

DIRECT MEASUREMENT OF THE INTERACTION BETWEEN PHOSPHATIDYLGLYCEROL BILAYERS IN AQUEOUS ELECTROLYTE SOLUTIONS

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ABSTRACT Results are presented of force measurements between deposited bilayers of dimyristoylphosphatidyl glycerol (DMPG) at $T > T_m$, and distearoylphosphatidyl glycerol (DSPG) at $T < T_m$. Below a bilayer separation of 100 nm, a repulsive double-layer force is measured, which can be explained through the combined screening and binding effect of the counterions in electrolyte solutions of NaCl, HCl, CaCl_2 , or mixtures of these. The binding of cations to bilayers in the fluid phase (DMPG) appears to be greater than to bilayers in the gel phase (DSPG). At shorter range, below ~ 3 nm, an attractive interaction is measured in solutions containing CaCl_2 , which was found to be slightly stronger than the theoretically expected van der Waals interaction. No hydration force was observed to exist in solutions containing CaCl_2 . In NaCl solutions, the measured interbilayer force can completely be accounted for by the electrostatic repulsion, down to a bilayer separation of at least 2 nm, below which no accurate measurements were possible anymore. Parallel measurements on PG monolayers show that the contraction of a DMPG monolayer following addition of CaCl_2 is significantly greater than what is predicted from the change in the double-layer free energy alone. This indicates that changes in the lateral interactions between the lipid headgroups probably involve Ca^{2+} -bridge binding and/or a possible dehydration of the lipid headgroups through Ca^{2+} binding. The results shed new light on both the interbilayer and intrabilayer interactions of PG and identify the possible factors responsible for the morphological behavior of PG aggregates.

INTRODUCTION

In the last decade, considerable progress has been achieved in the understanding of the physical principles of membrane structure and membrane interactions (1, 2). It is recognized that the organization of and physical interactions between membranes are closely related to the chemical composition of the lipids that constitute the membrane. Important experimental work in this area has been done on various liquid-crystalline structures (3, 4), especially lamellar systems (5, 6), and more recently on deposited bilayers (7, 8), whose physical properties can be investigated by a variety of techniques. Especially, parameters like the hydrocarbon chain length, unsaturation of hydrocarbon chains, the phase state of the lipids, the nature of the polar headgroup, and the aqueous electrolyte environment appear to play a crucial role in this respect. They are also important for explaining the often-observed phase separations in bilayers composed of mixtures of lipids (9). The investigation of bilayer interactions is of relevance for understanding phenomena involving intermembrane coupling, such as cell adhesion, membrane stacking, vesicle stability, and membrane fusion.

In a recent series of papers (7, 8, 10), the interactions between phosphatidylcholine (PC), phosphatidylethanolamine (PE), and galactolipid bilayers have been reported, using an experimental device capable of measuring directly the force between two deposited or adsorbed lipid bilayers as a function of the distance between them. All these lipids are intrinsically uncharged. An attractive van der Waals interaction and (below 25 Å separation) a short-range steric/repulsive hydration interaction were measured and related to headgroup properties and the phase state of the hydrocarbon interiors of the bilayers. The steric/hydration force, which is particularly important in membranes composed of PC lipids, prohibits the bilayers from coming into close contact and regulates the magnitude of the adhesion energy and the equilibrium bilayer separation. At the equilibrium bilayer separation, a balance exists between the van der Waals attraction and the steric/hydration repulsion.

Upon addition of millimolar amounts of divalent ions like Ca^{2+} or Mg^{2+} , an additional repulsive electrostatic double-layer force was identified between PC or PE bilayers. This electrostatic double-layer force arises from the adsorption of the cations to the bilayer surface, which gives the bilayers a net surface charge. A simple mass-action model, with charge regulation based on the Langmuir adsorption isotherm and the Poisson-Boltzmann

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equation, proved to be sufficient to explain the observed electrostatic repulsion as a function of the surface separation and the electrolyte concentration.

The results reported here represent an extension of this work to negatively charged bilayer membranes composed of distearoylphosphatidyl glycerol (DSPG) or dimyristoylphosphatidyl glycerol (DMPG). Phosphatidyl glycerol is the major negative lipid in bacterial and plant membranes (11). It carries a single charged phosphate group and has a phase behavior that is fairly well studied (9).

A few studies on PG monolayers at the air-water interface have been published (12,13), which have revealed the importance of the effect of the ionic species in solution on the lateral headgroup interaction. An important bilayer study on the adsorption of divalent ions to PG has been published by Lau et al. (14), using electrophoresis and NMR. From the measured ion adsorption, the electrostatic bilayer repulsion can be predicted in principle using the theory of the diffuse double-layer (15).

Cowley et al. (16) have measured the repulsive force between PG bilayers (without added electrolyte) using an osmotic stress technique. Apart from the expected double-layer force, they report the existence of an additional short-range hydration repulsion below a bilayer separation at 30 Å.

A full study on bilayer interactions should not only account for repulsive forces, but also for attractive and adhesion forces. Only repulsive interactions can be measured with the osmotic stress technique. The force measurement technique of Israelachvili on the other hand (17) allows both repulsive and attractive forces to be measured accurately, and is particularly convenient for the study of bilayer interactions when the bilayers are deposited on molecularly smooth mica surfaces using the Langmuir-Blodgett deposition technique (10). In the present study, an investigation is carried out to determine the binding affinity of Na^+ , H^+ , and Ca^{2+} to PG bilayers from the measured interaction force between the bilayers. This is done both for the bilayers in the gel phase (DSPG) and in the liquid phase (DMPG). Furthermore, the force of adhesion and the possible existence of a hydration force is examined. Finally, the contraction of a DMPG monolayer at the air-water interface, after addition of CaCl_2 , and its implication for the morphology of PG aggregates is given attention.

MATERIALS AND METHODS

High purity DSPG and DMPG lipids were purchased from Avanti Polar Lipids Inc. (Birmingham, AL) in powder form as the sodium salt. Water was purified by distillation, treatment overnight with activated charcoal, filtration, and a final distillation from an all-glass still. Potassium orthophosphate (KH_2PO_4), calcium chloride, and sodium chloride were all analar grade and used without further purification. Hexane and chloroform (AR) were further purified by double distillation.

