Area per Molecule and Distribution of Water in Fully Hydrated Dilauroylphosphatidylethanolamine Bilayers†

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ABSTRACT: The area per lipid molecule for fully hydrated dilauroylphosphatidylethanolamine (DLPE) has been obtained in both the gel and liquid-crystalline states by combining wide-angle X-ray diffraction, electron density profiles, and previously published dilatometry results [Wilkinson, D. A., & Nagle, J. F. (1981) *Biochemistry 20*, 187-192]. The molecular area increases from 41.0 ± 0.2 to 49.1 ± 1.2 Å^2 upon melting from the gel to liquid-crystalline phase. The thickness of the bilayer, as measured from the electron density profiles, decreases about 4 Å upon melting, from 45.2 ± 0.3 to 41.0 ± 0.6 Å. A somewhat unexpected result is that the fluid layer between fully hydrated bilayers is the same in both gel and liquid-crystalline phases and is only about 5 Å thick. From these data, plus the volume of the anhydrous DLPE molecule, it is possible to determine the number of water molecules per lipid and their approximate distribution relative to the lipid molecule. Our analysis shows that there are about 7 and 9 waters per DLPE molecule in the gel and liquid-crystalline phases, respectively. About half of the water is located in the fluid space between adjacent bilayers, and the remaining waters are intercalated into the bilayer, presumably in the head group region. There are significantly fewer water molecules in the fluid spaces between DLPE bilayers than in the fluid spaces in gel- or liquid-crystalline-phase phosphatidylcholine bilayers. This small fluid space in PE bilayers could arise from interbilayer hydrogen bond formation through the water molecules or electrostatic interactions between the amine and phosphate groups on apposing bilayers.

Three of the fundamental parameters used in describing lipid bilayers are (1) the area per molecule, (2) the amount of associated water per lipid molecule, and (3) the distribution of this water relative to the lipid molecules. The area per lipid molecule A is important in understanding or quantitating the asymmetric distribution of lipids in membranes (Israelachvili et al., 1980), lipid diffusivity (McCown et al., 1981), interactions between acyl chains and polar head groups (Kanbara & Sasaki, 1984), surface charge density (McLaughlin, 1977), theoretical models of lipid bilayers in the presence and absence of proteins (Mouritsen & Bloom, 1984), and nonbilayer structures such as the hexagonal phase (Seddon et al., 1983a). Knowledge of the number of water molecules per lipid molecule n is essential in obtaining lipid/water phase diagrams (Güldbrand et al., 1982) and in understanding the repulsive and attractive interactions between membranes (LeNeveu et al., 1976; Parsegian & Rand, 1983). Also the distribution of water molecules relative to the lipid bilayer is closely correlated to the potential energy profile across the membrane (Diamond & Katz, 1974) and the manner in which both extrinsic and intrinsic proteins associate with the bilayer (Engelman & Seitz, 1981).

Previously, the area per lipid molecule has been obtained primarily from two sources: monolayers, where A can be directly measured at a given surface pressure π, and X-ray diffraction of multilamellar bilayers, where A can be calculated from the lamellar repeat period d and the volume fraction of lipid ϕ_L in the dispersion. However, there are potential problems with both of these techniques in determining the area per molecule in a bilayer. For the monolayer technique the relationship between π and A for monolayers and bilayers is still controversial as is the question of whether monolayers at oil-water or air-water interfaces better represent the state of lipids in bilayers (Albrecht et al., 1978; MacDonald & Simon, 1985; Gruen & Wolfe, 1984; Phillips & Chapman, 1968). For the X-ray experiments, it is difficult to determine ϕ_L precisely because lipids are hygroscopic and trace impurities can affect the amount of water absorbed. Thus, it is not surprising to find the large variations reported for A. For example, for liquid-crystalline dipalmitoylphosphatidylcholine (DPPC)[1] bilayers estimates for A range from 56.8 (Inoko & Mitsui, 1978) to 71.2 Å^2 (Lis et al., 1982).

