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Molecular packing and area compressibility of lipid bilayers

(phospholipids/membranes/intermolecular forces/x-ray diffraction/neutron diffraction)

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ABSTRACT **Knowledge of the molecular packing of lipids and water in lipid bilayers is important for understanding bilayer mechanics and thermodynamics. Information on packing is most often obtained from x-ray or neutron diffraction measurements. Given the *d* spacing, composition, and partial specific volumes of the lipid and water, it is a simple matter to calculate the area per lipid molecule, bilayer thickness, and bilayer mass density. The partial specific volumes are commonly assumed to be those of bulk water and of lipid in excess water regardless of the degree of bilayer hydration. We present evidence here that these assumptions should be seriously questioned. At low hydrations, we find the head groups of egg and dioleoyl lecithin to be much less tightly packed than previously thought and the partial specific volume of water to be considerably smaller than 1 ml/g. Because the molecular packing affects the mechanical properties of bilayers, we use the results to reevaluate published experiments concerning the elastic area compressibility modulus of egg lecithin bilayers and the repulsive hydration force between bilayers.**

To describe fully the intermolecular forces in bilayers, one must understand the packing arrangements and constraints of the lipids and water comprising them. The molecular packing density of bilayers is revealed macroscopically by the partial specific volumes of the components as a function of composition. It has been commonplace to assume (*i*) that the partial specific volumes of water ($\bar{v}_W$) and lipids ($\bar{v}_L$) in bilayers are independent of the degree of hydration of the bilayer and (*ii*) that each equals 1 ml/g. Recent neutron diffraction experiments in our laboratory (1) using hexane molecules to probe the packing constraints of dioleoyl phosphatidylcholine (Ole_2PtdCho) bilayers suggest that these assumptions, which we shall call the common assumptions, are probably incorrect. We examine this issue further in the present paper and pay particular attention to its effect on the mechanical properties of bilayers.

The intermolecular forces that determine the molecular packing in bilayers are revealed when one changes the surface area of a bilayer of fixed mass by lateral compression or extension. The elastic area compressibility modulus has been measured for egg yolk phosphatidylcholine (EY-PtdCho) bilayers in two laboratories by using two different methods. Kwok and Evans (2) have used micropipet aspiration combined with video analysis to extend and measure the surfaces of single-walled vesicles. Parsegian *et al.* (3) have used osmotic and physical pressure to compress multilamellar liposomes. The structural equations of Luzzati (3–5) (see Fig. 3) are used to estimate the resulting changes in area per molecule. The moduli obtained in the two approaches differ by an order of magnitude. The method of Parsegian *et al.* (3) depends critically upon the assumptions that $\bar{v}_W = \bar{v}_L = 1$. We show here that failure of these assumptions is the likely explanation of the discrepancy.

As far as we are aware, neither $\bar{v}_W$ nor $\bar{v}_L$ have ever been measured for any lipid lamellar phase containing less than excess water. We do know, however, that the partial specific volume of water in extremely concentrated electrolyte solutions is considerably less than 1 ml/g (6, 7). For example, $\bar{v}_W$ in a nearly 100% sulfuric acid solution approaches 0.5 ml/g (8). Because the polar groups of lipids must surely form a very concentrated electrolyte solution, the water is likely to behave in a very nonideal way. This is exactly what is observed. Fig. 1 shows hydration data for Ole_2PtdCho and EY-PtdCho from several laboratories (9–11) plotted as relative vapor pressure of water (P/P_0) versus mol fraction of water (X_W). The dashed curve shows how an ideal solution should behave. The water in the lipid mixture exhibits a large negative deviation from ideality, as would be expected if the water is strongly attracted to the head groups. The arrows mark the water contents corresponding to 11–13 water molecules per lipid, which is the generally accepted range for the number of waters in the primary hydration shell of phosphatidylcholine lipids (see review by Hauser, ref. 12). The nonideality, which is clearly associated with the water of the polar group hydration shell, suggested to us that the partial specific volume of the water in bilayers at low hydrations might be <1 ml/g.

