MEASUREMENT AND MODIFICATION OF FORCES BETWEEN LECITHIN BILAYERS

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ABSTRACT We probe in two different ways the competing attractive and repulsive forces that create lamellar arrays of the phospholipid lecithin when in equilibrium with pure water. The first probe involves the addition of low molecular weight solutes, glucose and sucrose, to a system where the phospholipid is immersed in a large excess of water. Small solutes can enter the aqueous region between bilayers. Their effect is first to increase and then to decrease the separation between bilayers as sugar concentration increases. We interpret this waxing and waning of the lattice spacing in terms of the successive weakening and strengthening of the attractive van der Waals forces originally responsible for creation of a stable lattice.

The second probe is an "osmotic stress method," in which very high molecular weight neutral polymer is added to the pure water phase but is unable to enter the multilayers. The polymer competes for water with the lamellar lattice, and thereby compresses it. From the resulting spacing (determined by X-ray diffraction) and the directly measured osmotic pressure, we find a force vs. distance curve for compressing the lattice (or, equivalently, the free energy of transfer to bulk water of water between bilayers). This method reveals a very strong, exponentially varying "hydration force" with a decay distance of about 2 Å.

INTRODUCTION

The nature of the adhesive interactions between biological cells and between cells and artificial materials is a fundamental problem in cell biology. The most important of these interactions occur at contact between specific molecules on the cell periphery and are best probed by techniques of molecular biology. Underlying these short-range interactions are forces—particularly electrostatic and van der Waals forces—that can be felt at relatively large distances between cells and can influence the initial approach of cell membranes. The extent to which cell-cell and cell-substratum interactions depend on electrostatic repulsion and electrodynamic (van der Waals) attraction (1) has become increasingly amenable to rigorous theoretical analysis (2).

Recent experimental work (3–5) has shown that sufficiently strong electrostatic forces can prevent cell adhesion, while under reduced electrostatic repulsion, cells adhere to other cells as well as to metal/water and oil/water interfaces because of attractive physical forces. To have more than indirect evidence of these interactions, we seek to measure them directly as a function of the separation distance between the

interacting surfaces. Thus far we have been obliged to utilize bilayer lipid membranes. The current view of the structure of the cell membrane is that it is for the most part a lipid bilayer bearing proteins and saccharides. Our direct measurements are of the force required to bring bilayers to a given separation. We have also found changes in bilayer separation as added sugars modify electrodynamic forces.

In the model membrane systems of phospholipids in water, a balance between long-range attractive and repulsive forces operating over tens of Ångstroms is responsible for creating stable, ordered lamellar multilayers in which layers of water alternate with phospholipid bilayer membranes, whose structure has been described (6–9). Repulsion between electrically charged bilayers is due to the electrostatic double layers created by phospholipid polar head groups. Repulsion between zwitterionic lipids, such as electrically neutral lecithin, is caused by the competitive need for hydration of the polar groups, creating a repulsion between them. Attraction is believed to be caused by van der Waals interactions between the lamellae.

The lamellar multilayer system is convenient from an experimental point of view because the repeat distance can be measured by X-ray diffraction methods and hence the aqueous separation distance between lipid bilayers can be determined. Furthermore, the modern macroscopic theory of electrodynamic forces can readily be applied to the lamellar system (10–12). This theory has enabled us to predict (*a*) the force of attraction and (*b*) the way in which the force varies with the concentration of small sugars in the interbilayer aqueous phase. Since the attractive force is expected to be a function of sugar concentration (see Discussion below), the distance between bilayers should also show such dependence, as has been observed in X-ray studies (13). The essential feature, central to the macroscopic theory, is that modification of the absorption spectra of any of the interacting materials must modify the electrodynamic force.

It is possible to measure the force of repulsion between the bilayers of lecithin in water (14). Osmotic pressure π can be applied to the lamellae by means of dextran in the extralamellar water phase. This polymer is too large to diffuse into the interbilayer space; its osmotic effect is to remove water from between the lamellae, thereby forcing the lecithin bilayers together, i.e., in the same direction as the attractive force. If we write F_R for the interbilayer repulsive force and F_A for the interbilayer attractive (electrodynamic) force, we have $|F_A| + |\pi| = |F_R|$. From the observed π vs. separation curve and the theory of attractive forces, we expect that $|\pi| \gg |F_A|$ for separations a few Ångstroms less than those seen when F_R and F_A balance in the absence of dextran. Under most conditions, then, one may equate $|\pi|$ with $|F_R|$. The repulsive force is found to be exponential with separation. We extrapolate this exponential to the equilibrium separation (where $\pi = 0$). We then equate repulsion with attraction, $|F_R|$ (extrapolated value) $= |F_A|$, to infer the balancing attractive force.

MATERIALS AND METHODS

Lipids, Sugars, and the Preparation of X-Ray Samples

Chromatographically pure egg lecithin, isolated according to the procedures of Singleton et al. (15), was used throughout. Water used was doubly glass-distilled; ultrapure sucrose or glucose

(Schwarz-Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) was used in making the sugar solutions. Dextran of mol wt range 200,000–275,000 was obtained from British Drug Houses, Ltd. (Poole, Dorset, England).

X-ray samples of lecithin in various solutions were prepared in two ways. Dry lipid, which was to be allowed to imbibe as much water or sugar solution as it could, was placed into a large excess of glucose or sucrose solution (25 mg in 10 ml), dispersed with brief sonication (30 s at half power on a Biosonic III, Bronwill Scientific, Rochester, N. Y.) to hasten mixing, and recovered by centrifugation (150,000 *g* for 1 hr). It was therefore always in equilibrium with excess solution. Alternatively, X-ray samples for which the exact composition was required were prepared by adding the aqueous solution to the dry lipid gravimetrically in small weighing bottles and sealing them for a minimum of 48 h before mounting them for X-ray experiments. Random samples were chromatographed after the X-ray experiments to check for decomposition.

X-Ray Experiments

The X-ray measurements and the determination of structural data were done as previously described (16) (see also 17). Briefly, the X-ray camera is of the Guinier type operating *in vacuo* and using a bent quartz crystal monochromator which isolated the CuK_1 line (λ = 1.540 Å). The X-ray samples were sealed between mica windows 1 mm apart and their temperature in the camera controlled to ± 0.2°C with thermoelectric elements. The structures exhibited by material in sealed samples were checked to be stable over periods of days; any leakage of vapor from the sample holders is therefore negligible. The only structure of interest in this study is the lamellar phase, which gives several reflections, all integral orders of the single repeat distance d (± 0.5 Å) of the one-dimensional crystal. This structure has been shown to be alternating layers of water and bimolecular lipid leaflets (6–9).

When the X-ray sample is a single phase of known composition, the thickness d_l of the lipid layer containing all and only the lipid can be determined. Thus; $d_l = \phi d$, where ϕ is the volume fraction of lipid in the sample; $d_w = d - d_l$ is the thickness of the water layer between lipid layers; $\phi = \{1 + [(1 - C)/C][\nu_w/\nu_p]\}^{-1}$; C is the weight fraction of lipid in the sample; and ν_w, ν_p are the partial specific volumes of the aqueous phase and phospholipid, respectively. For the purposes of this study partial specific volumes of water and lipid were taken as equal to 1.00 (16) and those of the sugar solutions were taken from ref. 18. (Deviations from the $\nu = 1$ assumption are likely to be only of a few percent and will make a difference of these few percent or less in the estimates of d_l and d_w.)

