Molecular packing and area compressibility of lipid bilayers
(phospholipids/membranes/intermolecular forces/x-ray diffraction/neutron diffraction)

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Communicated by Harden M. McConnell, June 4, 1985

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 more 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 (v\text{w}) and lipids (v\text{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\text{2}PtdCho) 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 imaging to expand and measure the surface area 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 v\text{w} = v\text{L} = 1.

We show here that failure of these assumptions is the likely explanation of the discrepancy.

As far as we are aware, neither v\text{w} nor v\text{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, v\text{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\text{2}PtdCho and EY-PtdCho from several laboratories (9–11) plotted as relative vapor pressure of water (P/P\text{0}) versus mol fraction of water (X\text{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 (= f\text{w}−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 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\text{2}PtdCho 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 v\text{w} and v\text{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.

Abbreviations: EY-PtdCho, egg yolk phosphatidylcholine; Ole\text{2}PtdCho, dioleoyl phosphatidylcholine; Myr\text{2}PtdCho, dimyristoyl phosphatidylcholine; Pum\text{2}PtdCho, 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 Ole2PtdCho (ref. 9).

Neutron Diffraction Measurements on Ole2PtdCho

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

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

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 Ole2PtdCho 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 \( 28 \pm 1 \) Å. The volume of a single oleic acid chain is \( 475 \) Å\(^3\) (16), which leads to a value of \( A \) of \( 68 \pm 2 \) 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 \( (dA - 2V_{ac})/2 \) for \( d = 49.7 \pm 0.5 \) 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 Ole2PtdCho.

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 \( (d_{ph}) \) 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 $V_U = dA/2$. 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 $d_{ph}$, 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 (PamPtdCho) 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 $d_{ph}$ to arrive at $d_{ph}$ 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 hydrons used by Torbet and Wilkins. We applied our strip-function method (1) for determining $d_{ph}$ to his data and found $d_{ph} = 28.8$ 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 Ole2PtdCho 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 $V_L$ can be measured). Tardieu et al. (26) report a value of $V_L$ of 0.987 ml/g for EY-PtdCho liposomes in excess water where we assume $V_w$ must be 1 ml/g. Taking the molecular weight of EY-PtdCho as 770, we calculate $A$ to be 72.8 Å². The value we obtain from our analysis of Torbet and Wilkins’ data for excess water is 73.1 Å². The agreement among the various numbers is excellent. This gives us confidence in our conclusions.

Fig. 3 Upper shows the change in $d_{ph}$ with bilayer hydration compared to $d_1$ calculated from the Luzzati equations assuming $V_L = V_{wm} = 1$ ml/g. The change in $d_{ph}$ is quite modest compared to $d_1$ 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 (Mry2-

PtdCho) in the LB phase. Significantly, they too found $d_1$ to be much greater than $d_{ph}$ 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 $d_{ph}$ 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 $V_w$ is significantly smaller than 1. This is confirmed in Fig. 4.

The molecular volume of a lipid molecule and its associated water ($V_{lm}$) 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 15 waters per lipid. We do not know the exact equation for the points but we found we could accurately fit two straight lines to the data, which obey the equation $V_{lm} = V_{lm}^0 + V_{wm}n$, where $n$ is the number of waters per lipid. We interpret $V_{lm}$ as the partial molecular volume of the lipid and $V_{wm}$ as the partial molecular volume of the water. For $n < 10$, we find $V_{lm} = 1609 ± 2 Å^3$ and $V_{wm} = 6.66 ± 0.37 Å^3$ compared to the common assumption values of 1270 Å³ and 30 Å³. Assuming that the primary fatty acids of EY-PtdCho are palmitic and oleic, we estimate $V_{lm}$ to be 907 Å³. Subtracting this number from 1609 Å³ gives us a value comparable to the value of 736 Å³ obtained in the previous section for Ole2PtdCho. The molecular volume of water is, of course, the real surprise. It suggests very strong electrostatic 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 $24.8 ± 0.1 Å^3$, which are much closer to the expected values but still seem to be significantly different. If they are, it means that $V_L$ and $V_w$ may not reach unity until the excess water phase appears.

Reevaluation of Area Compressibility Data

Having obtained values for $V_{lm}$ and $V_{wm}$, we now reevaluate the data of Parsegian et al. (3) to examine the dependence of
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 $\mu_w$ 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 $V_{Ld}(n)$ (Fig. 4), from which we determined $A (= 2V_{Ld}/d_0)$, $d_{hc}$ (Eq. 1), and $d_0 (= d - d_{hc})$. $d_0$ should not be confused with $d_{pp}$. $d_0$ 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 $F_{LP} = Pd_{hc}/2$. Because $d_{hc}$ is ill-defined, we chose to replace it with $d_0$ and could justify doing so by using the same derivation as Parsegian et al. (3).

Fig. 5 shows a plot of $\log P$ 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 Å$^2$, 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 Kwo and Evans’ distension measurements (2), which should be continuous with Parsegian et al.’s compression measurement at the equilibrium area. However, Kwo 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 Kwo 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 $\mu$N) per 1% change in area, which corresponds to the elastic area
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 Ole2PtdCho 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.

We thank Dr. Benno P. Schoenborn and his staff for their advice and encouragement. Conversations with Dr. Russell Jacobs were very helpful in forming the ideas contained in this paper. Dr. James Hall’s insightful comments on the manuscript were invaluable. We are pleased to acknowledge the gift of deuterated Ole2PtdCho provided by Dr. J. Seelig. Parts of this research were carried out at Brookhaven National Laboratory under the auspices of the Department of Energy with the additional support of the National Science Foundation. This research was supported by grants from the National Science Foundation and the National Institutes of Health.