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Measurement of Repulsive Forces between Charged Phospholipid Bilayers†

A. C. Cowley, N. L. Fuller, R. P. Rand, and V. A. Parsegian*,‡

ABSTRACT: By using an osmotic stress technique (LeNeveu, D. M., et al. (1977) *Biophys. J. 18,* 209), we have measured the net repulsive force between egg lecithin bilayers containing various amounts of the charged lipids phosphatidylglycerol and phosphatidylinositol. At bilayer separations greater than about 30 Å, the repulsion is dominated by electrostatic forces; its variation with both bilayer separation and charge density is well described qualitatively by simple electrostatic double-layer theory. Quantitative agreement requires, however, that only about 50% of the phosphatidylglycerol polar groups be dissociated. At all charge densities, even for pure phosphatidylglycerol, and at bilayer separations less than about 30 Å, the repulsion is dominated not by the electrostatic force but by a strong "hydration force" (LeNeveu, D. M., et al. (1977) *Biophys. J. 18,* 209). We conclude that the hydration force demands more attention than it has enjoyed hitherto in attempts to understand bilayer membrane interaction and fusion.

Cell aggregation, cell fusion, and the fusion of lipid and membrane vesicles with cells depend on the interaction energies of the approaching membranes. Stable short-range interactions involve "receptors" or specific molecules on the cell surfaces. Long-range forces such as electrostatic repulsion and van der Waals attraction may well play a role by influencing the establishment of short-range interactions (Parsegian, 1973). In order to appraise these nonspecific forces we have been measuring the interactions between phospholipid bilayers, as model membranes, using a strategy capable of measuring the net repulsive force as a function of the distance between the bilayers (LeNeveu et al., 1976).

Our first measurements with egg lecithin bilayers gave an estimate of the van der Waals attractive force and, more importantly, showed that there is a very strong repulsive force between these electrically neutral bilayers, a force which at bilayer separations less than about 20 Å appears to be much stronger than electrostatic repulsion. This force decays very rapidly with bilayer separation but would, nevertheless, require a work of 20 kT to bring two parallel bilayers of area $(100\ \text{Å})^2$ within approximately 15 Å of each other. We have suggested that its origin is the work needed to remove water of hydration from the lipid polar groups. Barclay & Ottewill (1970) and Barclay et al. (1972), who have measured forces between colloidal particles, also identified a hydration force acting to keep the colloidal particles separated.

Such a force would prevent bilayer phospholipid vesicles from approaching other vesicles or cells closely enough to allow fusion. Indeed in current studies of vesicle fusion, where molecular diffusion (Papahadjopoulos et al., 1976a), molecular exchange (Pagano & Huang 1975; Poznansky & Lange 1976), adhesion without fusion, and true fusion have been distinguished experimentally (Weinstein et al., 1977), it appears that fusion is a very unlikely event. Even where vesicles have been targeted to specific cells through the use of antibodies or lectins, vesicle-to-cell adhesion (presumably through the short-range interactions of these receptor molecules) is far more likely than the distinguishable process of fusion. The strong hydration force that we have measured indicates that those areas of cell or vesicle membranes that are of bilayer structure are not likely to be important in stabilizing cell adhesion. Rather, regions that bear molecules projecting well out from the bilayer surface should be the source of mechanically strong contact. Fusion appears to depend on destabilization of the bilayer structure (Papahadjopoulos et al., 1976b). Our results on the hydration force suggest that destabilization of the bilayer structure would have to occur in a way that allowed the lipid hydrocarbon moieties to merge without requiring a large amount of work to remove water from the polar groups.