Composition of the Interbilayer Space

To determine the composition of the solution taken up by the lipid from the sugar solution, approximately 100 mg lecithin were suspended in 0.2 ml of sucrose solution of known concentration. The resultant lecithin phase was separated from excess solution by centrifugation at 50,000*g* for 3 h. The sugar concentration of the excess solution was determined by refractometry. Controls with pure water indicated whether or not the lipid was contributing to the index of refraction of the solution and sugar concentrations could be measured to ± 0.1%. This process was carried out with four lipid preparations over the sugar concentration range from 0 to 40%. Any deviation in sugar concentration from that of the suspending solution would reflect preferential uptake of sugar or water by the lipid. To describe this deviation (an apparent exclusion of sucrose by lipid), we imagine that the water taken up by dry lecithin from the 0.2 ml of sucrose solution can be divided into two compartments: one in which the sucrose concentration equilibrates with that in the sucrose solution; and a second compartment of water completely unable to dissolve sucrose. We denote the total amount in the second compartment as w, where $w = 0.2\rho_s\,[1 - (S_i/S_f)]$, where S_i and S_f are the initial suspending concentration and the final supernatant concentration of sucrose as weight fraction, and ρ_s is density of

the initial suspending sucrose solution. Mole ratios of the number of waters per lecithin were calculated from w and from the precise weight of lecithin added to the sucrose solution.

Osmotic Pressures of the Dextran Solution

Measurements of the osmotic pressure of the dextran solutions were made directly, with a water or mercury manometer for the lower pressures and Boindar gauges for the higher pressures. The dextran solutions were connected to the manometer or gauge and were in equilibrium with distilled water across a dialysis membrane.

RESULTS

Modification of Bilayer Interaction by Small Sugars

WEAK EFFECT ON THE THICKNESS OF THE LIPID BILAYER BY SUBSTITUTION OF SUGAR SOLUTIONS IN THE INTERBILAYER SPACE Figs. 1 and 2 show that the sugar solutions have practically no effect on the thickness of the bimolecular lipid leaflet. Fig. 1 shows that both d and d_l remain practically unchanged in samples that were mixed at 72 vol % lipid with increasing concentrations of sucrose solution up to 40 wt %.

The lines, fitted by linear regression, give $d_l = 38.93 + 0.026 \times (\%\ \text{sugar})$; $d = 54.14 + 0.031 \times (\%\ \text{sugar})$. The very small change in dimension with increasing sugar concentration may conceivably have some importance, but it will be neglected below.

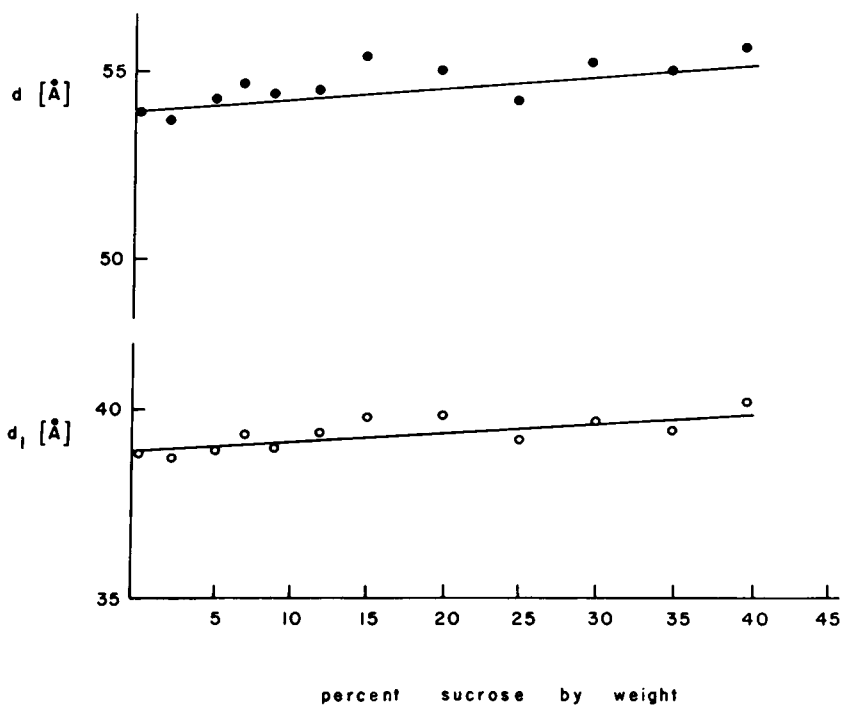

FIGURE 1 Lamellar repeat distance, d, and bilayer thickness, d_l, as a function of sucrose concentration in the water. The lipid constituted 72 vol % of each sample.

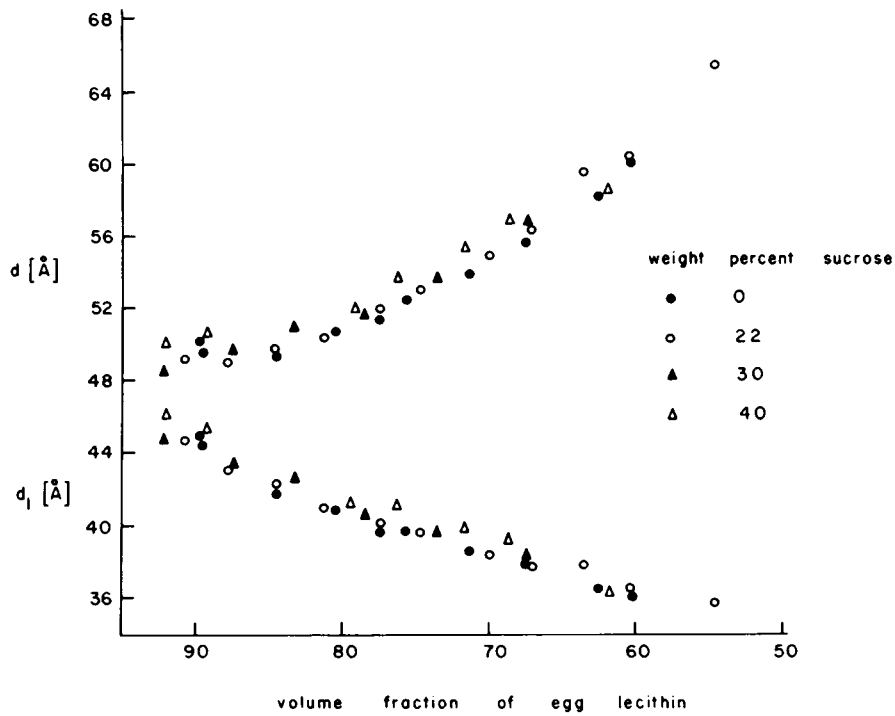

FIGURE 2 Repeat spacing d and bilayer thickness d_l vs. volume fraction lecithin mixed in solutions of different sucrose concentration (13).

That the samples were mixed at constant volume fraction of lipid ϕ is shown by the regression line $\phi = 0.72 + 0.000019 \times$ (% sugar).

Fig. 2 (from ref. 13) also shows that substituting 22%, 30%, or 40% sucrose solution for water does not detectably affect the thickness of the lipid bilayer over the normal swelling range from 90 to approximately 55 vol % lecithin. Over this range of volume percent of lipid in water, the thickness of the bilayer changes but the sucrose solutions give essentially the same bilayer thickness as pure water at any particular volume fraction.

From these observations we reach two conclusions: First, any change observed in the total spacing d of the lamellar phase of the lipid suspended in excess sugar solution is primarily a result of changes in the distance between bilayers and not in bilayer thickness. For this we assume that in going from nearly maximum separation (in the regime of one lamellar phase) to fully maximum separation (when there is a phase of excess water or sucrose solution), the bilayer thickness does not change significantly. Second, since the lecithin bilayer thickness is affected by a net repulsive force between adjacent leaflets (19), we expect that this net repulsive force is not changed enough by the substitution of sucrose solutions, up to 40 wt %, to cause a significant change in bilayer thickness.