We have solved the generally intractable problem of measuring the mass density ($= \bar{v}^{-1}$) of bilayers at low hydrations by using diffraction measurements to determine the area per molecule (A). Measurements of changes in area per molecule and d spacing with hydration lead immediately to the partial specific volume because the volume of a lipid molecule and the n water molecules associated with it is equal to $A \cdot d/2$. We discuss this approach in greater detail below and present compelling evidence that the partial specific volumes of the lipids and water in lamellar phases at low hydration are neither constant nor equal to 1.

We describe direct neutron diffraction measurements of the hydrocarbon thickness of Ole_2PtdCho at 66% RH and an analysis of x-ray diffraction data on EY-PtdCho published by Torbet and Wilkins (13). We calculate areas per molecule significantly different from those predicted from the common assumptions using the Luzzati equations (4). We calculate $\bar{v}_W$ and $\bar{v}_L$ as a function of hydration of the EY-PtdCho. We then reevaluate the data of Parsegian *et al.* (3) and compare it to the results of Kwok and Evans (2). Our reevaluation brings the two sets of data into excellent agreement, gives a new view of how water activity can affect bilayer mechanics, and reveals a repulsive force due to the bound water layer.

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Abbreviations: EY-PtdCho, egg yolk phosphatidylcholine; Ole_2PtdCho, dioleoyl phosphatidylcholine; Myr_2PtdCho, dimyristoyl phosphatidylcholine; Pam_2PtdCho, dipalmitoyl phosphatidylcholine; RH, relative humidity.

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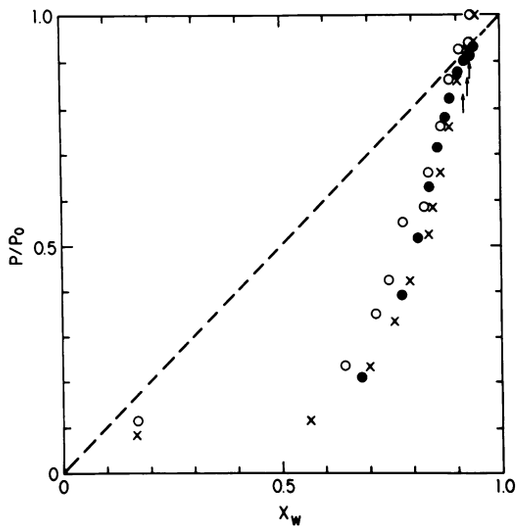

FIG. 1. Relative vapor pressure of water (P/P_0) as a function of the mol fraction of water (X_W) in lamellar lipid phases. Data have been replotted from measurements of water uptake as a function of relative humidity (RH). Plotted in this way, a large negative deviation from ideality for the water in the bilayers is revealed. The large negative deviations from the ideal curve (dashed line) are those expected from very strong attractive interactions between the water and head groups. The arrows mark positions corresponding to 11–13 waters per lipid, which comprise the primary hydration shell of the head groups (see ref. 12 for review). The nonideality range corresponds to the hydration shell. Data for EY-PtdCho are marked by ○ (ref. 9) and ● (refs. 10 and 11). ×, Data for $Ole_2PtdCho$ (ref. 9).