As part of our study of three nonspecific forces—van der Waals attraction, "hydration" repulsion, and electrostatic repulsion—we report here our measurements of the electrostatic repulsion between charged phospholipid bilayers. Although there has been an enormous amount of theoretical work on the electrostatic forces between charged planar layers and some indirect measurements of the surface potentials of cells and model membranes (McLaughlin, 1977; Castle & Hubbell, 1976), there is little work on measurement of electrostatic forces, particularly in aqueous systems. In their remarkable early demonstration of electrostatic forces between bilayers of brain lipids, Palmer & Schmitt (1941) found that lipid multilayers swelled indefinitely in distilled water but collapsed steadily upon exposure to solutions of progressively higher ionic strength. Kirschner & Caspar (1975) reported reversible shrinking of the myelin structure when exposed to a 10% or stronger dimethyl sulfoxide solution. Recently Israelachvili & Adams (1976) have measured the electrostatic repulsion between sheets of mica in aqueous solutions and have demonstrated agreement with theoretical prediction. They have also found (Israelachvili & Adams, 1978) "additional forces" which might be related to the hydration force. Gingell & Fornes (1976) have studied the interaction of red blood cells with a solid surface of controlled electrostatic potential.

Gulik-Krzywicki et al. (1969) showed that small quantities of charged phospholipids provide an electrostatic repulsion that can overcome long-range van der Waals attraction to cause egg lecithin bilayers to swell to large separations. In our present study we have added varying amounts of charged lipids to egg

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lecithin bilayers to measure the resultant net repulsive forces as the distance between the layers is varied from 10 Å to approximately 170 Å. We find that "hydration" repulsion dominates out to about 20–30 Å separation. The more slowly decaying electrostatic repulsion dominates only beyond about 30 Å; its variation with bilayer separation is well described by electrostatic double-layer theory. At bilayer separations, where hydration and electrostatic forces are comparable in magnitude, about 30 Å, there is often a separation of the lipid into two distinct lamellar phases.

Materials and Methods

Materials. Egg lecithin (PC)[1] was prepared by the method of Singleton et al. (1965). Sodium phosphatidylinositol (PI), extracted from pig erythrocytes, was a generous gift of Dr. D. O. Tinker of the University of Toronto. Sodium phosphatidylglycerol (PG), prepared from egg lecithin, was generously provided by Dr. D. Papahadjopolos of Roswell Park Memorial Institute. Lipid quality was monitored by thin-layer chromatography. Dextran (T2000, mol wt 2 000 000; and T250, mol wt 250 000) was purchased from Pharmacia (Sweden). Water, double-distilled in glass, was used for all the lipid and dextran solutions and had a conductivity of 1–2 μmhos/cm.

Preparation of X-Ray Samples. Pure lipids or lipid mixtures of the desired ratio were dried down from chloroform solution, first by a rotary evaporator and then by vacuum for several hours. If the dry weight percent of the x-ray sample was required, dry lipid and water were mixed by weight and allowed to equilibrate at least 48 h before being transferred to x-ray sample holders as previously described (LeNeveu et al., 1978).

Lipid that was to take up water against the osmotic pressure of a dextran solution was prepared in one of two ways. First the lipid was placed in direct contact with an excess of dextran solution (usually 20 mg of lipid in 0.2 mL of dextran solution) in small weighing bottles and allowed to come to equilibrium before being mounted along with some of the dextran solution in the x-ray sample holder. Alternatively the lipid was mounted in the sample holder, separated from the dextran solution by a dialysis membrane, sealed, and allowed to equilibrate therein. This latter method of preparation was necessary for those lipids that swelled to bilayer separations, d_w, greater than about 40 Å. For such lipids direct contact between the swelled lipid and the dextran solution had a disordering effect on the lipid lamellar structure which broadened the x-ray reflections as if the dextran were entering the multilamellar structure. For d_w less than about 40 Å the equilibrium spacing achieved did not depend on whether the lipid was in direct contact with the dextran solution or separated from it by a dialysis membrane. When the lipid was equilibrated within the sealed sample holder, it was necessary to ensure that even small hydrostatic pressure differences did not build up across the dialysis membrane; such pressures could greatly affect the ability of the lipid to swell, for reasons that will become clear below.

For either method of preparation, the final equilibrated dextran concentration was measured within ±0.2% with an Abbe refractometer. Its osmotic pressure was then obtained as described previously (LeNeveu et al., 1977). Powdered Teflon was included in every x-ray sample to provide an internal standard of known x-ray spacing, reducing the error in the measured lamellar phase spacing to ±0.2 Å. The single Teflon reflection at 4.87 Å was itself calibrated using beryllium acetate and was found to be thermally insensitive over the range of temperatures used.