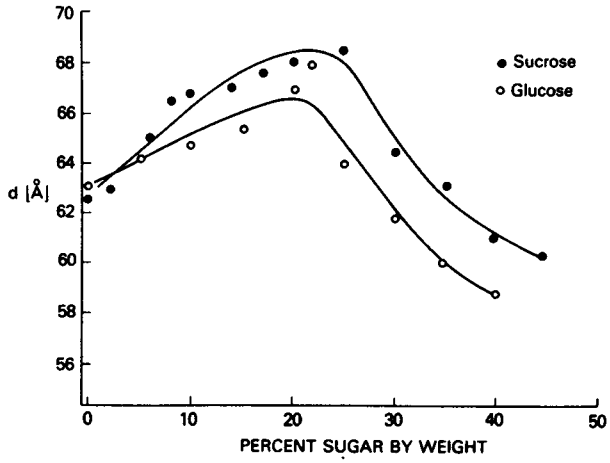

FIGURE 3 Repeat spacing of lamellar lattice immersed in excess quantities of various sugar solutions. Each spacing represents the spontaneous limit of swelling in the particular solution. Variations in repeat spacing reflect variation in bilayer separation.

EQUILIBRIUM BILAYER SEPARATION IN SUGAR SOLUTION Fig. 3 plotted from the data in Table I illustrates the total repeat distance d of the lamellar phase at maximal swelling formed by egg lecithin in equilibrium with a large excess of glucose or sucrose solution at various concentrations. The spacing increases by 6–7 Å to a maximum at approximately 22% sugar solution, and decreases thereafter. Since, as shown above, bilayer thickness is negligibly changed in these solutions, these changes in repeat spacing reflect changes in the bilayer separation; the bilayers first move further apart and then move back closer together as the sugar concentration increases. The effect is reversible in that each X-ray sample can be resuspended in pure water and recentrifuged. The spacing of lecithin swelled to a maximum in pure water is then obtained.

EQUILIBRATION BETWEEN BULK SOLUTION AND INTERBILAYER SPACE Fig. 4 shows the results of experiments done to determine the composition of the interbilayer space. The sugar concentration of the supernatant in equilibrium with the lamellar phase is always higher than that of the global sugar concentration, indicating a preferential uptake of water by the lecithin. This uptake occurs over the entire range of sucrose solutions and is practically constant, equivalent to 13 water molecules per lecithin molecule. The regression line is no. of water per lecithin = 13.37 − 0.049 × (% sugar). These results indicate that, given a choice between water and sugar, lecithin prefers water to the extent measured, and thus sugar is excluded from about 13 of the 34 or so molecules of water associated with each lipid. This number, 13, is close to the number of water molecules that appear not to undergo freezing (20) and are thought to compose the hydration shell of the polar group of the lecithin molecule. It is also close to the estimate of the sugar-excluding compartment of water inferred previously from partition coefficients (21). This hydration shell would occupy a layer about 5 Å thick on the polar groups, leaving a layer of approximately 17 Å of "free water" in the center of the inter-bilayer space free to exchange with the external solution (for d_w = 28 Å, a

TABLE I

VALUES OF LAMELLAR REPEAT DISTANCE *d* OF LAMELLAR PHASE FORMED BY EGG LECITHIN IN A LARGE EXCESS OF SUCROSE OR GLUCOSE SOLUTIONS OF VARYING CONCENTRATION. PLOTTED IN FIGURE 3.

Sugar	Glucose *d*	Sucrose *d*
wt %	*Å*	*Å*
0	63.0	62.5
2.0		63.0
5.0	64.1	
6.0		65.0
8.0		66.4
10.0	64.8	66.7
12.0		66.6
14.0		66.9
15.0	65.4	
17.0		67.6
20.0	67.0	68.1
22.0	68.0	
25.0	64.1	68.4
30.0	61.8	64.5
35.0	60.0	63.2
40.0	58.7	61.0

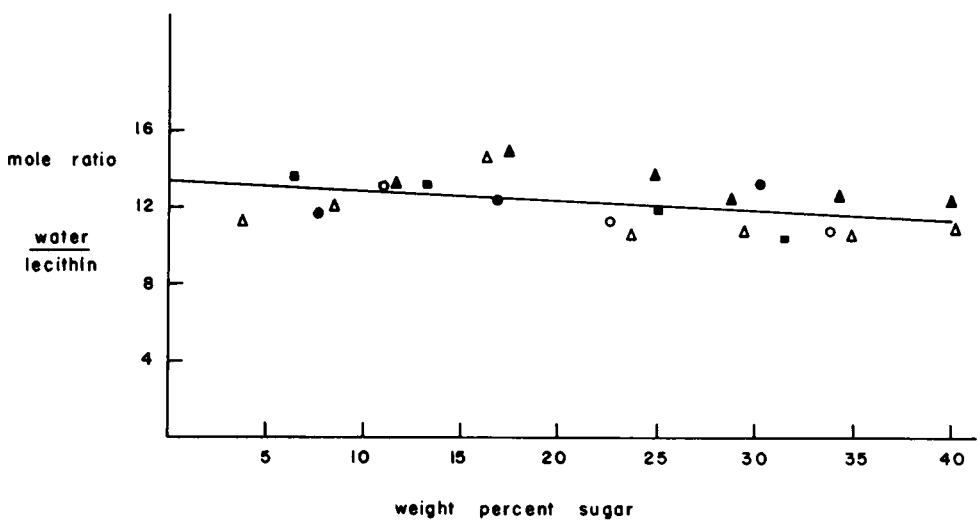

FIGURE 4 Number of water molecules between lipid bilayers that are apparently unable to dissolve sucrose. This is based on the assumption that the remaining inter-lamellar water and sucrose can equilibrate with the excess sugar solution outside the lattice.

region [13/34] × 28 = 11 Å excludes sugar). However, from the previous section it is interesting to recall (Fig. 2) that lecithin samples prepared gravimetrically and forced to mix with the sucrose solution with as high a sugar concentration as 40% behaved little differently than if pure water were used. At these sugar concentrations and particularly at volume fractions of lipid above 80% where the "bound water" layers would begin to make contact, sugars must occupy this hydration layer space. Thus sugars can penetrate into and substitute for this hydration shell without affecting the bilayer thickness.

Determination of the Interlamellar Force With Dextran Solutions

EGG LECITHIN IN WATER Fig. 5 provides structural data on the gravimetrically prepared samples of egg lecithin in pure water. The dependence of the repeat distance d, bilayer thickness d_l, and bilayer separation d_w are shown. These results and absolute dimensions are entirely consistent with those obtained many times before in

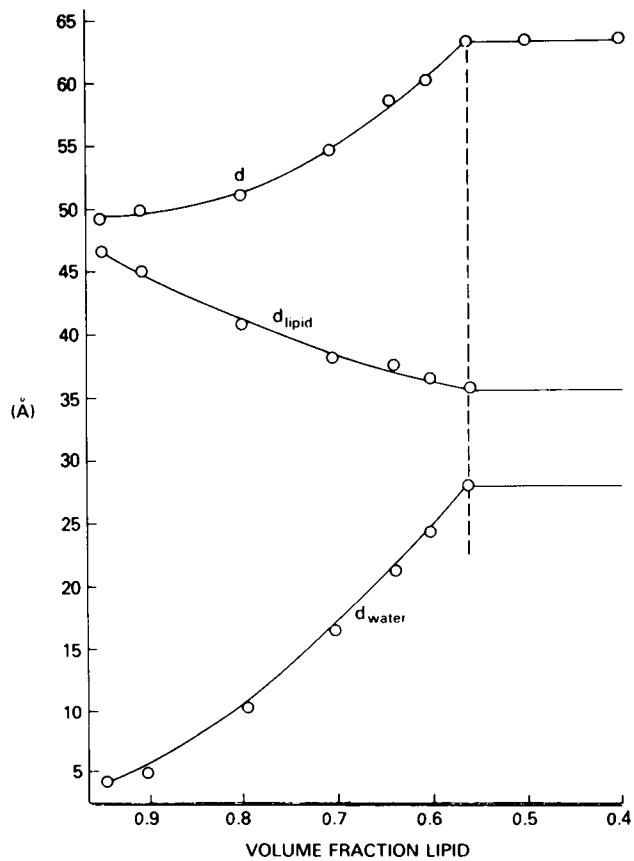

FIGURE 5 Lattice repeat, d, bilayer thickness, d_l, and separation d_w vs. concentration of lipid. At less than the lipid concentration (56% lipid) denoted by the dashed line, the lattice ceases to swell further, and a separate phase of pure water is formed.