Monolayer compression isotherms were measured in an all-Teflon Langmuir trough; the surface tension measured using the maximum pull on a vertical rod method (18). Both DSPG and DMPG lipids were spread from a chloroform solution on water.

To investigate the Langmuir-Blodgett desposition of the lipids onto mica, a series of calibration depositions were carried out on large mica sheets of 40 cm² surface area to obtain the transfer ratios at different deposition pressures. The transfer ratio is defined as the area per molecule in the monolayer on water to that actually being deposited onto the mica. To deposit a bilayer, two depositions have to be carried out consecutively. It was found in previous work (10) that the deposition of a first layer of dipalmitoyl-phosphatidylethanolamine (DPPE) on mica at a surface pressure of 32 dyne/cm, gives a very hydrophobic outer surface (head-groups down on the mica). This surface is particularly suitable for deposition of a second lipid layer, and in the present work, a PG layer was deposited at various surface pressures on top of a DPPE layer when the mica was slowly brought down into the water again. The deposited head group areas were chosen to be close to those pertaining to the phase state of the lipid bilayers in water. A detailed description of these procedures can be found in reference 10 where the deposition of bilayers of zwitterionic lipids is discussed.

Interactions between bilayers were measured using the direct force measurement technique of Israelachvili. This technique is described in detail elsewhere (17) and for the present purpose, a short summary will suffice. Two thin molecularly smooth mica surfaces are silvered on one side (with a 500 Å-thick highly reflecting silver coating) and glued down on two cylindrically curved silica-glass disks, with the silvered sides down (curvature radius $R \approx 1$ cm). Before these glass disks are mounted in the force-measurement apparatus, a bilayer is deposited on each mica surface, applying a surface pressure in the Langmuir trough that was found to give the desired transfer ratios of lipids on the large calibration sheets. Following this deposition, the glass disks with the mica plates attached are transferred under water in small glass beakers from the Langmuir trough to the force measurement apparatus, previously filled with water, where they are mounted. The bilayers have to remain immersed in water throughout since the bilayers lose their outer monolayer on being retracted from water.

To maintain equilibrium between the lipid molecules in the bilayers and free lipid monomers in solution, the water in the apparatus was always presaturated with lipid monomers by adding a lipid crystal to the water the previous day. This precaution is particularly important for fluid state DMPG bilayers, since their solubility in pure water appeared to be such that in case no presaturation with lipid monomers was established, the outer DMPG monolayers desorbed within a few hours. Presaturation from a lipid crystal for a time period of ~ 18 h was sufficient to carry out reproducible experiments (also in the presence of CaCl_2) with no indication of any bilayer desorption from the mica surfaces for at least one day. For gel state DSPG bilayers, these precautions were less important, probably because of the much lower solubility of DSPG monomers, and reproducible experiments could be carried out even in pure water.

In the force measurement apparatus, one of the glass disks is mounted on a rigid support, the other, facing the first one is positioned on a spring with a known spring constant. Now, when the bilayer surfaces are brought to close separation, the surfaces will experience an interaction from each other that can be measured through the deflection of the spring. The separation between the two surfaces is obtained by use of an optical technique using fringes of equal chromatic order or FECO interferometry (20). From the position and the shape of the FECO fringes seen in the spectrometer, the distance between the two bilayers can be measured to an accuracy of 1–2 Å. By measuring the surface force as a function of the surface separation down to surface contact, the full force law is obtained. Electrolyte was added by injection into the apparatus; the temperature was controlled by heating the whole room.

RESULTS

Monolayer Compression Isotherms

Fig. 1 shows the monolayer compression isotherms of DSPG (22°C) and DMPG (27°C) at various electrolyte concentrations.

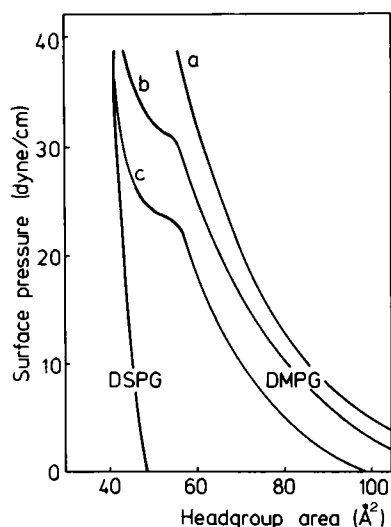


FIGURE 1 Monolayer compression isotherms of DSPG (22°C) and DMPG (27°C) at various electrolyte concentrations. (a) 1 mM phosphate buffer pH 6.9, 0.01 mM EDTA. (b) 100 mM NaCl + 1 mM CaCl_2 , pH 5.5. (c) 1 mM NaCl + 1 mM CaCl_2 , pH 5.5.

The DSPG monolayer is clearly in the gel phase, remains unaffected by changing the electrolyte concentration, and has a limiting headgroup area of 42 Å^2 .

The DMPG monolayer isotherm on a 1 mM phosphate (pH 6.8) subphase, is of the liquid-phase type and shows only a minor contraction of $\sim 1.5 \text{ dyne/cm}$ upon raising the electrolyte concentration to 100 mM NaCl (isotherm not shown). However, a pronounced contraction and a phase transition is observed following addition of 1 mM CaCl_2 (no buffer added; pH 5.5). Clearly, Ca^{2+} raises the phase transition temperature of the DMPG monolayer which is 24°C when no divalent ions are presented (9). The effect of Ca^{2+} furthermore depends on the NaCl concentration. A quantitative analysis of the effect of Ca^{2+} will be carried out at the end of this section when from an analysis of the force measurements, the binding affinities of Na^+ and Ca^{2+} to the DMPG headgroups have been determined.

Langmuir-Blodgett Deposition

Fig. 2 shows the deposited headgroup areas of DSPG and DMPG on a first layer of DPPE. In experiments where the interbilayer forces were to be measured, DSPG was deposited at a surface pressure of 32 dyne/cm giving a headgroup area of 42 Å^2 . DMPG was deposited at 38 dyne/cm ($T = 27^\circ\text{C}$) which leads to a deposited headgroup area of $62 \pm 2 \text{ Å}^2$.