X-Ray Diffraction. The lamellar phases, composed of alternating layers of water and bimolecular phospholipid leaflets, formed by lipids in these systems, have been characterized and analyzed by x-ray diffraction as previously described (Rand & Luzzati, 1968). Briefly, the x-ray diffraction reflections give the dimensions of the repeat distance d in the lamellar phase. For those samples whose composition (i.e., volume percent lipid ϕ) is known, d is divided into a lipid layer of thickness $d_l = \phi d$ and a water layer of thickness $d_w = d - d_l$. Thus the bilayer thickness and the separation between bilayers can be determined for all concentrations of lipid in water. For any lamellar phase formed in equilibrium with excess dextran solution, we equate its d spacing with the same d spacing of a sample mixed gravimetrically with water and attribute equivalent bilayer and water thicknesses to each (LeNeveu et al., 1977). We thus determine the bilayer separation d_w for all lipid lamellar phases that are in equilibrium with excess dextran solution.

Net Repulsion between Bilayers vs. Bilayer Separation. For those lipid lamellar phases that are in equilibrium with the dextran solutions, dextran is prevented from entering the multilamellar structure either by being too large or by the presence of the dialysis membrane. The chemical potential of the water between the lipid bilayers is equal to that of the dextran solution, and each is lower than that of bulk water. The chemical potential of the water in the dextran solution is lowered by virtue of its lower concentration and that of the water between bilayers is lowered because it is under negative pressure created by a net repulsive force between the bilayers. One may imagine that the lipid bilayers must swell with water against the osmotic pressure of the dextran solution. The net repulsion F_π between the bilayers is equal to the osmotic pressure of the dextran solution with which it has reached equilibrium. From the set of dextran concentrations and corresponding repeat spacings, d, we derive the net repulsive force, F_π, vs. bilayer separation, d_w.

Results

Lipid Bilayer and Water Layer Thicknesses of Lipid Lamellar Phases with Different Charge Density. X-ray diffraction of the various lipid mixtures showed that the structures of these lipid–water systems were, mostly, single lamellar phases. For all of these phases the variation of the repeat spacing d with the concentration of lipid is shown in Figure 1. Included also in Figure 1 are calculated values of the bilayer thickness d_l and the average area, S (Å^2), available to each head group at the water interface.

The surprising feature of the graphs shown in Figure 1 is that the bilayer thicknesses for 50 mol % PG in lecithin and for pure PG are higher than those for egg lecithin. Assuming that the partial specific volumes of PG and PC are equal, a higher d_l means a smaller S, i.e., a denser packing of head groups on the bilayer in spite of expected electrostatic repulsion between charged groups. This assumes that the PG has an acyl chain composition identical with that of the egg lecithin from which it was derived and has a partial specific volume equal to that of egg lecithin. The partial specific volume of PG would have to be 15% higher than that of egg lecithin in order for the areas to be equal at the higher levels of hydration.

Variation of Repeat Spacing d with Concentration of Dextran Solution. Each point in Figures 2 and 3 represents the d spacing found for a lipid lamellar phase in equilibrium with a particular concentration of dextran solution. In Figure 2, the variation of d with dextran concentration is shown when the same charged phospholipid (PG) is used, but the charge density in the bilayer is varied by mixing different molar