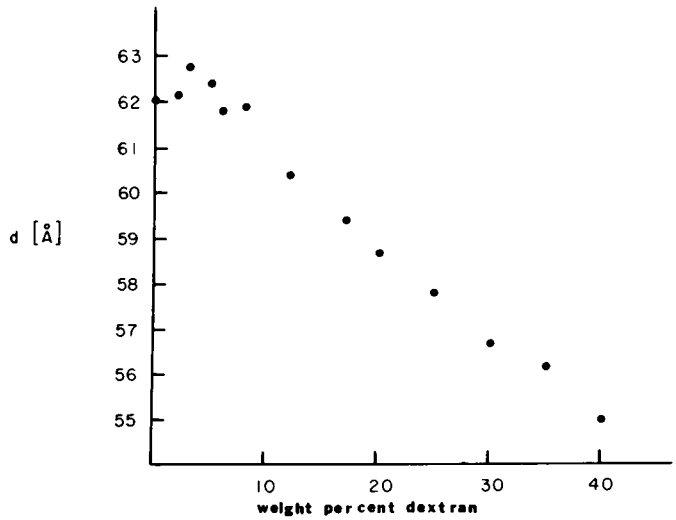

FIGURE 6 Decrease in the lamellar repeat d when lecithin is immersed in high molecular weight dextran solutions. Apparent scatter in points is within the experimental error ± 0.5 Å (14).

this laboratory and others (6–9, 17) and serve as a control for the studies using the same lecithin in dextran solutions.

EQUILIBRIUM SEPARATION OF EGG LECITHIN BILAYERS IN DEXTRAN SOLUTIONS Fig. 6 and Table II give data for lamellar phases formed by egg lecithin in dextran solutions of various concentrations. The lecithin is in equilibrium with a large excess of dextran solution. As the concentration of dextran increases, the repeat distance d of the lamellar phase decreases.

OSMOTIC PRESSURE OF THE DEXTRAN SOLUTIONS The measured osmotic pressures π of the dextran solutions are given in Table II, listed as $\log_{10}\pi$ where π is in dynes per square centimeter.

DETERMINATION OF THE INTERLAMELLAR FORCE A dextran molecule of mol wt 250,000 would have a radius of 42 Å if it were completely dry and spherical. The radius of gyration of hydrated molecules of dextrans follows the law (22–23) $R = 0.77 \times (\text{mol wt})^{0.431}$. This gives a radius of gyration of 138 Å. The aqueous space between lecithin bilayers in the maximally swelled state is about 27.5 Å. Dextran is too large to penetrate the interbilayer space and therefore remains in solution in a separate phase. We have in fact checked this by keeping a dialysis membrane between lecithin and dextran and ascertaining that the membrane has no effect, although equilibrium is achieved extremely slowly. Therefore, in the centrifuged samples, an egg lecithin-water phase coexists in equilibrium with the dextran solution and the interbilayer water is in equilibrium with the water of the dextran solution. Mild sonication, performed as a control on some of the dextran-water-phospholipid mixtures, had no effect whatsoever on the observed spacing.

Each d spacing of the lamellar phase in dextran solution (Fig. 6) can be equated to

TABLE II

DATA FROM FIG. 5 AND 6 ARE COMBINED TO GIVE LIPID BILAYER THICKNESS d_l AND WATER LAYER THICKNESS d_w TOGETHER WITH OSMOTIC PRESSURES π USED TO SHRINK THE LATTICE.

Dextran	d	d_l	d_w	$\log_{10} \pi$	$\log_{10}\lvert\mu_w\rvert$
wt %	*Å*	*Å*	*Å*	*dyn/cm²*	*cal/mol*
0	62.1	35.1	27.		
2	62.2	35.0	27.2	4.028	−2.34
3	62.8	34.9	27.9	4.204	−2.16
5	62.4	35.0	27.4	4.681	−1.69
6	61.8	35.2	26.6	4.853	−1.51
8	61.9	35.1	26.8	5.082	−1.28
12	60.4	35.6	24.8	5.415	−0.95
17	59.4	36.0	23.4	5.766	−0.60
20	58.7	36.3	22.4	5.943	−0.42
25	57.8	36.6	21.2	6.268	−0.092
28.5			(20.1)	6.511	+0.144
30	56.7	37.1	19.6		
32.5			(19.3)	6.671	+0.304
35	56.2	37.2	19.0		
39.3			(17.5)	6.994	+0.627
40	55	37.7	17.3		

Values of d_w in parentheses are interpolations. The quantity μ_w is the work of transfer of water from between bilayers to a region of pure water (see Appendix A).

the d spacing in the gravimetrically prepared samples shown in Fig. 2 (solid circles) and Fig. 5; the only difference is that water is denied to the lecithin osmotically rather than gravimetrically. (The erratic behavior at low dextran concentrations is due to error of about $\pm$ 0.5 Å in the repeat spacing when bilayers are near their spontaneously achieved minimum energy position.) With the data of Fig. 5, the d spacing of the lamellar phase in dextran solutions (Table II) was divided into bilayer thickness d_l and bilayer separation d_w. These values are listed in Table II. Also, the activity of the water in the interbilayer space, equal to that of the water in the dextran solution, is less than the activity of pure water. This reduction in activity can be viewed as resulting from a repulsive force between bilayers, putting the interbilayer water under negative pressure. The value of that repulsive force is equal to the osmotic pressure of the dextran solution, as listed in Table II. Derivation of the water activities and forces is given in Appendix A.

A plot of bilayer separation versus repulsive force is given by the solid circles in Fig. 7 (from ref. 14).

DISCUSSION

It is apparently possible to perturb a lamellar lattice of lipid membranes by addition of neutral solutes to the water without destroying the definition of the lamellar phase. Measurements on mixtures of lecithin with glucose or sucrose show a successive increase and decrease in spacing with increasing sugar concentration (Fig. 3). Observa-