The water distribution in phospholipid bilayers can be determined by neutron diffraction. However, due to limited resolution in the neutron diffraction patterns, water profiles across the bilayer are usually only obtainable for partially hydrated lipid multilayers (Worcester & Franks, 1976; Büldt et al., 1979).

In this paper, using the well-characterized lipid dilauroylphosphatidylethanolamine (DLPE), we develop a new technique for determining area per molecule and water distribution for the fully hydrated liquid-crystalline phase. The method involves first determining A for fully hydrated gel-state DLPE from the crystalline wide-angle X-ray reflection and then calculating the change in area in going from gel to liquid-crystalline phase by combining the change in volume for this

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[1] Abbreviations: DLPE, L-α-dilauroylphosphatidylethanolamine; DPPC, L-α-dipalmitoylphosphatidylcholine; DDPE, didodecylphosphatidylethanolamine; BPE, bacterial phosphatidylethanolamine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; OA, oleic acid; SD, standard deviation.

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transition obtained by dilatometry (Wilkinson & Nagle, 1981; Chang & Epand, 1983) with the change in bilayer hydrocarbon thickness obtained by electron density profiles. Using this method, we find that A increases from 41.0 to 49.1 Å^2 for DLPE in going from the gel (Lβ) to the liquid-crystalline (Lα) phase.

These values of A, combined with the electron density profiles, permit a calculation of n in both gel and liquid-crystalline phases, as well as the approximate distribution of these water molecules relative to the lipid head groups. The values obtained for n, 6.7 and 9.2 for the Lβ and Lα phases, respectively, are consistent with literature values (Seddon et al., 1983b; Cevc & Marsh, 1985). An important result from this analysis is that fully hydrated DLPE bilayers, in either the gel or liquid-crystalline phase, are separated by at most 5 Å, or about 2 water molecules. This value is much smaller than for phosphatidylcholines (PC), which are separated by about 5 water molecules (McIntosh & Simon, 1986). These differences may help explain why vesicles containing PE fuse under certain conditions and why PC cannot replace PE in promoting fusion (Düzgünes et al., 1981).

Materials and Methods

DLPE was purchased from Avanti Biochemicals and used as obtained. Dispersions of these lipids were made by swelling them in doubly distilled water and incubating them above their transition temperatures with periodic vortexing to prevent nonequilibrium phases resulting from inadequate hydration (Seddon et al., 1984; Mantsch et al., 1983).

X-ray diffraction data were collected and analyzed as previously described (McIntosh et al., 1980). Briefly, the specimens were sealed in quartz X-ray capillaries and mounted in a temperature-controlled specimen holder in a pinhole-collimated X-ray camera containing four sheets of Kodak DEF X-ray film in a flat-plate film cassette. Diffraction patterns were densitometered with a Joyce-Loebl Microdensitometer MK III, and integrated intensities were determined. The patterns for the gel phase were recorded at 20 ± 2 °C whereas those of the liquid-crystalline phase were recorded at 35 ± 2 °C. The main transition temperature of fully hydrated DLPE is 30 °C (Mantsch et al., 1983).

Results

Diffraction Patterns and Electron Density Profiles. Densitometer traces of typical X-ray patterns of fully hydrated DLPE in the gel phase and the liquid-crystalline phase are seen in parts A and B, respectively, of Figure 1. The gel-phase pattern contains 4 orders of a lamellar repeat period of 50.5 ± 0.1 Å (mean ± SD; N = 3 experiments) and a single sharp wide-angle reflection at 4.21 ± 0.01 Å (N = 3). This pattern is characteristic of the Lβ phase where the lipid chains are hexagonally packed and oriented perpendicular to the plane of the bilayer (Tardieu et al., 1973). The liquid-crystalline pattern contains four orders of a lamellar spacing of 46.1 ± 0.3 Å (N = 3) and a single broad wide-angle band centered at about 4.5 Å. These repeat periods and wide-angle spacings are similar to those previously reported (Seddon et al., 1986b; Chang & Epand, 1983). Parts A and B of Figure 2 show electron density profiles for the three samples of fully hydrated DLPE in the gel and liquid-crystalline phases, respectively. Clearly, the electron density profiles superimpose closely. The phase angles used in Figure 2A are equivalent to those used by Hitchcock et al. (1974) for DLPE crystals, and the phase angles used in Figure 2B are equivalent to those used by Simon et al. (1982) for the liquid-crystalline phase of bacterial phosphatidylethanolamine (BPE). These are the only