[1] Abbreviations used: PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

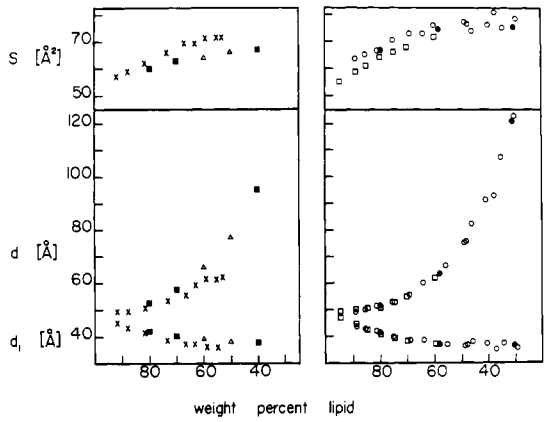

FIGURE 1: Structural parameters of the lamellar phase formed by lipid mixtures of various concentrations in water. d = lamellar repeat distance; d_l = bilayer thickness; S = area available to each lipid molecule at the bilayer–water interface. (X) One hundred percent egg lecithin (L); (△) 100% phosphatidylglycerol (PG); (■) 50 mol % PG; (●) 10 mol % PG; (□) 5 mol % PG; (○) 10 mol % phosphatidylinositol (PI).

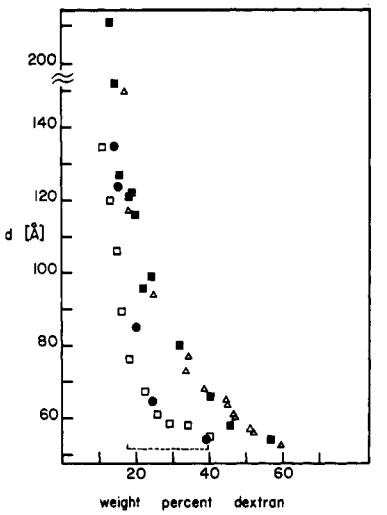

FIGURE 2: Variation of the repeat distance d of the single lamellar phases formed by phosphatidylglycerol–egg lecithin mixtures in different concentrations of dextran solution. (□) Five mole percent PG; (●) 10 mol % PG; (■) 50 mol % PG; (△) 100% PG. The dashed line indicates the limits, for all these mixtures, within which some samples showed the presence of two lamellar phases.

fractions of PG with lecithin. For all the charged systems studied, the curves are similar in general shape. In qualitative terms, for any given concentration of dextran, increases in d spacing are easily detected with large increases in bilayer charge density.

Figure 3 shows the experimental data obtained for lipid systems containing equal amounts (10 mol %) of different charged lipids. Again the general shape of curves formed by the experimental points for each system is very similar. At any given dextran concentration the d spacings for 10% PI and 10% PG are the same within the accuracy of our experiment.

As with the gravimetrically prepared lipid samples, each lipid system showed a number of samples containing two phases. The two phases appeared only at intermediate bilayer separations but not in every sample at those separations. We tried to eliminate external causes that might lead to separation of lipid into two phases. Ca^{2+} ions were at first suspected. Analysis of our dextran showed that the maximum amount of Ca^{2+} was 1 ppm by weight. Such low concentrations of Ca^{2+} would supply only one Ca^{2+} per 500 lipid molecules. A 20% dextran solution gave a conductivity of 90 μmhos/cm where the conductivity of doubly distilled water was 1–2 μmhos/cm. Deionizing a 20% dextran solution with a mixed bed deionizer reduced the conductivity to 20 μmhos/cm, but two phases still formed in contact with this solution.

The separation of lipid is not simply a separation of the charged lipids from lecithin. The lamellar phase with smaller d spacing is swollen beyond the limits of maximal swelling observed for pure lecithin. Futhermore, even pure PG segregates into two phases. We have not yet certified a rationale for this separation but its persistence convinces us that the phenomenon is real. Further investigation is required. For the present we have not used samples with two phases in our description of forces between bilayers.

Calculation of Forces Acting between the Lipid Bilayers. To translate the dextran concentration into physical pressure, we have used the osmotic pressure measurements of LeNeveu et al. (1977) supplemented by new osmotic pressure measurements up to $10^{8.08}$ dynes/cm^2 determined by a specially designed hydraulic press (to be described elsewhere). The force between bilayers as a function of bilayer separation is plotted in Figures 4 and 5. Figure 4 shows the force curves for 5, 10, 50, and 100 mol % PG in egg lecithin while Figure 5 shows the force curve for 10 mol % PG and PI in egg lecithin.