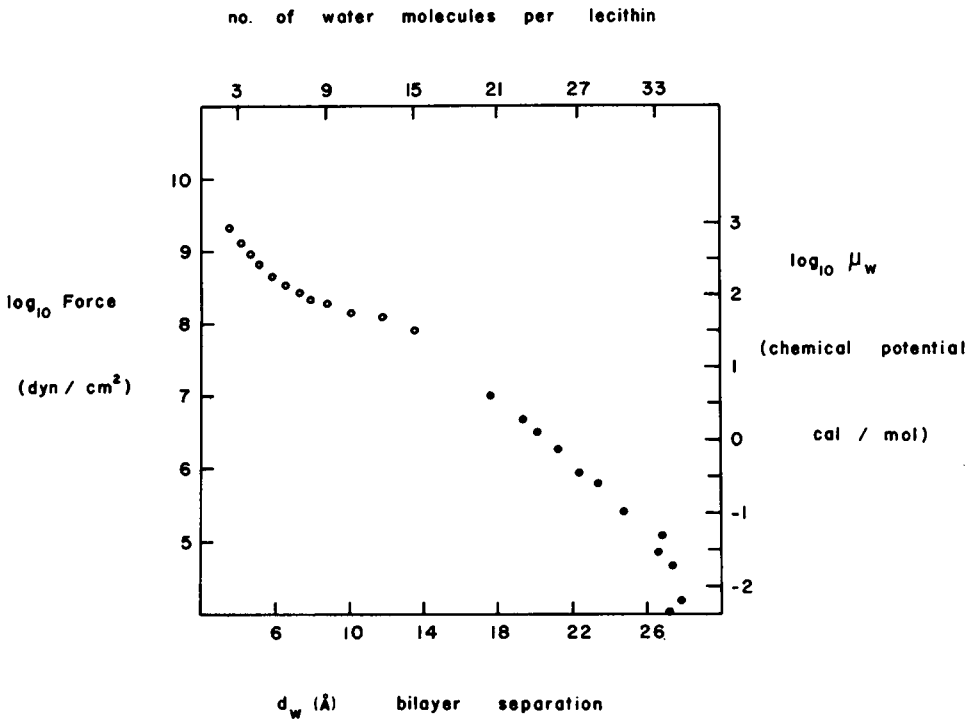

FIGURE 7 Interlamellar force vs bilayer separation. The force of pushing lamellae together can be described in terms of the work of transferring interbilayer water to a pure water phase. This work is given on the right-hand axis as the absolute value of the chemical potential μ_w relative to pure water. Instead of a bilayer separation, one may speak (top scale) of the number of waters remaining per lecithin molecule (14).

tions on lecithin swelling in solutions of large dextran molecules show a steady spacing decrease (Fig. 6). Under conditions of equilibrium with these aqueous phases, the observed spacings in all cases reflect a balance of attractive and repulsive forces between lamellae. We shall interpret the data for small sugars in terms of solute-induced modification of the attractive forces; the results with dextran illustrate and establish a general "osmotic stress" method for measuring the net repulsion between repelling bodies such as lipid membranes.

Modification of Attractive Forces by Small Sugars

The competing forces stabilizing a lamellar array of bilayers are apparently perturbed by the intrusion of small neutral sugars, sucrose and glucose, added to the aqueous medium. The multilayer system exists as a separate phase in equilibrium with a phase of pure sugar solution. The layers are given sufficient time to swell or shrink in response to any sugar-induced change in attraction or repulsion between them. The observations reported here show that the lattice swells to greater and then smaller bilayer separations (Fig. 3) as sugar concentration increases.

Why does this increase and decrease occur? Our original prediction, before actual

observation, was that bilayer separation would grow and shrink since the added sugar would successively weaken and then strengthen van der Waals attraction between lipid lamellae. Probably because of limitations in the early physical theory, people have tended to neglect the influence of solute on the electrodynamic or van der Waals interaction of macroscopic bodies suspended in a solvent medium. Since suspending media in most colloid and biological systems are in fact solutions of several components rather than pure solvents, it is worthwhile to recognize the influence of solutes and attempt to assess the magnitude of their effect on interactions.

According to the Lifshitz theory (10), the electrodynamic forces occurring at long distances between neutral macroscopic bodies are directly estimable from the polarizability of component substances described as continuous media (see Appendix B). There is no distinction in principle whether the component materials of the interacting bodies and intervening medium are chemically pure substances, composites, suspensions, or solutions, but, in that theory, the dimensions must be such that the materials can be treated as continua. Any modification of component media that alters spectroscopic properties will change electrodynamic forces.

To make the present computations, we have modeled the multilayer as bilayers of hydrocarbon bounded on either side by hydrated polar groups and alternating with layers of sugar water. The layer comprising the lipid polar groups is assumed to include the 13 molecules of water (Fig. 4) per lecithin that are free of small sugars. Because of present limitations in our knowledge of the structural and spectroscopic details of the polar region, the energy estimates are only qualitative. (A more detailed model of the bilayer could be developed mathematically but this would be inappropriate at this stage.)

Without resort to formulae (which appear in Appendix B), the weakening and strengthening of the electrodynamic force may be understood as follows:

Charge fluctuation forces between like planar bodies vary as the square of the difference in polarizabilities of the body substance and the medium substance. The total interaction involves a summation of contributions from fluctuations at all frequencies in the electromagnetic spectrum (10). The magnitude of the contribution from each frequency depends on the difference in polarizabilities at that frequency. Pure water is less easily polarized than aqueous solution or than hydrocarbon for a wide range of frequencies on either side of the visible spectrum from the mid infrared to near ultraviolet. Addition of sugar to the water increases its index of refraction and consequently decreases the difference in polarizabilities, thus decreasing the contribution of visible frequencies to the total electrodynamic interaction. At sufficiently high sugar concentrations, aqueous polarizability begins to exceed that of the hydrocarbon or polar layers and the total interaction increases with added sugar.

This behavior can be visualized from the plots of dielectric permeability $\epsilon(i\xi) - 1)$ vs. frequency shown in Fig. 8. The total interaction energy depends on a sum of the squares in differences in susceptibilities of the two substances Fig. 9 (see Appendix B).

There is a decrease and increase in the electrodynamic attraction of the layers as the sugar concentration of the aqueous layer increases. The sugar concentration at which

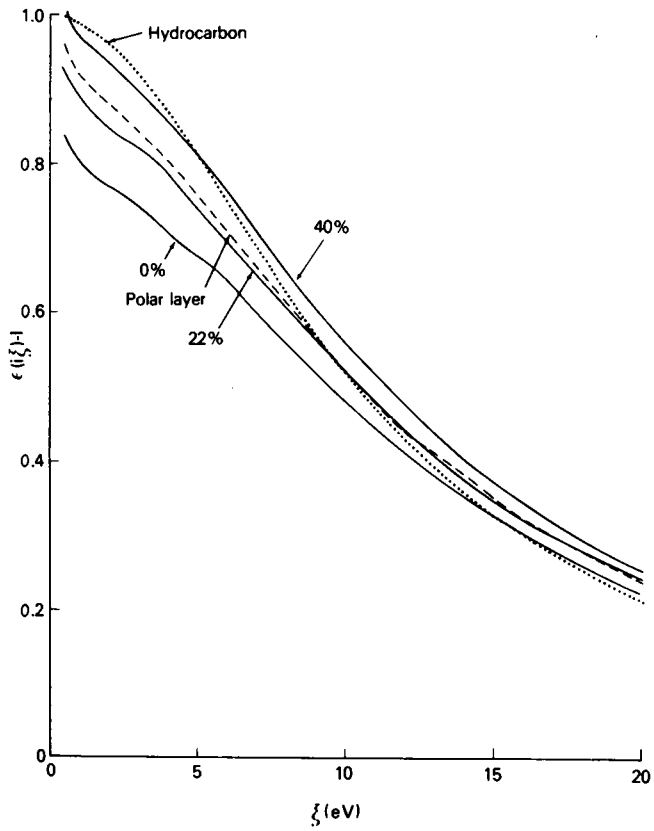

FIGURE 8 Dielectric permeabilities (Eq. B1 and Table IV) vs. frequency ξ. For compactness frequency is given in units of photon energy $\hbar\xi$. Note that as the sugar concentration increases the aqueous solution polarizabilities ϵ_a (solid lines) approach and then depart from the polar layer permitivity ϵ_p (dashed line). These are the two polarizabilities that determine the coefficient A_1 (Eq. B4*a*), which dominate the force as A_1/d_a^3 (Eq. B3).

this turnaround occurs will depend, of course, on specific properties of the spectra of all materials over the entire frequency spectrum, and its estimate necessarily depends on the assumptions we make, given the lack both of full spectral information and of detailed structure of the lipid-water interface. For present purposes it is enough to ascertain qualitatively that a successive weakening and strengthening of interaction occurs for bilayers in sugar water and that this modulation can be large enough to cause Ångstrom changes in bilayer spacing (Fig. 10, line).