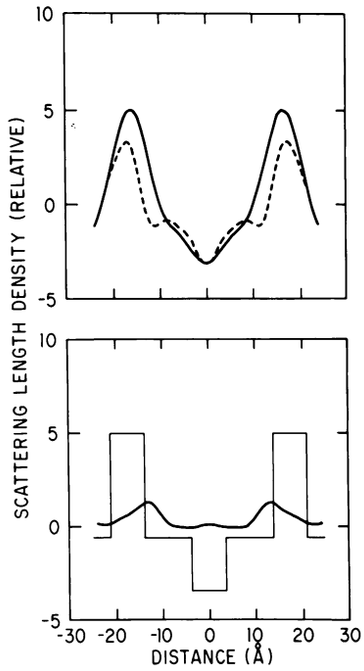

FIG. 2. Determination of the hydrocarbon thickness of $Ole_2PtdCho$ bilayers. (*Upper*) Structures of $Ole_2PtdCho$ (dashed curve) and $Ole_2PtdCho$ deuterated at the C-2 positions of acyl chains (solid curve) based upon eight orders of diffraction data. The difference of these two structures, shown as the heavy curve in *Lower*, reveals the locations of the C-2 carbons that are taken as the markers of the hydrocarbon core. Superimposed on the difference structure in lighter lines is a strip-function model, which can also be used to determine the hydrocarbon thickness of bilayers accurately (1). The hydrocarbon thickness derived by either method is 28 ± 1 Å. All measurements were performed at 22.5°C and 66% RH. The scattering length-density scales are arbitrary; the scales chosen for *Upper* and *Lower* are different from one another.

Neutron Diffraction Measurements on $Ole_2PtdCho$

There is abundant evidence in the literature that the packing of the acyl chains in phospholipid bilayers is that expected of bulk alkyl liquids (1, 4, 14, 15). This means that if the thickness of the hydrocarbon layer (d_{hc}) can be accurately determined, the area per phospholipid can be simply calculated from the equation

$$A = 2V_{ac}/d_{hc}, \quad [1]$$

where V_{ac} is the average combined total molecular volume of the two acyl chains of the phospholipid. This volume can be calculated from the individual volumes of the olefin, methylene, and methyl groups derived from the densities of bulk alkyl liquids (4, 16). The carbonyl groups are generally assumed to be part of the head group and are excluded from the calculation. Thus, C-2 carbons are taken as the inclusive boundaries of the hydrocarbon region. Lewis and Engelman (17) have used this method to determine the areas of a number of different phospholipids.

We have developed a method (ref. 1; unpublished data) for accurately determining the hydrocarbon thickness using strip-function models fitted to neutron diffraction data. A more direct approach, and the one reported here, is to selectively label the phospholipid with deuterium at the C-2 positions and use difference neutron scattering-length-density profiles to determine the distance between the C-2 carbons on opposite sides of the membrane, which is taken as equal to d_{hc}. This difference–structure method has been described in detail elsewhere (19–21).

Fig. 2 shows the difference structure of oriented $Ole_2PtdCho$ bilayers at 66% RH. For comparison, a strip-function model determined as described in ref. 1 has been included. Both the difference structure and the strip model yield a hydrocarbon thickness of 28 ± 1 Å. The volume of a single oleic acid chain is 475 $Å^3$ (16), which leads to a value of A of 68 ± 2 $Å^2$. The Luzzati equations, on the other hand, predict a value of 60 $Å^2$ assuming there are six waters per phospholipid (ref. 9) *and* that the partial specific volumes of both lipid and water are 1 ml/g. The molecular volume occupied by a head group and its associated water can be easily calculated to be 736 $Å^3$ from $(d \cdot A - 2V_{ac})/2$ for d = 49.7 ± 0.5 Å. The value calculated by using 60 $Å^2$ for the area is 540 $Å^3$. This strongly suggests that the partial specific volumes of the lipid and water must be different from 1 ml/g and that the molecular packing in the head group must be different than predicted from estimates based on the crystalline volume of phosphocholine (22). If we had measurements of d_{hc} as a function of hydration, it would be easy to calculate the partial molecular volumes. Lacking such data, we analyzed the x-ray data of Torbet and Wilkins (13) for EY-PtdCho and found it to be consistent with our single result for $Ole_2PtdCho$.

Analysis of X-Ray Diffraction Data From EY-PtdCho

Torbet and Wilkins (13) have published d spacings and structures for oriented EY-PtdCho multilayers at different hydrations and for EY-PtdCho liposomes in excess water. Our analytical procedure was to determine the transbilayer phosphate-to-phosphate distance (d_{pp}) from the structures, subtract twice the phosphate-to-C-2 distance to obtain d_{hc}, and calculate A using Eq. **1**, where we took V_{ac} = 907 $Å^3$ assuming one palmitic and one oleic chain per EY-PtdCho molecule. This was done for each hydration. The amount of water per lipid was determined from the data of Fig. 1. The "unitary" volume (V_U, the volume occupied by one lipid and

its water molecules) was calculated from $V_U = d \cdot A/2$. We then plotted V_U against the number of water molecules per lipid and thus obtained the partial molecular volume of the water in the bilayers as a function of hydration. Before describing the results, which are summarized in Table 1, it is necessary to provide some additional information on the calculations.