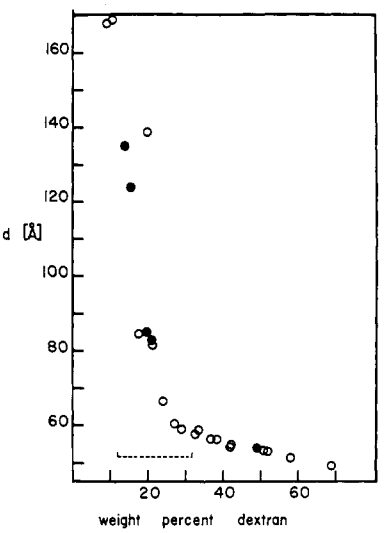

FIGURE 3: Variation of the repeat distance d of the single lamellar phases formed by 10 mol % PG (●) and 10 mol % PI (○) in egg lecithin in different concentrations of dextran solution. The dashed line indicates the limits within which some samples showed the presence of two lamellar phases.

Interpretation and Discussion

Balance of Forces. The equilibrium spacing of bilayers in contact with the dextran solutions represents the balance of four forces. Forces tending to decrease bilayer separations are the van der Waals attractive force F_A between bilayers and the externally applied osmotic pressure F_π of the dextran solution (which pushes bilayers together by withdrawing water from the interbilayer space). Those forces tending to increase bilayer separation are the "hydration force", F_H, and electrostatic repulsion F_{ES}. At equilibrium the known external force F_π is equal to the net internal force ($F_H + F_{ES} - F_A$).

F_A, the van der Waals attraction between bilayers, has been described in detail (Parsegian, 1973; LeNeveu et al., 1977). Its magnitude has been inferred experimentally at one particular separation between egg lecithin bilayers (LeNeveu et al., 1977). From this measurement one may extract an estimate

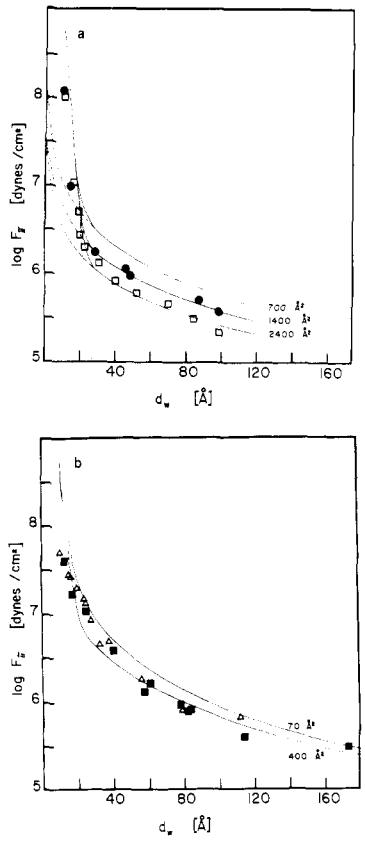

FIGURE 4: Relation between the net repulsive force between phosphatidylglycerol–egg lecithin bilayers, F_π, and their separation d_w. Experimental values are given as follows: (□) 5 mol % PG; (●) 10 mol % PG; (■) 50 mol % PG; (△) pure PG. The solid lines give the theoretical net repulsive force, described in the text, for the areas per unit charge shown. (- - -) The contribution of F_H, the hydration repulsive force; (— — —) the contribution of F_{es} the electrostatic repulsion to the total force. F_A (van der Waals attraction) is not shown. Electrostatic repulsion dominates at large separations and hydration repulsion dominates at separations below 20 Å.

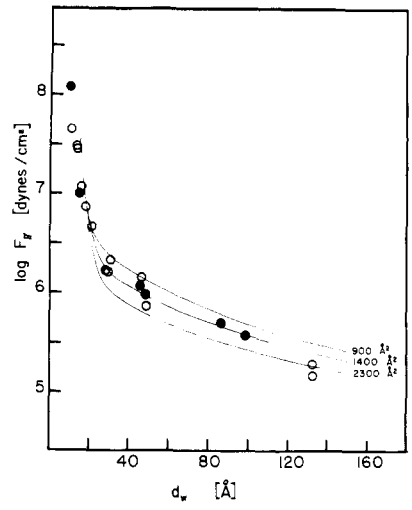

FIGURE 5: Relation between the net repulsive force F between egg lecithin bilayers containing (●) 10 mol % PG and (○) 10 mol % PI and their separation d_w. Solid lines give the theoretical net repulsive force for the different areas/unit charge shown.