Although the exact equilibrium headgroup areas of these lipids in bilayers are not known, a chosen headgroup area of 42 Å^2 for deposited DSPG lipids ensures that the bilayer is in the gel phase, since it is consistent with the closest possible packing of these lipids without a chain tilt. The equilibrium headgroup area of fluid state DMPG lipid bilayers in a monovalent electrolyte solution at pH 6.9 is most likely somewhere in between $60\text{--}70 \text{ Å}^2$ (16).

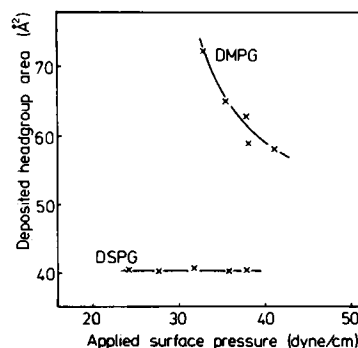


FIGURE 2 Deposited headgroup areas on a hydrophobic DPPE monolayer of DMPG (27°C , pH 6.9) and DSPG (22°C , pH 5.5), as a function of the applied surface pressure in the Langmuir trough.

In previous work on deposited phosphatidylcholines (7), it was demonstrated that a bilayer asymmetry on mica is unimportant. The deposition of a fluid state PC monolayer on a gel state PE monolayer (deposited as a first layer on mica) was shown to leave the outer PC monolayer in the fluid phase, and the measured forces were essentially those between fluid state PC bilayers. Hence, we are confident that also the deposition of a fluid phase DMPG monolayer from the air/water interface in a 1 mM phosphate buffer solution pH 6.9, at a headgroup area of $\sim 62 \text{ Å}^2$, leaves the outer deposited monolayer in the fluid state as long as the temperature is above the transition temperature $T_m = 24^\circ\text{C}$ (9).

Forces between DSPG Bilayers

Location of the Outer Helmholtz Plane and the Van der Waals Plane. Forces between deposited DSPG bilayers were measured in electrolyte solutions with concentrations up to 100 mM for NaCl and 10 mM for CaCl_2 . This range includes the physiologically interesting ionic environment of 100 mM monovalent electrolyte with about 1 mM divalent electrolyte.

Fig. 3 gives the forces in 10^{-4} M to 10^{-1} M NaCl as a function of the bilayer separation. In Fig. 4, the forces in the presence of $10^{-5}\text{--}10^{-3} \text{ M}$ CaCl_2 are given. Fig. 5

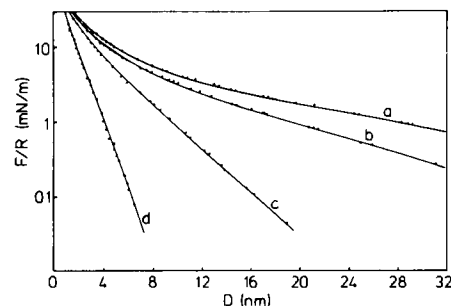


FIGURE 3 Measured forces between two DSPG bilayers (22°C , pH 6.9) at various NaCl concentrations. See also Table I. The solid lines are the theoretically predicted forces assuming fully charged bilayers and a Hamaker constant $A = 6 \times 10^{-21} \text{ J}$. (a) 0.3 mM NaCl. (b) 1.1 mM NaCl. (c) 9.2 mM NaCl. (d) 100 mM NaCl.

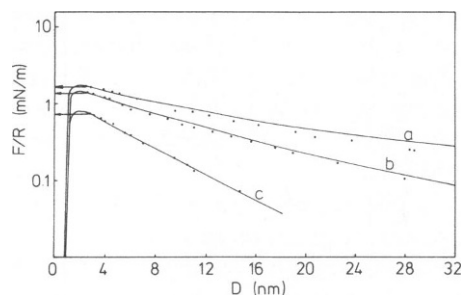


FIGURE 4 Measured forces between two DSPG bilayers (22°C, pH 5.5) at various CaCl_2 concentrations. The arrows give the surface separations from where a jump into adhesive contact occurred. The solid lines are the theoretically predicted force curves using the binding constants given in Table I and assuming a Hamaker constant $A = 6 \times 10^{-21} \text{ J}$. (a) 0.01 mM CaCl_2 . (b) 0.12 mM CaCl_2 . (c) 1.2 mM CaCl_2 .

represents the forces measured in mixtures of NaCl and CaCl_2 . An important problem in these experiments is the determination of $D = 0$: the outer Helmholtz plane (OHP), or the plane from where the diffuse double-layer originates. It is evident from Fig. 3 that contact at $D = 0$ cannot easily be attained in a monovalent electrolyte solution at the present concentrations. At surface separations $< 20 \text{ \AA}$, the double-layer repulsion becomes so strong, that the supporting curved mica surfaces locally begin to flatten. However, when at the end of an experiment a small amount of CaCl_2 was added, there was a drastic reduction of the double-layer force (see Figs. 4 and 5) and at close separation a sudden jump into adhesive contact. This contact was chosen as $D = 0$ for the following reasons:

(a) When forcing the two adhering surfaces further together under a large load, they appear to behave essentially as a hard wall: no hydration force was observed.

(b) Slowly draining the apparatus of water removes the outer DSPG monolayers from the mica surfaces, exposing only the hydrophobic DPPE layers, which can be brought into contact again in air. A comparison of the surface contacts before (in the presence of CaCl_2) and after the drainage with the FECO technique gave for the thickness

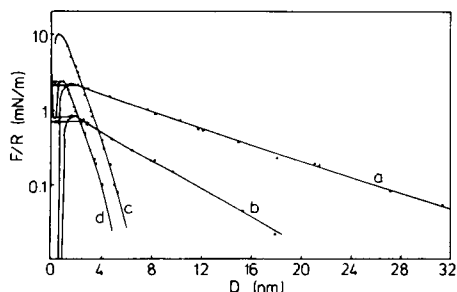


FIGURE 5 Measured forces between two DSPG bilayers (22°C) in mixtures of NaCl and CaCl_2 . See also legend of Fig. 4. In a 100 mM NaCl + 1 mM CaCl_2 solution, an outward jump from $D = 4 \text{ \AA}$ to $D = 20 \text{ \AA}$ was observed. (a) 1 mM NaCl + 0.1 mM CaCl_2 . (b) 1 mM NaCl + 1.2 mM CaCl_2 . (c) 100 mM NaCl + 0.1 mM CaCl_2 . (d) 100 mM NaCl + 1 mM CaCl_2 .