To obtain d_{hc}, we needed a reasonable estimate for the phosphate-to-C-2 distance for phosphatidylcholine lipids in the liquid crystalline state. Excellent neutron diffraction data on specifically deuterated dipalmitoyl phosphatidylcholine ($Pam_2PtdCho$) in the liquid crystal state at 10 and 25 weight% water have been published (23, 24). Labeling was done in the region of the phosphate group and the C-4 carbon. Because we needed the position of the C-2 carbon, we subtracted 1 Å per carbon (ref. 17) from the phosphate-to-C-4 distance to establish a phosphate-to-C-2 distance of 7 Å. Because this number was the same at 10 and 25 weight% water, it seems unlikely that this distance depends upon the degree of hydration. We therefore subtracted 14 Å from d_{pp} to arrive at d_{hc} regardless of hydration.

We found two tests for our analysis of the data. Worcester (25) has published neutron diffraction data for EY-PtdCho at 66% RH, which is one of the hydrations used by Torbet and Wilkins. We applied our strip-function method (1) for determining d_{hc} to his data and found $d_{hc} = 28$ Å. We obtained a value of 28.1 Å from Torbet and Wilkins' data. The values are in excellent agreement with each other and with our directly measured value for $Ole_2PtdCho$ reported above. The other test was to compare the value of A obtained from Torbet and Wilkins' data for the excess water case with that calculated from Eq. 1 (which must give the *correct* answer because excess water is present and $\bar{v}_L$ can be measured). Tardieu *et al.* (26) report a value of $\bar{v}_L$ of 0.987 ml/g for EY-PtdCho liposomes in excess water where we assume $\bar{v}_W$ must be 1 ml/g. Taking the molecular weight of EY-PtdCho as 770, we calculate A to be 72.8 Å^2. The value we obtain from our analysis of Torbet and Wilkins' data for excess water is 73.1 Å^2. The agreement among the various numbers is excellent. This gives us confidence in our conclusions.

Fig. 3 *Upper* shows the change in d_{pp} with bilayer hydration compared to the change in d_l calculated from the Luzzati equations assuming $\bar{v}_L = \bar{v}_W = 1$ ml/g. The change in d_{pp} is quite modest compared to d_l and indicates that the bilayer is much less deformable than expected on the basis of the common assumptions. A similar observation was made by Janiak *et al.* (27) on dimyristoyl phosphatidylcholine (Myr_2-PtdCho) in the $L\beta'$ phase. Significantly, they too found d_l to be much greater than d_{pp} at low hydrations and speculated that the partial specific volume of the lipid might depend upon hydration. Fig. 3 *Lower* shows the change in A calculated from d_{hc} compared to the change in A calculated from Eq. 1 by using the common assumptions. This figure reveals very clearly two things about the bilayer at low hydration. First, the membrane is much stiffer than permitted by the common assumptions. Second, the volume change of the bilayer with increasing hydration is very small, suggesting that $\bar{v}_W$ is significantly smaller than 1. This is confirmed in Fig. 4.

