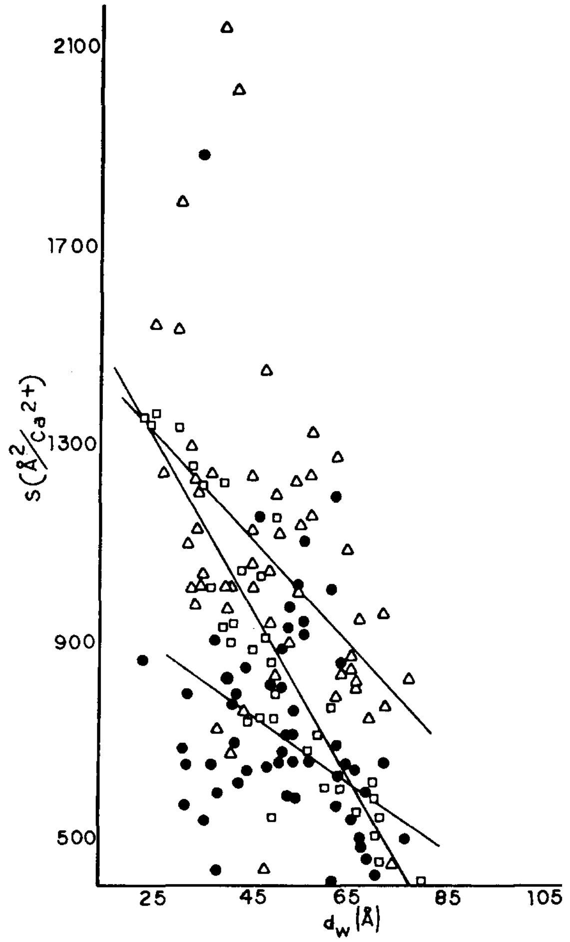

Fig. 5. Effective surface area, S, per Ca^{2+} on the bilayer surface as a function of d_w for calcium acetate (●), calcium chloride (□) and calcium nitrate (△). The solid lines represent least-squares fits of the data.

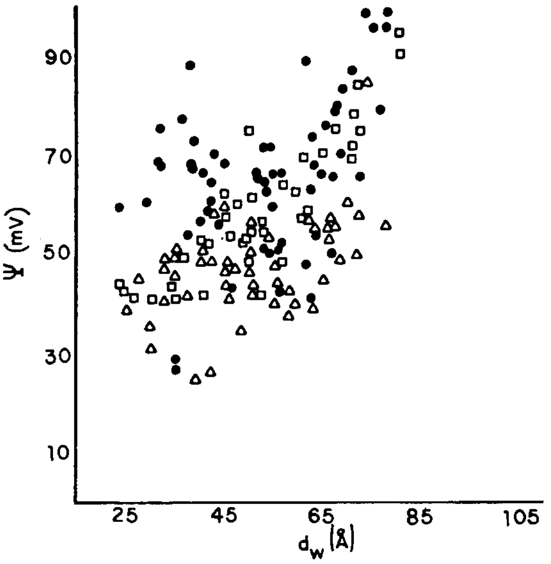

Fig. 6. Surface potential (ψ) as a function of d_w for DPPC bilayers in calcium acetate (●), calcium chloride (□) and calcium nitrate (△).

nally similar, but significantly different from that observed with the acetate and nitrate salts. The calcium salt produces a larger electrostatic repulsion than the magnesium salt with the chloride anion present.

The area per Ca^{2+} and Mg^{2+} at the bilayer surface as a function of bilayer separation, and the surface potential were calculated from the data presented in Figs. 1 and 2 using the solution of the non-linear Poisson-Boltmann equation as described by Lis et al. [7]. We used a dielectric constant of 80 and integrated the Poisson-Boltzmann equation to the bilayer surface. A summary of these calculations is presented in Figs. 3, 4, 5, and 6. The general trend of the data suggests that as the anion species is changed, Mg^{2+} and Ca^{2+} bind to the bilayer surface in the order acetate$^-$> chloride$^-$> nitrate$^-$ with a identical ordering for the increase in surface potential. We can also infer that Ca^{2+} binds more than Mg^{2+} in the presence of a given anion.

Discussion

Divalent cations were shown to exhibit a small but measurable binding affinity for the zwitterionic head groups of DPPC bilayers through NMR, Raman spectroscopic and electrophoretic tests [11–18]. Anion binding was not shown at molarities greater than 100 mM, but the change in the effective potential at lower molarities leads to the possibility that anion species bind at those molarities.

There are in fact three possible explanations for the previously observed changes in the surface potential: (1) the cations affiliated with the DPPC head group desorb with no effective screening between the bilayers, (2) the anions come to be affiliated with the DPPC head groups, consequently reducing the surface charge density on the DPPC bilayers and hence the surface potential as well, or (3) the cations affiliated with the DPPC head groups desorb with screening from anions in the interbilayer region.

The first possibility is not plausible except in the case of anions of great bulk: anions too large to fit within the inter-bilayer region. The only way in which no screening could occur is if there were no anions present between the bilayers. But the cations present in the area attract anions which in turn attract more cations and so forth. No anion used in this report was too large to fit in between the bilayers.

Lis et al. [7] have shown that the decay of the logarithm of applied pressure versus bilayer separation is linear in the region where the electrostatic force is prevalent, $d_w > 25$ Å. If the anions were to bind to the bilayer surface, we would expect to see a change in the surface charge density and a corresponding change in the electrostatic pressure decay. We do observe a change in the surface charge density of Mg^{2+} and Ca^{2+} with a change in anion species, namely, the surface charge density decreases in the order acetate > chloride > nitrate. Thus, if the anions do bind to the bilayer surface, we may infer that NO_3^- binds more than Cl^-, which binds more than $(C_2H_3O_2)^-$. This would be consistent with observations of anion binding as reported by Hauser et al. [18] and Westman and Eriksson [17].

If we assume that screening is the dominant role played by the anions, then by considering the surface potential as a function of bilayer separation we can conclude that NO_3^- screens better than Cl^-, which screens better than $(C_2H_3O_2)^-$.

It is interesting to note that the decays of surface charge density with decreasing bilayer separation (Figs. 3 and 5) appear similar for the acetate and nitrate salts, but differ for the chloride salts. No statistical evaluation of these results was performed, due to the inherent propagation of error through the electrostatic equations [7]. Thus, the decay of the electrostatic repulsive pressure reflects the decay of the surface charge density and is a good measure of it.

An alternative approach to distinguish between binding and screening of the anions is to recognize that the chloride anion is a single atom, much smaller in volume then the bulkier, polyatomic acetate and nitrate anions. Thus, it would be more likely for the chloride anion to adsorb to the bilayer surface in the region of the DPPC choline group than the acetate or the nitrate anions. Our data of the electrostatic pressure versus bilayer separatetion are consistent with this hypothesis. We would then infer that the chloride anion binds, along with calcium and magnesium, to the bilayer surface, and the acetate and nitrate anions effectively screen the surface potential of the bilayers.

Conclusion

The anion species present in calcium or magnesium salt solutions do affect the magnitude of the electrostatic repulsive pressure acting between DPPC bilayers. In the region where the electrostatic force is prevalent, $d_w < 25$Å, the magnitudes of the electrostatic force in either calcium or magnesium salt solutions decrease in the order acetate > chloride > nitrate. These salts exhibit a complex behavior which is consistent with anion binding and screening; however, the chloride anion definitely appears to bind to the surface of DPPC bilayers, whereas the major effect of the acetate and nitrate anions appears to be screening of the surface potential of DPPC bilayers.

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