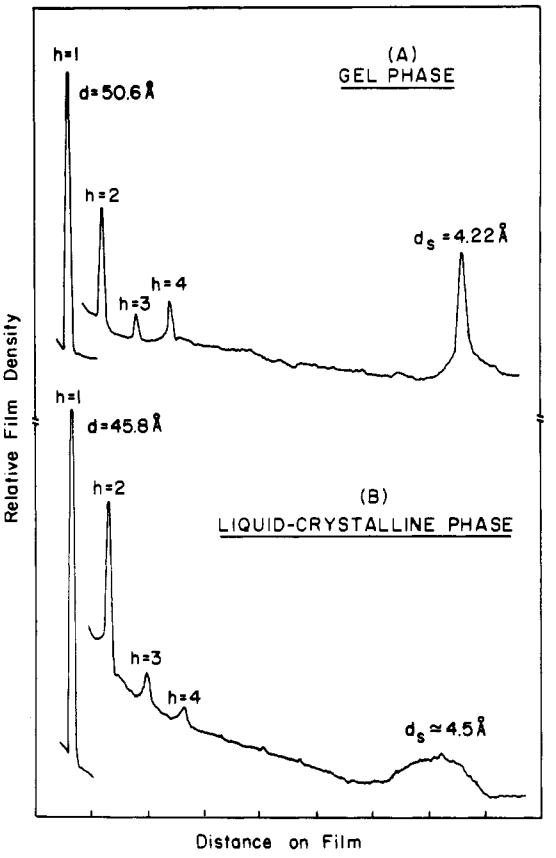

FIGURE 1: Densitometer traces of fully hydrated dilauroylphosphatidylethanolamine bilayers in (A) the gel phase (20 °C) and (B) the liquid-crystalline phase (35 °C). For both phases, 4 orders (h = 1–4) of a lamellar repeat period, d, are observed. For both phases orders 2–4 and the wide-angle reflections (d_s) are from traces of the first films in the cassette, whereas first orders (h = 1) are from traces of the third films in the pack.

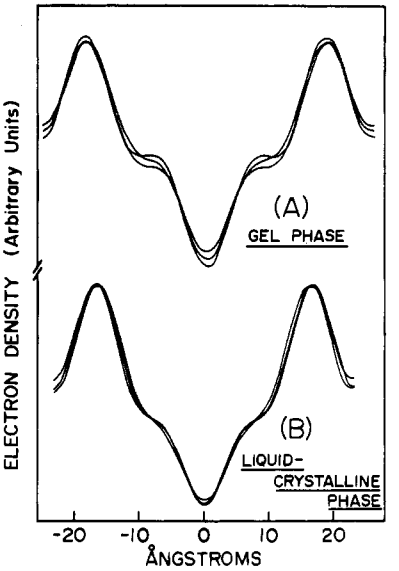

FIGURE 2: Electron density profiles for three separate experiments for DLPE in (A) the gel phase and (B) the liquid-crystalline phase.

phase-angle combinations that give profiles consistent with a bilayer structure. In both profiles the highest electron density peaks correspond to the polar head groups, and the trough in the geometric center corresponds to the terminal methyl region. The medium-density regions between the head group peaks and the terminal methyl trough correspond to the methylene groups, and the narrow medium-density regions at the outer