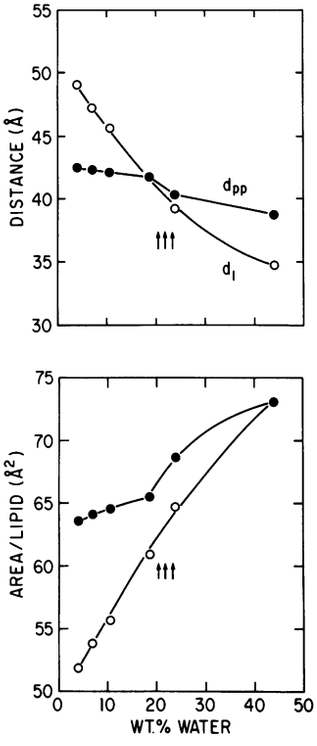

FIG. 3. Structural parameters for EY-PtdCho bilayers as a function of water content. (*Upper*) Variation in the phosphate-to-phosphate distance (d_{pp}) (●) compared to d_l (○) calculated from $d_l = \phi d$, where $\phi = [1 - (1 - c)\bar{v}_W/c\bar{v}_L]^{-1}$, assuming the partial specific volumes of water and lipid are each 1 ml/g independent of water content. d_l is a hypothetical bilayer thickness, which assumes the water forms a pure separate layer of thickness $d_w = d - d_l$. d_{pp} changes relatively little with hydration, suggesting that $\bar{v}_W$ is smaller than expected. (*Lower*) Area per lipid (A) calculated from the hydrocarbon thickness by using Eq. 1 (●) compared to A calculated from $A = 2MW_L\bar{v}_L/\phi d N_0$ (○). The arrows in *Upper* and *Lower* indicate the water contents corresponding to the 11–13 hydration shell waters per lipid. In the Luzzati equations above (ref. 4), c is the dry-weight percent of lipid, N_0 is Avogadro's number, and MW_L is the molecular weight of the lipid.

Table 1. Summary of the structural parameters of EY-PtdCho bilayers at various water contents

RH, %	n, waters per lipid	d, Å	d_{pp}, Å	d_{hc}, Å	d_p, Å	A, Å^2	V_U, Å^3
23	1.8	51.0	42.5	28.5	22.5	63.6	1622
47	3.2	50.8	42.3	28.3	22.5	64.1	1628
66	5.1	51.0	42.1	28.1	22.9	64.5	1645
92	9.8	51.1	41.7	27.7	23.4	65.5	1674
100*	13.4	51.4	40.4	26.4	25.0	68.7	1766
Excess*	33.6	62.0	38.8†	24.8	37.2	73.1	2266

The data were derived from those of Torbet and Wilkins (13). n, Number of water molecules per lipid; d, d spacing; d_{pp}, phosphate-to-phosphate distance; d_{hc}, thickness of the hydrocarbon region; d_p, thickness of the polar region ($d - d_{hc}$); A, area per molecule; V_U, unitary volume, which is the molecular volume of one phospholipid and its associated water molecules. V_U is plotted against n in Fig. 4 to obtain the partial molecular volumes of the lipid and the water.
*The water contents as derived from the d spacings (ref. 11) are not the same for excess water and 100% RH because it is difficult to achieve a precise hydration via the vapor phase at RHs near 100%.
†Corrected from Torbet and Wilkins' data (13) to take into account Fourier termination errors.

The molecular volume of a lipid molecule and its associated water (V_U) is plotted in Fig. 4 as a function of the number of waters per lipid. There is a large change in slope between 10 and 13 waters per lipid. We do not know the correct equation for the points but we found we could accurately fit two straight lines to the data, which obeyed the equation $V_U = \bar{V}_{Lm} + \bar{V}_{Wm}n$, where n is the number of waters per lipid. We interpret $\bar{V}_{Lm}$ as the partial *molecular* volume of the lipid and $\bar{V}_{Wm}$ as the partial *molecular* volume of the water. For $n < 10$, we find $\bar{V}_{Lm} = 1609 \pm 2$ Å^3 and $\bar{V}_{Wm} = 6.66 \pm 0.37$ Å^3 compared to the common assumption values of 1270 Å^3 and 30 Å^3. Assuming that the primary fatty acids of EY-PtdCho are palmitic and oleic, we estimate V_{ac} to be 907 Å^3. Subtracting this number from 1609 yields 702 Å^3, which is comparable to the value of 736 Å^3 we obtained in the previous section for $Ole_2PtdCho$. The molecular volume of water is, of course, the real surprise. It suggests very strong electrostrictive effects in the polar head groups. At hydrations above 10 waters per lipid, we find lipid and water volumes of 1432 ± 2 Å^3 and 24.8 ± 0.1 Å^3. These are much closer to the expected values but still seem to be significantly different. If they are, it means that $\bar{v}_L$ and $\bar{v}_W$ may not reach unity until the excess water phase appears.