Lacking complete information for computation, we must keep in mind other possible causes of the spacing changes and suggest some here.

First, to correlate spacing changes with direct interbilayer forces, one usually assumes that the small sugars are free to distribute uniformly in the aqueous regions between lipid layers. This condition is not completely satisfied in the present system. Sugar is excluded from 13 of the 33 or so molecules of water associated with each lipid

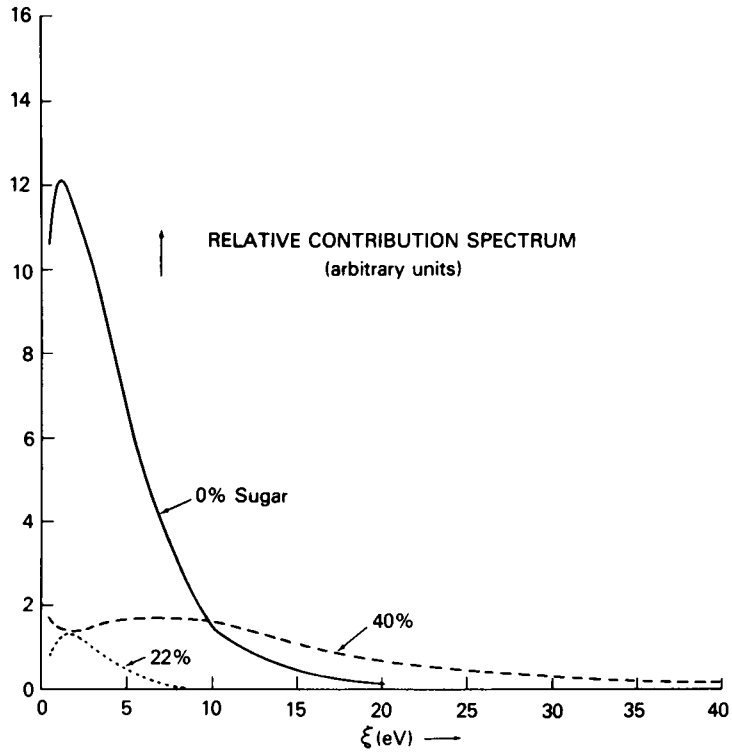

FIGURE 9 Relative contribution to the van der Waals force between bilayers. These curves are the functions being summed in Eq. B4, weighted by the distance functions appearing in brackets in Eq. B3. The curves are dominated by the difference, $[(\epsilon_a - \epsilon_p)/(\epsilon_a + \epsilon_p)]^2$, summed to give the coefficient A_1, Eq. B4a.

(Fig. 4). However, exclusion of sugars between the lamellae cannot create an osmotic force to bring them together (as it does in the case of dextran, which is completely excluded), since the sugar and water can exchange between the bulk solution and interbilayer space and can reach equilibrium. The most reasonable interpretation of the sugar exclusion is that there are two compartments of water lying in between the lamellae: one, excluding sugar, is hydrating the polar groups at the lipid-water interface; another, in the middle of the aqueous layer, is equilibrating with sugar in the bulk solutions. In spite of this exclusion an indirect effect on the bilayer is unlikely, since the bilayer thickness is constant over the entire concentration range of sugars (Fig. 2).

Second, it is possible that the small sugars interfere with repulsive rather than the attractive forces. Direct repulsive forces have been inferred (19) and here directly observed between lecithin bilayers. It has been suggested that electrostatic interactions can occur between the zwitterionic polar groups (24, 25). Both sucrose and glucose are known to decrease slightly the dielectric constant of water (26), and might thereby alter electrostatic repulsion. However, it is not obvious how this could explain how the addition of sugar should first increase then decrease the repulsion.

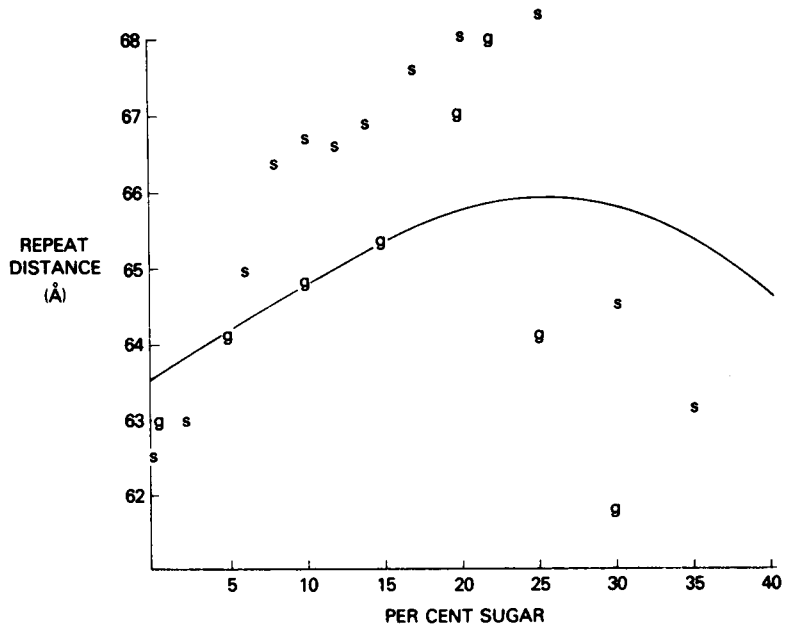

FIGURE 10 Expected shift in repeat spacing d (solid line) with change in sucrose concentration. Letters indicate data points for sucrose (s) and glucose (g) solutions.

We, therefore, favor the explanation described above and in Appendix B, that the small sugars modify the electrodynamic forces and cause the waxing and waning of bilayer separation.

Measurements of Forces between Bilayers Using the Osmotic Force of Dextran Solutions

Bilayers in water come to an equilibrium spacing at which the long-range forces of repulsion and electrodynamic attraction are equal. This equilibrium can be perturbed by exerting an osmotic stress on the lamellar phase by means of dextran in the extralamellar phase. Dextran is too large (22–23) to penetrate between the lipid bilayers, so it tends to extract water from the aqueous layers separating the bilayers, thereby decreasing the thickness of the water layers. Equilibrium is reached when the forces tending to compress the water layers equal those expanding them. Those tending to compress them are the long-range attractive force between bilayers and the osmotic pressure π of the dextran solution. These are balanced by the repulsion between bilayers. Thus $|\pi| + |F_A| = |F_R|$.

If, in dextran, $|F_A| \ll |\pi|$, then $|F_R| \approx |\pi|$. On the basis of this assumption (discussed below), one can describe the relation between π and bilayer separation d_w by the function $F_R(d_w)$. The results for 17.3 Å $< d_w <$ 26.6 Å can be accurately fitted to a curve of the form $F_R = P_0 e^{-d_w/\lambda}$, with the constants $P_0 = 10^{11}$ dyn/cm^2 and $\lambda =$ 1.93 Å determined by a least squares best fit. The experimental points must depart

from this curve as d_w approaches the 27.5 Å equilibrium separation, i.e. as $|F_A|$ becomes comparable to, and then equals $|F_R|$. At $|F_A| = |F_R|$, $\pi = 0$; the $\log_{10}(\pi)$ must diverge toward $-\infty$. The estimate of $\lambda \approx 2$Å suggests that significant changes in F_R occur with changes in separation on the order of dimensions of water molecules.

To supplement the picture derived from our osmotic stress measurements, we have used the data of Elworthy (27, 28)[1] for the amount of water imbibed by lecithin exposed to different water-vapor pressures p_w. The chemical potential of water in the resulting mixture is given by $\mu_w = RT\ln(p_w/p_w^{sat})$. The repeat distances and layer thicknesses were not determined in Elworthy's samples. We used our structural parameters that correspond to the lipid/water weight ratios reported by Elworthy. The results are plotted as the open circles in Fig. 5. The conversion of data is described in Appendix A.