edges of each profile correspond to the fluid layers between adjacent bilayers. In the profiles of the gel-state bilayer, the terminal methyl trough is deeper, indicating more ordered hydrocarbon chains in this phase as compared to the liquid-crystalline phase. In going from the gel to liquid-crystalline phase, the distance between head group peaks decreases from 37.2 ± 0.3 (N = 3) to 33.0 ± 0.6 Å (N = 3). Thus, the decrease in distance between the head group peaks upon melting is, within experimental uncertainty, equal to the decrease in lamellar repeat period, 50.5 Å − 46.1 Å = 4.4 Å. Since most of the volume change observed on chain melting occurs in the hydrocarbon region of the bilayer (Wilkinson & Nagle, 1981), this agreement provides excellent evidence that (1) the thickness of the fluid layer between bilayers remains nearly constant upon melting and (2) the conformation of the phosphate moiety–glycerol backbone portion of the DLPE head group is approximately the same in the gel and liquid-crystalline phases. At this resolution, the electron density profiles are relatively insensitive to the position of the amine portion of the PE head group. However, the calculation of area per molecule (below) does not depend on the position of the amine moiety.

The electron density profiles can be used to estimate the width of the bilayer L and the width of hydrocarbon core of the bilayer H for both gel and liquid-crystalline phases. Since the boundaries between lipid and fluid space and between lipid head group and lipid hydrocarbon core are not molecularly smooth (Hauser, 1981) and are characterized by thermal undulations (Marra & Israelachvili, 1985), any definition of these boundaries is somewhat arbitrary. However, an advantage of the Fourier synthesis approach over other methods used to obtain A is that the electron density profiles provide structural information on particular regions of the lipid molecule. Thus, we use definitions of these boundaries based on the best structural data available. It is known from the analysis of Hitchcock et al. (1974) that, at the resolution of the profiles in Figure 2, the head group peak is located between the phosphate moiety and the glycerol backbone. From the crystal structure of DLPE (Hitchcock et al., 1974), this would place the head group peak in the electron density profiles about 4 Å from the outer edge of the DLPE molecule and about 5 Å from the deeper carbonyl group, which we use to delimit the edge of the hydrocarbon region of the bilayer. This definition of the edge of the hydrocrbon layer is consistent with the previous analysis of Simon et al. (1982), who found that water penetrates 5.5 Å into the bilayer from the head group peak in profiles of BPE bilayers. Using these definitions for the edges of the bilayer and the bilayer hydrocarbon region, we find for gel and liquid-crystalline bilayers, respectively, L = 45.2 and 41.0 Å and H = 27.2 and 23.0 Å.

Calculation of Area per Molecule. Since the electron density profiles give an accurate estimate for the relative thickness change of the hydrocarbon region, $\Delta H/H_G$, in going through the phase transition, and since dilatometry provides the relative volume change, $\Delta V/V_G$, the relative area change, $\Delta A/A_G$, can be calculated from

$$\Delta V/V_G = \Delta A/A_G + \Delta H/H_G$$

Therefore:

$$\Delta A = A_G(\Delta V/V_G - \Delta H/H_G) = A_G[\Delta V/V_G - (H_{LC} - H_G)/H_G]$$

Since the hydrocarbon chains in the gel state of saturated PEs are oriented perpendicular to the bilayer and packed in a hexagonal array (McIntosh, 1980), A_G can be calculated from the wide-angle spacing d_s by $A_G = 2(2/\sqrt{3})(d_s)^2 = 41.0 \pm 0.2$ Å^2. Using $\Delta V/V_G = 0.043$ (Wilkinson & Nagle, 1981) and our values of H_G = 27.2 Å and H_{LC} = 23.0 Å, we calculate that the partial molal area increases 8.1 Å^2 upon melting. Therefore, the area in the liquid-crystalline phase is A_{LC} = 49.1 Å^2. This value of A_{LC} of 49.1 Å^2 can be compared to the values of 55 Å^2 for didodecyl-PE (DDPE; an ether analogue of DLPE) (Seddon et al., 1983b), 75 Å^2 for egg PE (Lis et al., 1982) obtained by X-ray measurements that make use of ϕ_L and d (see above), and 48 Å^2 obtained from DLPE monolayers at a surface pressure of about 35 dyn/cm (Phillips & Chapman, 1968). Since the temperature dependence of the relative area change in the liquid-crystalline phase is about (2×10^{-3})/°C (Kwok & Evans, 1981), no significant corrections need to be made over the temperature ranges presented in this paper.