Reevaluation of Area Compressibility Data

Having obtained values for $\bar{V}_{Lm}$ and $\bar{V}_{Wm}$, we now reevaluate the data of Parsegian *et al.* (3) to examine the dependence of

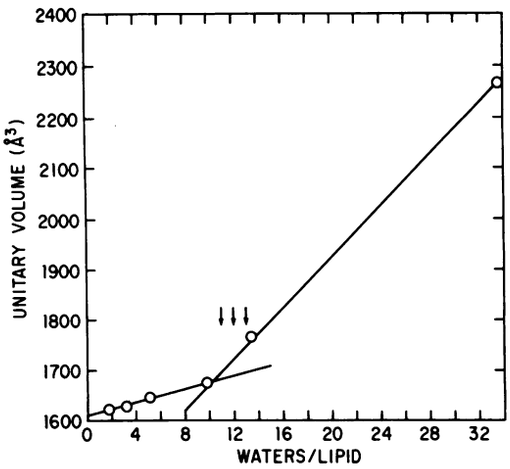

FIG. 4. Molecular volume of an EY-PtdCho "molecule" and its associated waters (unitary volume, V_U) as a function of the number of waters per lipid (derived from ref. 13). The arrows indicate the approximate number of waters per lipid in the primary hydration shell. At low hydrations, the lipid has a volume of 1609 ± 2 Å³ and the water has a volume of 6.66 ± 0.37 Å³. At high hydrations, the numbers are 1432 ± 2 and 24.8 ± 0.1 Å³. The common assumptions lead to 1270 Å³ and 30 Å³ for all hydrations.

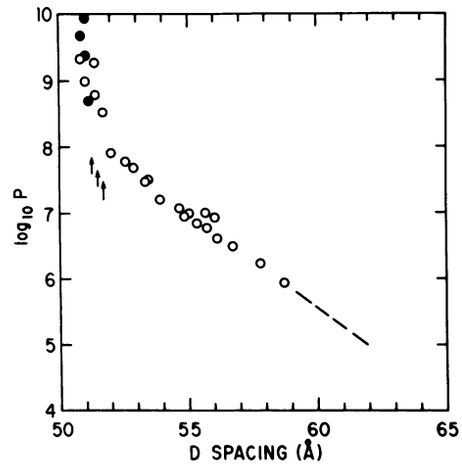

FIG. 5. $\log_{10}$ of equivalent applied pressure (P) as a function of d spacing. $P = -\mu_W/\bar{V}_W$. ○, Derived from data of refs. 3 and 11; ●, derived from data of ref. 13. The arrows indicate the hydration shell of 11–13 waters per lipid. Note the strong repulsive force due to the hydration shell. Once the shell is filled, the hydration force of Parsegian *et al.* (3) prevails (curve to the right of the arrows).

the equivalent pressure P on bilayer spacing and of the lateral pressure (F_{LP}) on A. The former provides information on the repulsive hydration force, whereas the latter provides information on the elastic area compressibility modulus (2). We proceeded as follows. We first constructed a table of corresponding values of d spacing, n, and P from the data of Parsegian *et al.* (3) and Torbet and Wilkins (13). We determined n from Fig. 1 or from plots of d versus weight% lipid (11). For $n < 10$, where the bilayers were equilibrated with saturated salt solutions, we calculated the equivalent pressure from $P = -\mu_w/\bar{V}_W$, where μ_w and $\bar{V}_W$ are, respectively, the chemical potential and partial *molar* volume of the water. $\bar{V}_W$ was calculated from our value of $\bar{V}_{Wm}$ and was found to be 4 ml/mol. Because $\bar{V}_W$ is now much smaller than 18 ml/mol, the equivalent pressure at a given d will be much greater than calculated by Parsegian *et al.* For $n > 10$, the calculated pressures differed little from those calculated by Parsegian *et al.* (3). Having established data sets of d, n, and P, we calculated $V_U(n)$ (Fig. 4), from which we determined A ($= 2V_U/d$), d_{hc} (Eq. 1), and d_p ($= d - d_{hc}$). d_p should not be confused with d_{pp}. d_p is the thickness of the polar region consisting of the head groups plus water. We calculated F_{LP} slightly differently than did Parsegian *et al.* (3), who took $F_{LP} = P \cdot d_W/2$. Because d_W is ill-defined, we chose to replace it with d_p and could justify doing so by using the same derivation as Parsegian *et al.* (3).