of the two outer monolayers (one DSPG bilayer) a value of $59 \pm 1 \text{ \AA}$. A theoretical calculation for the thickness was carried out using Tanford's formula (20) for the volume v of an alkane hydrocarbon chain in the gel phase containing n carbon atoms: $v = (2 \times 27.4 + n \times 26.9) \text{ \AA}^3$. Choosing the PG headgroup volume as 300 \AA^3 (21) and using the deposited headgroup area of 42 \AA^2 , the thickness T of the bilayer is calculated as $T = 2 \times [2(27.4 + 26.9 \times 17) + 300]/42 = 60 \text{ \AA}$. This value agrees with the experimental thickness, which indicates that the observed hard wall is due to the lipid headgroups and that, at least in the presence of CaCl_2 , there is almost no hydration region near the bilayer surfaces. Assuming that the PG headgroup is laying flat on the surface when two bilayers adhere to each other, the charged phosphate groups must be exposed to this surface, which leads us to locate the position of $D = 0$ (the OHP) at the contact between the two bilayer surfaces.

To be able to quantitatively analyze the bilayer attraction at short bilayer separations in Figs. 4 and 5, we also need to consider the location of the van der Waals plane. The location of the van der Waals plane, defined as the effective lipid/water interface with respect to the van der Waals interaction, will depend on the degree of headgroup penetration into the aqueous phase as has been shown in earlier work on uncharged lipid bilayers (7, 8, 10). This arises because the van der Waals interaction originates not only from the bilayer hydrocarbon interior but also from the less well defined headgroup region of the bilayer. However, when there is little headgroup hydration, as seems to be the case for DSPG bilayers in the presence of CaCl_2 , the PG headgroup penetration into the aqueous phase cannot be expected to be large. For this reason, it appears to be justified to take the OHP position $D = 0$, where all the hydration water has been removed from the bilayer surfaces (i.e., the anhydrous bilayer/water interface) also as the effective van der Waals plane.

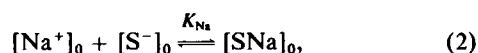
Double-layer Forces in the Presence of NaCl. An analysis of the force curves in Fig. 3 can be carried out by fitting the curves to the theoretical double-layer repulsion with charge regulation (22). This in particular enables us to obtain the surface potential ψ_0^∞ and the surface charge density σ_0^∞ , which exist when the bilayers are well separated (the surface potential and the surface charge change, to some extent, at shorter separations due to regulation of the ion binding as a function of the surface separation [23]).

It is well known that in an aqueous 1:1 electrolyte solution, the ψ_0^∞ and σ_0^∞ are related to each other via

$$\sigma_0^\infty = \frac{\sqrt{C}}{273} \left[2 \cosh \left(\frac{e\psi_0^\infty}{kT} \right) - 2 \right]^{1/2}, \quad (1)$$

where σ_0^∞ is expressed in (absolute) electronic charges per \AA^2 and the electrolyte concentration C in moles/liter. When only binding of Na^+ ions takes place (at $\text{pH} \approx 7$, the

binding of H^+ ions can be ignored; see below) the binding to the surface can, as a first approximation, be described through an intrinsic binding constant K_{Na} , using a mass-action law relating the ion concentration in the bulk $[Na^+]_\infty$ to the number density of bound ions on the surface $[SNa]_0$ (23). The association constant K_{Na} for the reaction



where $[S^-]_0$ is the surface density of charged unbound lipid headgroups, is given by

$$K_{Na} = \frac{[SNa]_0 \exp\left(\frac{e\psi_0}{kT}\right)}{[S^-]_0 [Na^+]_\infty}, \quad (3)$$

where use is made of the Boltzmann distribution of ions in a potential field. Taking $\sigma_0 = [S^-]_0$ together with the total lipid density $[S]_0 = [S^-]_0 + [SNa]_0$, we derive

$$K_{Na} = \frac{([S]_0 - \sigma_0) \exp(e\psi_0/kT)}{\sigma_0 [Na^+]_\infty}, \quad (4)$$

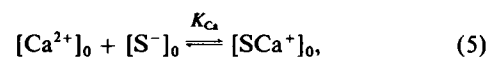
valid for all bilayer separations.

Eq. 4 provides a relation between σ_0 and ψ_0 in terms of K_{Na} and $[Na^+]_\infty$. When the experimental force curves are analyzed with the double-layer force theory (22, 23), it is possible to fit a value of K_{Na} which gives at all surface separations values for ψ_0 and σ_0 consistent with Eq. 4 and the measured double-layer repulsion. In doing so it became evident from Fig. 3 that K_{Na} was very low ($<0.4 M^{-1}$) and the force curves can be fitted adequately by assuming a fully charged bilayer surface. Both the range and magnitude of the measured double-layer forces are in excellent agreement with theory. However, with the present technique an accurate determination of very small binding constants is not possible, since in the limit of high charge

density, the double-layer force becomes fairly insensitive to a small reduction of the surface charge (through ion binding). The results given in Table I for the values of ψ_0^∞ and σ_0^∞ existing at very large bilayer separations, should therefore not be taken too literally and are probably somewhat too high.

Down to a bilayer separation of 20 Å, below which no accurate measurements could be made anymore, no additional short-range repulsive forces need to be invoked to explain the experimental force laws. These observations, however, do not exclude the possible presence of a short-range hydration force in NaCl solutions below $D = 20$ Å (see reference 16).