It appears that our osmotic (solid points) and the vapor pressure (open points) can be connected as one curve, but that the exponential behavior is lost below about 15 Å separation. Given the uncertainties in this extension, particularly the nonidentity of the lecithins, it is probably unwise to press comparison further.

There appear to be at least two possible sources contributing to bilayer repulsion across an aqueous layer: (*a*) Coulombic interaction between charges on the zwitterionic head group; (*b*) work of removal of waters of hydration around the polar groups. In an earlier analysis (25) of electrostatic interaction acting between bilayers with limited amounts of water, it was found that no static, ordered arrangement of zwitterionic groups gave an adequate description for lipid repulsion (in fact, static arrays are likely to feel coulombic attraction). Rather surprisingly, a model of the zwitterions pictured as comprising an electrostatic double-layer (25) gave a good rationale for observed changes (7,9) in d_w and d_l with removal of water. However, compared to our present measurements of lamellar repulsion, this double-layer model gives poor results. Marcelja and Radic (29) have suggested an order parameter expansion to describe the exponential force in terms of modification of water structure.

Attractive Forces between Bilayers

As emphasized above, the coexistence of a lamellar phase with a separate water phase is evidence of an attraction between lecithin bilayers; with no attractive force the bilayers would separate indefinitely. At $d_w = 27.5 \pm 0.5$ Å there is an observed balance between repulsive and attractive forces. Formally, $\pi = 0$, and $F_R = F_A$. In using our π vs. d_w data to estimate F_A at the point of balance, we note that $F_R(d_w)$ varies much more rapidly with d_w than does $F_A(d_w)$. If, as we expect, F_A is due to an electrodynamic van der Waals interaction, it will vary roughly as the one-third power of the bilayer separation. Such a power dependence on separation is much slower than the $e^{-d_w/1.93A}$ variation observed for net force π just below the equilibrium separation. We may then say that F_A is of negligible magnitude compared to F_R below the equilibrium separation.

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[1] Elworthy, P. H. 1962. Personal communication.

We have already used this assumption to extract the relation $F_R = P_0 \exp(-d_w/\lambda)$. We now say that F_R maintains this form out to the equilibrium separation and extrapolate the exponential law to the point of equilibrium, $d_w = 27.5 \pm 0.5$ Å. In that range of separations the extrapolated F_R takes on the values 5–8.3 $\times 10^4$ dyn/cm^2, which we take to be an estimate of F_A at equilibrium separations. As shown in Appendix B, this magnitude of the inferred attraction is expected for a van der Waals force between bilayers made of a hydrocarbon core bounded by wet polar group layers.

To check for consistency we may calculate the ratio F_A/F_R near $d_w = 27.5$ Å, while F_A is given by an inverse cube law

$$|F_A/F_R| = (27.5/d_w)^3 e^{(d_w - 27.5)/1.93 \text{ Å}}.$$

At 26.5 Å the ratio is 0.66, while by 25.0 Å F_A/F_R is only 0.36.

Since the lamellar phase is known to occur for a great many phospholipids, one can now use osmotic stress to probe the interactions of a wide variety of systems. In particular the lamellar lattice can incorporate charged lipids which will create electrostatic double-layer forces. Using the osmotic stress technique, one may now measure double-layer interactions between model membranes of deliberately varied charge immersed in aqueous reservoirs of any pH and salt concentration. Recent studies on charged membranes (30) show that their repulsion follows that expected from double-layer theory at separations greater than 30 Å but resembles repulsion between zwitterionic lecithin (solid points, Fig. 7) at smaller separations.

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APPENDIX A

Osmotic Pressure Data

The water activities, a_w, of the swelled lipid phase and the dextran solution with which it is in equilibrium are equal. The chemical potential, μ_w, relative to pure water is written in terms of the relative activity a_w. The chemical potentials of the dextran solutions have been determined from their osmotic pressures, π, against pure water; thus $\pi \bar{V}_1 = -RT \ln a_w$, where $\bar{V}_1$ is the partial molar volume of water (18 cm^3/mol) and π (cm Hg $\times$ 1.33 $\times 10^4$ for units of dyn/cm^2) is the pressure that must be applied to the dextran solution to maintain it in equilibrium with pure water.

Hence $-\mu_w = \pi \bar{V}_1$ ergs/mol ($\times$ 2.39 $\times 10^{-8}$ to get cal/mol) gives the work of transferring water from the interbilayer space to pure water.

The chemical potential of the water between lecithin bilayers must be reduced to that of the dextran solution with which it is in equilibrium. This reduction can be considered as resulting from the negative pressure that the water is under as a result of the repulsive force between lipid bilayers. This force is just equal to the osmotic pressure π of the dextran solution. The resultant values are plotted in Fig. 7 and listed in Table II.

Vapor Pressure

Professor P. H. Elworthy[1] supplied us with tabulated values of relative vapor pressures p_w that exist over "natural" lecithin-water mixtures of known composition. Assuming that the lipid formed the same dimensions of lamellar phase as does egg lecithin of similar composition, from our X-ray data of Fig. 5 and Table III we calculated the bilayer separation d_w of each sample.

From the relative vapor pressure p_w/p_w^{sat} we calculated the chemical potential of the water between bilayers as $\mu_w = +RT\ln(p_w/p_w^{sat})$ in cal/mol. The absolute value of μ_w is the work of moving water from the interbilayer space to bulk water. By dividing this work per mole by the molar volume of water (18 cm^3/mol), the force per unit area between bilayers is calculated.

The values obtained are shown in Table III and the resultant force law is plotted in Fig. 7 (open circles).

APPENDIX B

As indicated in Fig. 3 and Table I, the lamellar repeat distance d goes from 63 to 68 Å, then decreases when sucrose or glucose is added to the water. Is this change in spacing in accord with shifts in the balance point of forces? In this appendix we suggest the affirmative by a plausibility argument.

If one imagines that the exponential repulsion law is unaffected by small sugars and also that the law holds even out to a repeat spacing d of 68 Å (both tenuous assumptions), one may check whether the expanded spacings can result from expected changes in the van der Waals attraction.

From the relation $F_R = P_0 e^{-d_w/1.93A}$, one expects a decrease in F_R by a factor of $e^{-(32.5-27.5)/1.93} = 1/13.3 = 0.075$ in the lamellar repulsion (and balancing attraction) as bilayers move apart. For the attractive force one can expect a change only of the order of 10% in the "Hamaker coefficient" A_H of hydrocarbon across water. If one used an attractive force of the form $F_A = -A_H/6\pi d_w^3$ (for two infinitely thick planar hydrocarbon bodies across a space d_w), the decrease in attraction would be by a factor of only $0.9 \times (27.5)^3/(32.5)^3 = 0.54$. This would suggest that the expected increase in bilayer separation as a result of adding small

TABLE III

REDUCTION OF DATA SUPPLIED BY PROFESSOR P. H. ELWORTHY (27, 28)[1] AS DESCRIBED ABOVE.

a	Relative vapor pressure	Lipid	d_w	$\log_{10}$(Force)	$\log_{10}\lvert\mu_w\rvert$
g H_2O/100 g lipid		*wt %*	*Å*	*dyn/cm²*	*cal/mol*
5.0	0.207	95.2	3.5	9.34	2.97
8.0	0.390	92.6	4.1	9.11	2.75
10.0	0.517	90.9	4.6	8.96	2.59
12.0	0.628	89.3	5.1	8.81	2.44
14.0	0.714	87.7	5.8	8.67	2.30
16.0	0.780	86.2	6.5	8.53	2.17
18.0	0.823	84.7	7.3	8.42	2.06
20.0	0.856	83.3	7.9	8.33	1.96
22.0	0.877	82.0	8.8	8.26	1.89
25.0	0.899	80.0	10.1	8.17	1.80
30.0	0.912	76.9	11.8	8.10	1.74
35	0.943	74.1	13.5	7.91	1.54