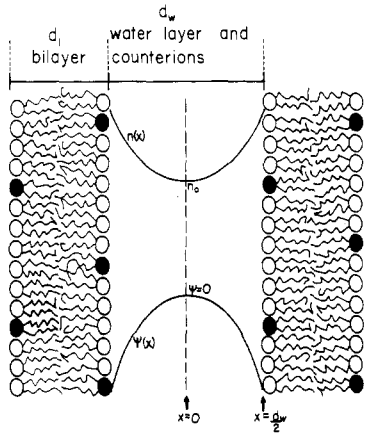

FIGURE 6: Diagrammatic representation of lipid bilayers separated by an aqueous solution. The ellipses represent the lipid polar groups (open, neutral lipids; solid, charged lipids) while the wavy lines represent fluid hydrocarbon chains. In the aqueous region between bilayers, the functions $\psi(x)$, the electrostatic potential, and $n(x)$, the number density of counterions, are indicated. The variable x is the distance measured from the midpoint between the surfaces of the bilayers and n_0 is the ion concentration at the midpoint.

of the Hamaker coefficient A_h defined as

$$F_A = \frac{A_h}{6\pi d_w^3}$$

F_H, the hydration force measured for egg lecithin (LeNeveu et al., 1976, 1977), followed the relation $F_H = 10^{11}e^{-d_w/1.93}$. We have assumed that such a relation holds for the egg lecithin–PG mixtures and for pure PG. This assumption is of little consequence for bilayer separations greater than about 40 Å where this force is negligible but may be significant for smaller separations. (By speaking of F_H in this way we do not imply that the origin of this force, other than being a work of removal of water from soluble polar groups, is understood. The mathematical formalism of Marcelja & Radic (1976) suggests an exponential force whose physical basis is obscure.)

F_{ES}, the electrostatic force between the charged bilayers, has been derived from the theory of the electrostatic double layer using the scheme in Figure 6.

The one-dimensional Poisson equation relating the electrostatic potential $\psi(x)$ to charge density $\rho(x)$ and dielectric constant ϵ,

$$-\frac{d^2\psi}{dx^2} = \frac{4\pi\rho(x)}{\epsilon} \quad (1)$$

is solved in the aqueous region between bilayers. The variable x is the distance measured from the midpoint between the two parallel planar charged surfaces of separation d_w. The charge density

$$\rho(x) = en(x) \quad (2)$$

where e is the charge of a univalent cation and $n(x)$ is the number density of counterions dissociated from the bilayer ionizable groups. The distribution $n(x)$ is assumed to follow a Boltzmann law in the electrostatic potential $\psi(x)$ as

$$n(x) = n_0e^{-e\psi/kT} \quad (3)$$

where k and T are the Boltzmann constant and absolute temperature. The value of the coefficient n_0 will be found from equating the ions per unit area to the density α/A of ionized groups

$$\left(\frac{A}{\alpha}\right)\int_0^{d_w/2} n(x)dx = 1 \quad (4)$$

where A is the area per dissociable phospholipid and α the fraction of groups actually dissociated.

The Poisson–Boltzmann equation is solved under the boundary conditions

$$\text{(a)} \quad \left.\frac{d\psi}{dx}\right|_{x=0} = 0 \quad \text{(by symmetry)} \tag{5a}$$

and

$$\text{(b)} \quad -\left.\frac{d\psi}{dx}\right|_{x=d_w/2} = \frac{4\pi\sigma}{\epsilon} \tag{5b}$$

where $\sigma = \alpha e/A$. Its solution for the present case is

$$\psi(x) = 2\frac{kT}{e}\ln(\cos(Kx)) \tag{6}$$

which satisfies the first boundary condition (eq 5a) implicitly and the second (eq 5b) when K is defined by