Double-Layer Forces in the Presence of Mixtures of NaCl and $CaCl_2$. As in the case of monovalent ion binding, also the binding of Ca^{2+} to the bilayer can be described by a mass-action law. Two binding modes of Ca^{2+} to the lipids are possible. The 1:1 binding mode and the 2:1 binding mode where a Ca^{2+} ion bridges two adjacent lipids. For simplicity, we will assume only 1:1 binding to occur. Then (at pH ≈ 7 , where the bilayer surface is fully dissociated from protons)



and the intrinsic association constant K_{Ca} is given by

$$K_{Ca} = \frac{[SCa^+]_0 \exp\left(\frac{2e\psi_0}{kT}\right)}{[Ca^{2+}]_\infty [S^-]_0}. \quad (6)$$

Now, when both Na^+ and Ca^{2+} binding takes place, the equation for the absolute value of the surface charge density becomes

$$\sigma_0^\infty = [S^-]_0 - [SCa^+]_0, \quad (7)$$

TABLE I
DOUBLE LAYER AND ADHESION RESULTS FOR DSPG BILAYERS AT 22°C; ($K_{Na} = 0$)

Electrolyte concentration		Surface potential ψ_0^∞	Surface Charge		Binding constant K_{Ca}	Measured adhesion energy E_0
NaCl	$CaCl_2$		σ_0^∞			
mM	mM	mV	C/m^2	$\text{\AA}^2/\text{charge}$	M^{-1}	dyne/cm
0.3	—	-310	-0.381	42	—	—
1.1	—	-265	-0.381	42	—	—
9.2	—	-212	-0.381	42	—	—
100	—	-154	-0.381	42	—	—
~0.03	0.01	-93	-0.0075	2130	60	0.48
~0.03	0.12	-64	-0.0080	2000	48	0.56
1	0.1	-69	-0.0109	1470	40	0.40
~0.03	1.2	-37	-0.0083	1930	40	0.64
1	0.1	-69	-0.0109	1470	40	0.40
1	1.2	-37	-0.0088	1820	40	0.64
100	0.1	-65	-0.0623	257	40	—
100	1.0	-38	-0.0315	508	40	—
100	10.0	—	—	—	—	0.80

assuming that no charge reversal takes place. For the total lipid density we have

$$[S]_0 = [S^-]_0 + [SNa]_0 + [SCa^+]_0 \quad (8)$$

By solving Eqs. 1, 3, and 6–8 simultaneously, we can express σ_0 and ψ_0 in terms of K_{Na} and K_{Ca} , and both binding constants can be fitted by analyzing the forces measured in solutions that contain a mixture of NaCl and CaCl₂ (the value of K_{Na} is obtained independently of K_{Ca} from measurements in solutions containing NaCl only).

Unlike what was found in Fig. 3, where the measured forces could be explained by taking into account only the double-layer forces between fully charged bilayer surfaces, the analysis of the results in Figs. 4 and 5 indicates that substantial binding of Ca²⁺ ions to the PG headgroups occurs also, which reduces the surface charge density. Values for the various double-layer parameters K_{Ca} , ψ_0^∞ , σ_0^∞ , and the surface area that carries one net elementary charge for various electrolyte solutions, are given in Table I.

In Fig. 4, it was noted that in a 10⁻⁵ M CaCl₂ solution, the experimental decay of the double-layer force with distance is somewhat more rapid than according to theory. This observation is in agreement with earlier work on the double-layer force between mica surfaces (17) in dilute CaCl₂ solutions and might reflect inadequacies of the theory.

From Fig. 5, it is evident that following addition of 0.1 M NaCl to the CaCl₂ solution, the double-layer force becomes of much shorter range but increases in magnitude at small bilayer separations. This is due to the enhanced screening of the surface charge, which displaces many of the adsorbed Ca²⁺ ions from the bilayer surface and increases the surface charge (see Table I). Addition of more CaCl₂ causes the surface charge and the net repulsive force to become smaller: in a 100 mM NaCl + 10 mM CaCl₂ solution, no repulsion could be measured anymore.

Van der Waals Forces and Adhesion Forces. In Figs. 4 and 5, the magnitude of the repulsive forces is much reduced compared to those in a NaCl solution. Below about $D = 25$ Å, a van der Waals attraction becomes apparent which, at closer separation, dominates the total interbilayer force. The bilayers jump into adhesive contact at $D = 0$ from a distance where the gradient of the interaction force equals the spring constant of the spring on which the lower mica plate is mounted, as indicated by the inward arrows in Figs. 4 and 5. In Fig. 5 curve c, the bilayer separation became too small to accurately measure an inward jump.

Taking into account a van der Waals force law between two curved surfaces having the expected form

$$F/R = -\frac{A}{6D^2}, \quad (9)$$

where A = the nonretarded Hamaker constant and $D = 0$ is the effective van der Waals plane, and adding this force

to the double-layer force (DLVO theory [15]), the forces at small bilayer separations can be fitted when Hamaker constants of $8\text{--}10 \times 10^{-21}$ J are used. These values are somewhat higher than the theoretically expected value of $A = 5\text{--}7 \times 10^{-21}$ J for two hydrocarbon layers interacting across water (24). Only in a 100 mM NaCl + 1 mM CaCl₂ solution can the measured force maximum be predicted exactly by using the value $A = 6 \times 10^{-21}$ J. Note that most inward jump distances are large compared to atomic dimensions, hence it seems unlikely that calcium bridging between two apposing bilayers contributes to the jump into adhesive contact.

Upon pulling the bilayers out of adhesive contact, an adhesion force F_0 is needed to separate the surfaces. This measured adhesion force is related to the interfacial free energy per unit area, E_0 , of two planar bilayers at their equilibrium separation D_0 by

$$E_0 = \frac{F_0}{2\pi R}, \quad (10)$$

where R is the radius of curvature of the surfaces. Values for E_0 at various electrolyte concentrations are given in Table I. We see that E_0 has a limiting value of ~ 0.8 dyne/cm when the double-layer force becomes small. Using Eqs. 9 and 10 with $A = 6 \times 10^{-21}$ J, a calculation shows that the corresponding equilibrium bilayer separation should be $D_0 \sim 4$ Å. This agrees with the measured $D = 3\text{--}5$ Å where the curved bilayers jumped apart from each other under a pull-off force. Whether this nonzero distance can be called a hydration layer or not remains subjective. After all, when two bilayers are at their equilibrium separation, a layer of Ca²⁺ ions is present between the surfaces and is expected to have an effect on D_0 through the finite size of the Ca²⁺ ions.

Proton Dissociation from the Bilayer Surface. The proton dissociation from the DSPG surface was investigated by measuring the interbilayer forces at different pH values. The measured forces are given in Fig. 6.