TABLE IV
SPECTRAL CONSTANTS

Region	w_D	C_D	w^{ir}	C^{ir}	w_w^{uv}	C_w	w_s	C_s
	eV		eV		eV		eV	
Aqueous solution	6.5×10^{-5}	74.8	0.021	1.464	12.7	0.78	8.82	$n_s^2 - n_w^2$
			0.067	0.737				
			0.092	0.153				
			0.2	0.014				
			0.42	0.075				
							w_p, eV	C_p
Polar layer	6.5×10^{-5}	74.8	0.021	1.464	12.7	0.78	7	0.22
			0.069	0.737				
			0.092	0.153				
			0.2	0.014				
			0.42	0.075				
Hydrocarbon core		0				10.4		1

sugars to the interbilayer space would be much less than observed. As in studies of interactions across bilayers (30 *a*), a more careful analysis which includes more features of the bilayers is required.

Following Tinker et al. (31), we divide the repeat distance d into a hydrocarbon layer, d_h, bounded on either side by polar group layers d_p and separated by an aqueous sugar solution d_a, $d = d_h + 2d_p + d_a$. To be consistent with the sugar uptake measurements, we assume that the aqueous layer is in equilibrium with the bathing sucrose solution while there are 13 water molecules per lecithin in the polar group layer. (This division is somewhat arbitrary but reasonable. The computations below should therefore be considered illustrative rather than definitive.) Following ref. 31, we assign the thicknesses d_h = 24.8 Å, d_p = 10.9 Å, and d_a = 16.4 Å when d = 63 Å for lecithin in pure water.

In computing van der Waals forces we use methods described previously (32): a single ultraviolet oscillator model without bandwidth for the polarizability of each component in each layer. As elaborated elsewhere (32, 33), for use in computation one may write dielectric permeabilities of each layer in the form

$$\epsilon(i\xi) = 1 + \Sigma\{C_D/[1 + (\xi/w_D)]\} + \Sigma\{C_l/[1 + (\xi/w_l)^2]\} \qquad \text{(B1)}$$

The constants w_l were absorption resonance frequencies and the C's measure the strength of absorption. The quantities w_D and C_D describe the orientation polarization of polar molecules at microwave frequencies, which we take to occur at only one frequency. For C_l we will use data from indices of refraction and for w_l estimates of mean absorption frequency from ionization potentials. (Much better data are available for water [34] and hydrocarbons [35] and are being prepared for sugar solutions.[2] Information about the phosphoryl-chlorine glycerol polar group region, critical if the present illustrative calculations are to be made rigorous, is not known to us at all.)

While aware of the crude simplification it entails in the context of the present calculation, we consider the polarizability of both polar layer and sucrose solution as that of liquid water plus a single oscillator at ultraviolet frequencies. Thus the properties of these regions are described (Table IV) by the spectrum of water (first six columns of Table IV plus an additional oscillation of Eq. B1, $C_s/[1 + (\xi/w_s)^2]$ for sucrose in water and $C_p/[1 + (\xi/w_p)^2]$

[2]Painter, L. R. Personal communication.

for the polar layer. The frequency w_s for sugar is taken from the ionization potential for sucrose while C_s is the measured difference in the square of the refractive indices of sucrose solutions and pure water. The corresponding constants w_p and C_p for the polar group layer are chosen arbitrarily.

Formulae employed for computation are given in ref. 36 and are used in the effective pairwise sum approximation (Eq. 16 of that paper). For the attractive force per unit area, F_a, of two bilayers we have

$$-6\pi F_A = A_1\left[\frac{1}{d_a^3} - \frac{2}{(d_a + 2d_p + d_h)^3} + \frac{1}{(d_a + 4d_p + 2d_h)^3}\right]$$

$$+ 2A_2\left[\frac{1}{(d_a + d_p)^3} - \frac{1}{(d_a + d_p + d_h)^3} - \frac{1}{(d_1 + 3d_p + d_h)^3} + \frac{1}{(d_a + 3d_p + 2d_p)^3}\right]$$

$$+ A^3\left[\frac{1}{(d_a + 2d_p)^3} - \frac{1}{(d_a + 2d_p + d_h)^3} + \frac{1}{(d_a + 2d_p + 2d_h)^3}\right] \quad \text{(B3)}$$

where

$$A_1 = \frac{3}{2} kT \sum_{n=0}{}' \left(\frac{\epsilon_a - \epsilon_p}{\epsilon_a + \epsilon_p}\right)^2 \quad \text{(B4}a\text{)}$$

$$A_2 = -\frac{3}{2} kT \sum_{n=0}{}' \left(\frac{\epsilon_a - \epsilon_p}{\epsilon_a + \epsilon_p}\right)\left(\frac{\epsilon_h - \epsilon_p}{\epsilon_h + \epsilon_p}\right) \quad \text{(B4}b\text{)}$$

$$A_3 = \frac{3}{2} kT \sum_{n=0}{}' \left(\frac{\epsilon_h - \epsilon_p}{\epsilon_h + \epsilon_p}\right)^2 \quad \text{(B4}c\text{)}$$

with k and T being the Boltzmann constant and absolute temperature. The sums Σ' are taken over the integers $n = 0, 1, 2, \ldots$ where the $n = 0$ term is multiplied by 1/2. The permeabilities in Eq. B1 are evaluated at eigenfrequencies $\xi_n = [(2\pi)^2 kT/h]n$, $n = 0, 1, 2, \ldots = 0.159n$ (eV) where h is Planck's constant.

Most of the van der Waals attraction comes from the first term in each series in square brackets in Eq. B3. The force is most sensitive to the difference in permeabilities $(\epsilon_a - \epsilon_p)$ between the polar group layer (ϵ_p) and aqueous solution (ϵ_a). The functions ϵ_a, ϵ_p, and ϵ_h are plotted in Fig. 8 for three concentrations of sucrose, 0%, 22%, and 40%. The calculations show that the difference $(\epsilon_a - \epsilon_p)^2$ decreases, then increases with increasing concentration. The leading term in the force, A_1/d_l^3 (Eqs. B3 and B4*a*), involves squares of the difference $(\epsilon_a - \epsilon_p)$ where the sum $(\epsilon_a - \epsilon_p)^2/(\epsilon_a + \epsilon_p)^2$ (Eq. B3) goes through a minimum. cf. Fig. 9 for plots of the contribution to the force (Eqs. B3 and B4) from charge fluctuations at each frequency over the entire spectrum.

For each sucrose concentration we compute an attractive force and find the separation at which it balances repulsion. We find (Fig. 10) a variation of the order of Ångstroms. The location and size of the maximum spacing depends on the dielectric properties assigned to the polar and aqueous layer. It is relatively insensitive to the properties of the hydrocarbon core or to the infrared terms in ϵ_a and ϵ_p. The qualitative correspondence between observations of these computations does not rigorously establish our interpretation but only makes plausible the idea that spacing changes may be due to weakening and strengthening of the van der Waals forces.

Note Added in Proof

Using a mechanical pressure apparatus, we have extended the measurements of repulsive pressure up to 630 atm (or $10^{8.8}$ dyn/cm^2) and bilayer separations of 9 Å. The exponential repulsion law is obeyed at least until this point and the experimental points are consistent with those of Elworthy (Fig. 7). Such enormous repulsive forces suggest that the fusion of neutral phospholipid bilayer vesicles is highly improbable without distortion of the phospholipid packing.

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