$$\frac{Kd_w}{2}\tan\frac{Kd_w}{2} = \alpha\frac{\pi e^2}{kT}\frac{d_w}{A} \tag{7a}$$

or with $q = Kd_w/2$

$$q\tan q = \alpha\frac{\pi e^2}{kT}\frac{d_w}{A} \tag{7b}$$

Using this form of the potential, we note that the ion concentration n_0 at the midpoint between bilayers is

$$n_0 = \frac{\alpha}{A}\frac{K}{\tan(Kd_w/2)} = \frac{2\alpha}{Ad_w}\frac{q}{\tan q} \tag{8}$$

Since the bilayers are being pushed together in an ion-free medium the osmotic pressure at the midplane, kTn_0, must be compared with the osmotic pressure exerted by the dextran solution for each particular separation d_w.

We test whether the pressure–distance curve follows the relation

$$F(d_w) = kTn_0 = kT\frac{2\alpha}{Ad_w}\frac{q}{\tan q} \tag{9}$$

where q is given by eq 7. The degree of dissociation α is not known a priori.

Finally then, the net repulsion between bilayers is equal to

$$F_\pi = 10^{11}e^{-d_w/1.93} + \frac{kT2\alpha}{Ad_w}\frac{q}{\tan q} - \frac{A_h}{6\pi(d_w)^3}$$

In order to indicate the contribution of F_H and F_{ES} to F_π, these forces are plotted separately in Figure 4a. F_A is a negligible force on the scale of the ordinate. At bilayer separations greater than approximately 30 Å, F_H too is negligible, and the repulsion between bilayers is dominated by the electrostatic force alone. The theoretical electrostatic force, given in eq 9, has been fitted to all the experimental points at these large separations by adjusting A/α, the apparent area per charge. These selected areas are indicated on the curves. As discussed below, these areas are consistently greater than those expected from the lipid composition on the assumption that each phospholipid occupies 70 Å^2. At bilayer separations less than about 25 Å the experimental points deviate considerably from F_{ES} and fall near the F_H curve. These characteristics are independent of the kind of lipid contributing the charge (Figure 5).

Two important lessons are apparent. First, the spatial variation of the electrostatic repulsion between charged lipid membranes at large separations is remarkably well described by the most straightforward theory of the electrostatic double layer. Second, at membrane separations less than 20 to 30 Å (depending on charge density) electrostatic repulsion becomes overwhelmed by the shorter range repulsive force previously detected between zwitterionic lecithin bilayers.

The best-fit lines to the experimental points at large bilayer separations are made with the areas per charge indicated in Figures 4 and 5. The discrepancy between fitted charge densities (Figures 4 and 5) and those expected from lipid composition suggests that fewer than 100%, in fact only about 50% of the PG molecules in each lipid mixture, are dissociated in the multilamellar phase. A recent monolayer study of synthetic PG shows that its apparent pK varies around 5 depending on the salt concentration of the subphase (Sacre & Tocanne, 1977). The pH of the unbuffered dextran solutions used in this study was 5.8–6.2. In order to ensure that conditions in the bulk phase, with which the lipid was in equilibrium, were such as to expect complete dissociation, lipid samples of 10% PG were equilibrated with dextran solution prepared at pH 6, 7, 8, and 10. All gave spacings similar to those equilibrated with dextran prepared in pure water. On the basis of the monolayer studies, conditions were such as to expect 100% dissociation of the PG. Because F_{ES} varies slowly with surface charge density for double layers of high charge densities, a change in area per charge by a factor of 2 would be difficult to detect. However, at the lower densities the force is quite sensitive to the amount of charge. Even with scatter due to error the experiments here do suggest clearly that not all the lipid is dissociated from protons or perhaps from the Na counterions.

Incomplete charge dissociation may be only apparent. If the charge-bearing phosphates, in fact, protrude into the water layer rather than sit precisely on the hydrocarbon water interface, then counterions may percolate *behind* the fixed-charge groups as well as diffuse into the region between bilayers. The surface charge density creating the electrostatic double layer will be lowered by such percolation (Parsegian, 1974).