The same formalism, used to describe the binding of Na⁺ ions was used to evaluate the binding of protons. At pH = 4.7 and 4.2 the intrinsic binding constants K_H were $K_b = 129$ M⁻¹ and 150 M⁻¹, respectively. These values should be compared with the literature value (25) for the dissociation of H₂PO₄⁻ in solution (25°C), i.e. $K_H = 132$ M⁻¹ ($pK_H = 2.12$).

At pH > 5, the proton concentration becomes very low and the difference between the force curve of the fully charged bilayer and the partly protonated bilayer becomes relatively small. Under those circumstances, the constants K_H cannot be determined accurately any more. Addition of 10 mM NaCl to a pH 4.7 solution caused the force curve to be already indistinguishable from the force curve in 10 mM NaCl at pH 6.8. Below pH = 4.0, it should be

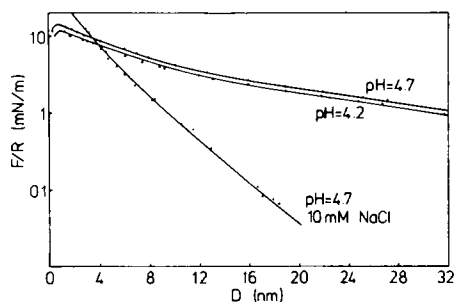


FIGURE 6 Measured forces between two DSPG bilayers (25°C) at pH 4.7 and 4.2. The solid lines are the theoretically predicted forces using a binding constant $K_H = 120 \text{ M}^{-1}$ ($\psi_0^* = -221 \text{ mV}$) or $K_H = 150 \text{ M}^{-1}$ ($\psi_0^* = -191 \text{ mV}$) at pH 4.7 and pH 4.2, respectively. Background electrolyte concentration $\approx 10^{-5} \text{ M}$ monovalent electrolyte. In a 1 mM NaCl, pH 4.7 solution, the experimental (and theoretical) force curve is virtually indistinguishable from the predicted force curve between two fully charged bilayers. No adhesion was observed.

anticipated that the phosphate groups become hydrolyzed from the bilayer surface.

Forces Between DMPG Bilayers

Phase State of the Deposited DMPG Monolayers. A DMPG bilayer in a monovalent electrolyte solution (pH ≈ 7) exists in the fluid state above $T = 24^\circ\text{C}$ (9). The present experiments were performed at $T = 27^\circ\text{C}$. Following addition of CaCl_2 , the phase transition temperature rises through the decrease of the electrostatic free energy of the surface (26). Findley and Barton (9) have shown that isolated DMPG bilayers have their phase transition temperature T_m increased by $\sim 20^\circ\text{C}$ on addition of CaCl_2 and also show complex mesomorphism.

When the bilayer goes through a phase transition, it should contract to a smaller headgroup area and thicken at the same time. However, since the DMPG layer is deposited on top of a DPPE monolayer, the DMPG layer cannot contract to a headgroup area of 42 \AA^2 without exposing a large hydrophobic DPPE surface area to water, which is energetically extremely unfavorable. A possible flip-flop process in the PE monolayer where some of the lipids turn their headgroup towards the water phase to reduce the large hydrophobic surface free energy seems unlikely or at least a very time consuming process, since the PE monolayer is close packed and the PE headquarters are adsorbed and must be largely immobilized on the mica surface. Of course, extra DMPG monomers from solution can be incorporated in the outer monolayer but in regard to the very small DMPG equilibrium concentration ($< 10^{-6} \text{ M}$), this also must be a very slow process. The experiments were carried out in a matter of hours and the headgroup area is expected to be fixed at about 62 \AA^2 , leaving the DMPG layers to remain in the fluid phase whether this is the equilibrium phase or not. Indeed, no indication of a thickening of the bilayers became apparent during the experiments. This is reinforced by the finding that in the

presence of CaCl_2 , where adhesive contact between the DMPG monolayers could be established, the measured thickness of the DMPG bilayer was $40 \pm 2 \text{ \AA}$, i.e. consistent with the calculated thickness $T = 39 \text{ \AA}$ for a fluid phase DMPG bilayer with a headgroup area of 62 \AA^2 (using a fluid hydrocarbon chain density of 0.76 gram/cm^3 and a headgroup area of 300 \AA^3). Together with the observed absence of any anomalous bilayer interaction at short distances (see below), it also shows that there are no complications arising from the possible formation of nonbilayer structures with the lipids of the outer monolayer through the presence of Ca^{2+} .

Double-layer Forces and Van der Waals Forces. Fig. 7 gives the forces between two DMPG bilayers in 1 mM NaCl and mixtures of NaCl and CaCl_2 , all at about pH = 5.5. As was found for the DSPG bilayers, also the DMPG bilayers showed no short-range hydration force in the presence of CaCl_2 when they came into adhesive contact at $D = 0$. Results from the analysis of the double-layer force curves are presented in Table II.

It is apparent that the binding of both Na^+ and Ca^{2+} to DMPG is stronger than to DSPG. The forces between DMPG bilayers in a 1.5 mM NaCl solution are smaller than those expected between fully charged bilayers and can be well fitted with an intrinsic binding constant $K_{\text{Na}} = 0.6 \text{ M}^{-1}$, a value often quoted in the literature (14) for phosphatidylglycerol bilayers. A consequence of the stronger ion binding is that in a 100 mM NaCl + 1 mM CaCl_2 solution, a significant adhesion energy of $E_0 = 0.25 \text{ dyne/cm}$ was measured, whereas no adhesion was observed between two DSPG bilayers in the same electrolyte solution. Using the binding constants in Table II, excellent agreement with theory is obtained at large surface separations, but like what was found for DSPG bilayers, the theoretically predicted force using a Hamaker constant $A = 6 \times 10^{-21} \text{ J}$ is slightly too repulsive at small bilayer separations (i.e. experimentally too attractive). The values

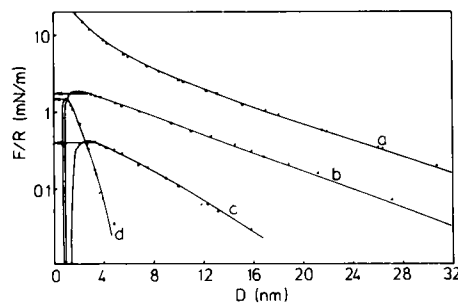


FIGURE 7 Measured forces between two DMPG bilayers (27°C) in a 1.5 mM NaCl solution (pH 6.9) or in solutions containing a mixture of NaCl and CaCl_2 (pH 5.5). The solid lines give the theoretical force curves using the binding constants given in Table II and assuming a Hamaker constant $A = 6 \times 10^{-21} \text{ J}$. The arrows give the surface separations from where a jump into adhesive contact occurred. (a) 1.5 mM NaCl. (b) 1.5 mM NaCl + 0.08 mM CaCl_2 . (c) 1.5 mM NaCl + 1 mM CaCl_2 . (d) 100 mM NaCl + 1 mM CaCl_2 .