Fig. 5 shows a plot of $\log_{10}P$ versus d. For d spacings greater than those corresponding to 11–13 waters per lipid we obtain a curve representing the repulsive hydration force, which is not significantly different from that of Parsegian *et al.* (3). However, for d spacings corresponding to <11–13 waters per lipid, the curve becomes much steeper. This segment of the bilayer repulsion curve must correspond to the work required to remove water from the hydration shell of the head groups. Its characteristic decay length is about 0.1 Å rather than the ≈2.5 Å corresponding to the hydration force.

Fig. 6 shows a plot of lateral pressure (F_{LP}) against area per molecule (A). Also shown, for comparison, are a few of the points calculated by Parsegian *et al.* (3). It is quite clear that the lateral repulsive force is much steeper than previously calculated. A basic feature of the result of Parsegian *et al.* is still retained, however. At the equilibrium area (73 Å², open triangle) where $F_{LP} = 0$, the slope of the curve, which is a measure of the elastic area compressibility modulus, is much smaller than that expected from Kwok and Evans' distension measurements (2), which should be continuous with Parsegian *et al.*'s compression measurement at the equilibrium area. However, Kwok and Evans performed their measurements in 0.1 M NaCl, whereas Parsegian *et al.* used pure water. We thus calculated the osmotic pressure of a 0.1 M NaCl solution and determined from LeNeveu *et al.*'s measurements (11) the corresponding d spacing. Following the procedure outlined earlier, we then calculated A and F_{LP}. This point, corresponding to Kwok and Evans' equilibrium point, is plotted in Fig. 6 as a "+." We then constructed a straight line through this point having a slope of 1.40 dyn/cm (1 dyn = 10 μN) per 1% change in area, which corresponds to the elastic area

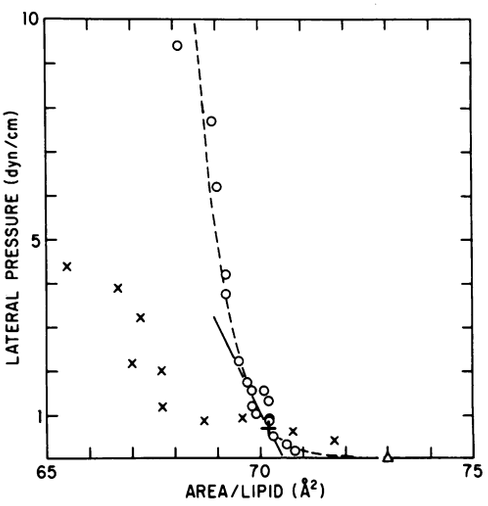

FIG. 6. Lateral repulsive pressure (F_{LP}) versus area per lipid. $F_{LP} = P \cdot d_p/2$, where d_p is the thickness of the polar region defined as $d - d_{hc}$. ×, Data of Parsegian *et al.* (3) obtained from pure water as originally presented by them; ○, the same data reevaluated as described in the text; △ at 73 Å², the equilibrium area per lipid in pure water. The heavy "+" represents the equilibrium point of Kwok and Evans (2), who did their measurements of the elastic area compressibility in 0.1 M NaCl. The line drawn through the + has a slope of 1.4 dyn/cm per 1% change in area corresponding to Kwok and Evans' elastic area compressibility modulus of 140 dyn/cm.