Figure 4 shows that the electrostatic force between bilayers does increase with increasing concentrations of PG in the lipid bilayer, and presumably with increasing surface charge density.

While the validity of traditional electrostatic double layer theory is satisfying, the importance of electrostatic forces between closely approaching membranes may now be questioned. Hydration forces apparently overwhelm electrostatic interactions at some tens of angstroms separation. Screening of electrostatic forces by added salt, especially at physiological concentrations, would only make hydration forces relatively stronger. Below 20–30 Å separation, electrostatic surface charge density makes little difference to the measured forces. The hydration force apparently extends down to only a few angstroms (Fuller, Rand, & Parsegian, in preparation) and deserves most careful attention. This force may in fact be the barrier that prevents rapid fusion of vesicles with membranes and whose effect is deleted by ion-specific processes during synaptic depolarization (Parsegian, 1977).

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Exchange Mechanisms for Hydrogen Bonding Protons of Cytidylic and Guanylic Acids†

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ABSTRACT: The pH dependence of buffer catalysis of exchange of the C-4 amino protons of cyclic cytosine 2′,3′-monophosphate (cCMP) and the N-1 proton of cyclic guanosine 2′,3′-monophosphate (cGMP) conforms to an exchange mechanism, in which protonation of the nucleobases at C(N-3) and G(N-7) establishes the important intermediates at neutral to acidic pH. Rate constants for transfer of the G(N-1) proton to H_2O, OH^-, phosphate, acetate, chloroacetate, lactate, and cytosine (N-3) were obtained from ^1H nuclear magnetic resonance line width measurements at 360 MHz and were used to estimate the pK or acidity of the exchange site in both the protonated and unprotonated nucleobase. These estimates reveal an increase in acidity of the G(N-1) site corresponding to 2 to 3 pK units as the G(N-7) site is protonated: At neutral pH the G(N-1) site of the protonated purine would be ionized (pK = 6.3). Determinations of phosphate, imidazole, and methylimidazole rate constants for transfer of the amino protons of cCMP provide a more approximate estimate of pK = 7 to 9 for the amino of the protonated pyrimidine. A comparison of the intrinsic amino acidity in the neutral and protonated cytosine is vitiated by the observation that OH^- catalyzed exchange in the neutral base is not diffusion limited. This leads to the conclusion that protonation of the nucleobase effects a *qualitative* increase in the ability of the amino protons to form hydrogen bonds: from very poor in the neutral base to "normal" in the conjugate acid.

A dominant characteristic of the biological function of monomeric and polymeric nucleotides is the ability of their nucleobase protons to participate in hydrogen bonding with a high degree of specificity that is crucial to their biological effect. Therefore, one might expect that the evolution of the purine and pyrimidine structures in biomolecules contained a selective element that would govern the characteristics of hydrogen bond formation peculiar to nucleic acids. This element would be perceived through the mechanism of hydrogen exchange, which describes two related aspects important for hydrogen bonding: proton lifetime and acidity.

For this reason, it was of particular interest to find that exchange of the $-NH_2$ protons of aqueous adenine, cytosine, and guanine nucleotides were accessible to direct study by NMR (McConnell & Seawell, 1972, 1973). Adenylic acid-NH_2 proton exchange at neutral and acidic pH occurs almost exclusively by a two-step mechanism, in which transfer of the $-NH_2$ proton to solvent is preceded by protonation of the basic endocyclic nitrogen of the purine (N-1) (McConnell, 1974). Confirmation of this mechanism in the adenine mononucleotide was achieved by stopped-flow methods (Cross et al., 1975). Although there is $-NH_2$ exchange from the unprotonated form of the nucleotide as well, this becomes significant only well above neutrality. It appears that the important mechanism at physiological pH involves the ability of the conjugated purine structure to communicate the effects of ring protonation as a regulatory aspect of proton exchange at a separate site on the molecule, the exocyclic $-NH_2$. This two-step exchange mechanism accounts for properties of hydrogen exchange in

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