TABLE II
DOUBLE LAYER AND ADHESION RESULTS FOR DMPG BILAYERS AT 22°C

Electrolyte concentration		Surface Potential ψ_0^{∞}	Surface charge σ_0^{∞}	$\text{\AA}/\text{charge}$	Binding constant		Measured adhesion energy E_0
NaCl	CaCl ₂				K_{Na}	K_{Ca}	
<i>mM</i>	<i>mM</i>	<i>mV</i>	<i>C/m²</i>		<i>M⁻¹</i>	<i>M⁻¹</i>	
1.5	—	-191	-0.0970	165	0.6	—	—
1.5	0.08	-60	-0.0088	1820	0.6	100	0.64
1.5	1	-28	-0.0056	2857	0.6	100	0.96
100	1	-32	-0.0260	615	0.6	60	0.25

for K_{Ca} decrease almost a factor of two when the NaCl concentration is increased.

The Electrical Double-layer Free Energy of a DMPG Monolayer

With the results on the surface potentials and binding constants of DMPG bilayers, we can now return to the monolayer compression isotherms in Fig. 1 and analyze the observed changes in the surface pressure Π as a function of the electrolyte concentration. Because the compression isotherms give an insight about the lateral interactions existing between adjacent lipid molecules in a monolayer or bilayer, this analysis should provide an idea about the importance of electrostatic interactions on the total lateral interaction.

From standard double-layer theory (27), the electrical double-layer contribution Π^{el} to the surface pressure Π of a charged monolayer is given by

$$\Pi^{el} = \int_0^{\psi_0^{\infty}} \sigma_0^{\infty} d\psi_0^{\infty}. \quad (11)$$

The general expression for σ_0^{∞} in terms of ψ_0^{∞} is

$$\sigma_0^{\infty} = \frac{\text{Sgn}(\psi_0^{\infty})e}{273} \left\{ \sum_j C_j \left[\exp\left(\frac{-ez_j\psi_0^{\infty}}{kT}\right) - 1 \right] \right\}^{1/2}, \quad (12)$$

where C_j stands for the bulk concentration of the j th ionic species (in mol/liter) with valency z_j , and σ_0^{∞} is expressed in $C/\text{\AA}^2$.

In the general case of a mixture of 1:1 and 2:1 electrolyte, Eq. 11 has to be integrated numerically. When only a 1:1 electrolyte is present or when the 2:1 electrolyte concentration is much smaller than the 1:1 electrolyte concentration, Eq. 1 can be used rather than Eq. 12 and Eq. 11 is integrated readily to

$$\Pi^{el} = \frac{4kT\sqrt{C}}{273} \left[\cosh\left(\frac{e\psi_0^{\infty}}{2kT}\right) - 1 \right], \quad (13)$$

where Π^{el} has dimensions $J/\text{\AA}^2$.

With the data in Table II, the values of ψ_0^{∞} can be calculated for the monolayers in Fig. 1 at a headgroup area of $\sim 62 \text{\AA}^2$. The results on the differences in the Π^{el} at various electrolyte concentrations are given in Table III

and a comparison is made with the experimentally observed changes in Π .

It is apparent that good agreement with theory is only obtained when the subphase does not contain CaCl_2 . When CaCl_2 is added, the observed decreases of the surface pressures are significantly larger, especially at low NaCl concentrations, than the theoretical predictions in which only the double layer contribution is taken into account. Subject to the validity of the theoretical analysis, the results indicate that changes in the chemical interactions between lipids accompanying ion binding are important as well and can be much larger than the change in the double-layer interactions.

DISCUSSION

Forces Operating between PG Bilayers

The interbilayer force measurements have shown that, at least in solutions containing Ca^{2+} , there are only two types of forces operating between PG bilayers; at long range the repulsive double-layer force and at shorter range the attractive van der Waals force. In these solutions, there is no evidence for the existence of a hydration force between PG bilayers, although Cowley et al. (16) have reported a hydration force in an electrolyte free solution with a distance range of $\sim 25 \text{\AA}$. Because no reliable force measurements could be performed in NaCl solutions below

TABLE III
MONOLAYER DATA OF DMPG (27°C)

Electrolyte concentration		Π^{el} (theoretical)	$\Delta\Pi^{el}$ (theoretical)	$\Delta\Pi$ (experimental)
NaCl	CaCl ₂			
<i>mM</i>	<i>mM</i>	<i>dyne/cm</i>	<i>dyne/cm</i>	<i>dyne/cm</i>
1	—	4.7	1.3	1.4
100	—	3.4		
100	—	3.4	3.0	5
100	1	0.4		
1	—	4.7	4.6	13
1	1	0.07		
100	1	0.4	0.3	6
1	1	0.07		

$D = 20 \text{ \AA}$, it can not be concluded from the present experiments whether indeed a hydration force exists in the absence of Ca^{2+} ions.

It is interesting to compare the present results with the work on hydration forces between mica surfaces (23). Experiments on bare mica surfaces have shown that in those systems, the hydration forces are intimately associated with the adsorption of ions on the surfaces. Both monovalent and divalent ions apparently remain hydrated when they adsorb, since they cause a hydration force when the mica surfaces are brought together. In the present system, although almost 50% of the lipids carry a Ca^{2+} ion (see Tables I and II), there is certainly no hydration force arising from the mere adsorption of Ca^{2+} . A possible explanation is that Ca^{2+} bridge binds between adjacent lipid headgroups, which effectively dehydrates the Ca^{2+} ions (and possibly the headgroups as well), whereas on the mica surface, no such bridge binding between two lattice sites is possible.