compressibility of 140 dyn/cm determined by Kwok and Evans (2). The slope of $F_{LP}(A)$ at the Kwok and Evans point is clearly very close to 140 dyn/cm. The $F_{LP}(A)$ curve seems to represent accurately, then, the surface pressure versus area equation of state curve for EY-PtdCho and places Kwok and Evans' data on an absolute scale. The thermodynamic basis for $F_{LP}(A)$ has been described in detail by Evans and Skalak (18). An immediate implication of the curve is that A depends strongly on water activity at physiological ionic strengths. The area per molecule changes by about 4.5% upon going from pure water to 0.1 M salt.

Conclusions and Discussion

The basic hypothesis that comes from our neutron diffraction measurements (above and ref. 1) and our analysis of Torbet and Wilkins' data (13) is that the partial molar volumes of the lipid and water in bilayers depend strongly on the degree of hydration. For Ole_2PtdCho at 66% RH, the molecular volume of the head group and its six water molecules is about 736 Å^3 rather than the 540 Å^3 predicted by using the common assumptions. For EY-PtdCho, the molecular volume of the head group approaches about 700 Å^3 as the number of waters per lipid approaches zero. Allowing for possible error, the number is unlikely to be smaller than 650 Å^3. Based on Small's arguments (22), which start with various crystalline and liquid molecular volumes for head group constituents, a value of about 380 Å^3 would be expected, which is almost a factor of 2 smaller than seems to be observed. To place the numbers in a slightly different context, one can calculate the volume swept out by a phosphocholine group rotating about one end in a plane parallel to the interface. By using Corey–Pauling–Koltun models, this volume can be estimated to be about 1300 Å^3. This suggests that a major factor in determining head group packing may be an excluded volume effect.

The conceptual model that arises from the above line of thought is as follows. At very low hydrations (1 or 2 waters, for instance), the head groups are not efficiently packed, perhaps due to an excluded volume effect. As additional waters enter the head groups, they bind tightly to the head groups within the existing excluded volume spaces and consequently cause minor changes in volume (partial molecular volume, about 7 Å^3 rather than 30 Å^3). As more waters are added after the filling of the hydration shell, they behave more normally and, we assume, osmotic pressure is driving the head groups apart. The further apart they are, the smaller excluded volume effects will be. Our analysis suggests that the partial specific volume of water might remain somewhat smaller than 1 between end of hydration shell filling (13 waters per lipid) and the beginning of the excess water phase (33 waters per lipid). There may be a lingering effect of head group group volume exclusion. It occurs to us that this could well be tied in with the hydration force (3).

Our conceptual model ignores packing changes in the hydrocarbon region. In the absence of solutes that may enter the hydrocarbon core selectively (1), we agree with Lewis and Engelman (17) that the head groups must predominate in determining the area per molecule. We note that the areas per molecule we calculate for EY-PtdCho, which range from 64 to 73 Å^2 with increasing hydration, are in complete accord with Lewis and Engelman's (17) values of 65–70 Å^2. The agreement improves when one considers that they did their experiments in 0.1 M salt, which, according to Fig. 6, should cause a decrease in A. Including the effect of the water activity reduction by the salt, the range of A becomes 64–70 Å^2.

We have not failed to consider the possibility that the ideas discussed in this paper could have implications for statistical mechanical models of bilayers, for membrane fusion, and for the interactions of proteins with bilayers. In the latter case, seemingly subtle changes in either the polar region or the hydrocarbon region could alter the behavior of the other region. The work of McIntosh and Simon and their colleagues (29–31) shows that the addition of solutes soluble in the polar region can cause interdigitation of the acyl chains of opposing monolayers under some circumstances. Our studies (1) of the interaction of hexane with Ole_2PtdCho at low hydrations suggest that the hexane causes a significant loosening of the polar group packing and tightening of the hydrocarbon packing densities. We wonder if the regulation of the insertion and transport of proteins into and across bilayers might not be mediated in part by such effects.

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