It was noted in Figs. 4, 5, and 7 that at low concentrations of CaCl_2 and short bilayer separations, the repulsion predicted by theory is somewhat too strong. Several factors can be responsible for this, i.e., breakdown of continuum theory, inadequacies of the simple ion adsorption model, etc. It may be worthwhile here to mention the recent theoretical work of Guldbrand et al. (28) and Kjellander et al. (29), who have found deviations from the standard Poisson-Boltzmann treatment of the double-layer force, in particular for divalent ions at small surface separations and high charge densities. They attribute these deviations to correlations between the counterions in the double-layer and correlated fluctuations in the ionic layer adsorbed on both surfaces, leading to an extra attractive interaction (of a van der Waals dispersion force type) that has a strength comparable to the conventional van der Waals attraction, and is screened in large concentrations of monovalent electrolyte. In Figs. 4, 5, and 7, the force maxima can be predicted correctly using a Hamaker constant which is $\sim 50\%$ larger than the theoretical hydrocarbon/water Hamaker constant, indicating that an extra attractive interaction might be at work here. The magnitude of the adhesion force can roughly be explained from the sum of the van der Waals force and the double-layer force at the equilibrium bilayer separation.

Ion Binding and Its Effect on the Lateral Headgroup Interactions

The analysis of the measured double-layer forces in Figs. 3–7 has given values for the surface charge and surface potential (listed in Tables I and II) of PG bilayers in various electrolyte solutions. Binding constants are evaluated according to a simple Langmuir adsorption model assuming a 1:1 binding mode.

A phase transition in a PG bilayer appears to influence the binding affinity of ions. Bilayers in the fluid phase

(DMPG) bind ions stronger than bilayers in the gel phase (DSPG). This is also apparent from the observation that the adhesion between DMPG bilayers is stronger than the adhesion between DSPG bilayers in the same electrolyte solution.

The intrinsic binding constants K_{Ca} in 100 mM NaCl (40 M^{-1} and 60 M^{-1} for DSPG and DMPG, respectively) are larger than the binding constant $K_{\text{Ca}} \approx 8.5 \text{ M}^{-1}$ in a 100 mM NaCl solution, (PG in the fluid phase) reported by Lau et al. (14), who used the same adsorption model. Indeed, their reported zeta potential of -41 mV , measured by electrophoresis in a 100 mM NaCl + 1 mM CaCl_2 solution is also higher than the value of $\psi_0^{\infty} = -32 \text{ mV}$ at the OHP, found for DMPG bilayers in the present study. The latter comparison can be made because the OHP should be very close to the plane where the zeta potential is measured, a view held by most colloid scientists (30).

A possible source of the discrepancy might be that Lau et al. used PG derived from egg PC, which has a different hydrocarbon composition than synthetic DMPG. It is difficult to say to what extent a different hydrocarbon interior (unsaturation etc.) changes the properties of the headgroup region, and a straightforward comparison between the two studies might not be possible. Another circumstance that should be considered is that in the present study, the potential ψ_0^{∞} at the OHP has been obtained using only the diffuse double-layer theory. It is not obvious that this theory holds very close to the surface (where the zeta-potential is measured), since discrete ion effects are not accounted for (i.e., Stern layer effects). This might affect the decay and magnitude of the potential up to a few angstroms from the surface.

It is expected that the soundness of the applied Langmuir adsorption model with a 1:1 binding mode will be reflected in the constancy of K_{Ca} with the electrolyte concentration. Inspection of Table I shows that for a DSPG bilayer, the values for K_{Ca} are independent of the electrolyte concentration (except in 10^{-5} M CaCl_2 but that value is somewhat uncertain since the experimental double-layer force does not agree with theory), suggesting that the Langmuir adsorption model can be used here.

Table II shows that the Langmuir adsorption model holds less well for the fluid DMPG bilayers. The values K_{Ca} decrease with an increase in the electrolyte concentration, a trend also found by Lau et al. (14). An explanation might be that the assumed 1:1 binding mode only, is too simple and that, as mentioned before, also 2:1 binding occurs where Ca^{2+} bridges two neighboring lipids. A 2:1 binding mode on phospholipids bilayers has been shown to exist by Altenbach and Seelig (31), but a 1:1 binding mode is certain to occur as well since by electrophoresis, PG membranes have been shown to reverse charge in concentrated CaCl_2 solutions (14).

An adsorption model in which both binding modes are considered is readily developed (but increases the number of adjustable parameters). This has been done recently by

Ohki and Ohshima (32) who analyzed their binding studies on PS monolayers with such a model. Like what was found with PG monolayers, they also found that upon addition of CaCl_2 , the surface pressure of a PS monolayer decreased much more than could be accounted for by pure double-layer effects only. They attempted to account for this discrepancy by introducing a nonelectrostatic contribution from the ion binding to the change in the surface pressure accompanying ion binding. According to this analysis, the main cause of the surface pressure decrease is a conformational change of the lipid headgroups due to the bridge-binding of divalent ions. With regard to the experimental data reported here, which indicate an absence of hydration forces in the presence of CaCl_2 , it is suggested that this bridge-binding results in a dehydration of the PG headgroups, which should substantially decrease the short-range lateral repulsion between the headgroups and hence the surface pressure. Unfortunately, it cannot be unambiguously concluded from the present experiments that a short-range hydration force exists in the absence of CaCl_2 .

Of interest in this respect is the work of Verkley et al. (33), and Van Dyck et al. (34, 35) who studied the morphological characteristics and phase transitions of synthetic PG aggregates. They found a drastic increase in the transition temperature near surface neutralization by Ca^{2+} and a morphological change from liposomal structures to cylindrical or lamellar structures.

These observations also indicate that Ca^{2+} has a marked effect on the nature of the bilayer surface. The present results on PG monolayers indicate that apart from double-layer interactions, other more specific factors like bridging, conformational changes, and/or headgroup dehydration might play an important role.

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