Calculation of Number of Water Molecules per Lipid. The total number of waters associated with the lipid above and below the main transition temperature T_m can be obtained by subtracting the volume of the anhydrous molecule from the unit cell dimensions of the hydrated lipid. The volume of fully hydrated DLPE and its associated water molecules in the Lβ phase is equal to $A_G(d/2)$ = (41.0 Å^2) × [(50.5 Å)/2] = 1035 Å^3. The volume of an anhydrous DLPE molecule in the Lβ phase can be calculated from the volume of crystalline DLPE, which is obtainable from two sources. Using the data of Hitchcock et al. (1974) for DLPE/acetic acid crystals, we obtain 797 Å^3, by subtracting the volume of the acetic acid molecule from the volume of the unit cell. The data of Seddon et al. (1983b) for the monohydrate form of DLPE give a volume of 808 Å^3 for the anhydrous DLPE molecule when the volume of a single water molecule is subtracted. In our calculations, we use a volume of 800 Å^3 for anhydrous DLPE. The volume of the anhydrous DLPE molecule in the Lβ phase is obtained by correcting this volume for the difference in volume between chains in the crystalline state and the Lβ phase. The difference in volume per CH_2 group in these two phases is about 1.5 Å^3 (Small, 1984). Therefore, one must add 33 Å^3 (2 × 11 × 1.5 Å^3) to the crystal volume of 800 Å^3 to obtain the volume of the anhydrous DLPE molecule in the Lβ phase. We did not include the carbonyl carbon in the hydrocarbon region as previously mentioned. Therefore, the total volume of water associated with gel-state DLPE is (1035 Å^3) − (833 Å^3) = 202 Å^3. Assuming the volume of a water molecule is 30 Å^3, we obtain a value of 6.7 water molecules per lipid in the Lβ phase. In the fluid (Lα) phase the volume of a fully hydrated DLPE molecule is (49.1 Å^2) × [(46.1 Å)/2] = 1132 Å^3. Assuming the anhydrous DLPE volume increases upon melting by the same percentage as the volume of fully hydrated DLPE (2.8%), we obtain a volume of 856 Å^3 for the anhydrous melted lipid and 276 Å^3 for the associated water. Thus, in the liquid-crystalline phase we calculate that there are 9.2 waters per lipid. These values for the number of waters per lipid can be compared to (1) the calorimetric data of Cevc and Marsh (1985), which showed that for DLPE T_m becomes independent of water content at about 7 water molecules per lipid, (2) the NMR results of Seddon et al. (1983a) for DDPE, which has 6 waters per lipid, and (3) the X-ray data of Seddon et al. (1984), which showed that for diarachinoylphosphatidylethalomanine d becomes independent of water content at about 9 water molecules per lipid for both gel and liquid-crystalline phases.

Distribution of Water Molecules. To determine the distribution of these water molecules relative to the polar head group, we use the calculated A and the bilayer thickness, L, as estimated from the electron density profiles (Figure 2).

Consider the volume of the water space between adjacent bilayers. The approximate width of this space, $F = d - L$, is 5.3 Å for the gel phase and 5.1 Å for the liquid-crystalline phase. The volume of the fluid space, $V_F = AF$, is 217 Å^3 for the gel phase and 250 Å^3 for the liquid-crystalline phase. Again, assuming the volume of a water molecule is 30 Å^3, we find that there are 7.2 and 8.3 water molecules in the fluid space between adjacent gel and liquid-crystalline DLPE bilayers, respectively. Dividing these numbers by 2 and subtracting the values of n reported above, we obtain 3.1 and 5.1 for the number of water molecules per lipid located within the gel- and liquid-crystalline-phase bilayers, respectively. Therefore, for the Lβ phase there are 6.7 waters per lipid molecule with about half of these in the fluid space between bilayers and half intercalated in the lipid head group. In the Lα phase there are about 9 waters per lipid molecule with about 4 in the fluid space and 5 intercalated into the bilayer head group region.

Discussion

Our calculation of A_{LC} depends on three measurements: the gel-state wide-angle spacing used for calculating A_G, the published dilatometry values of $\Delta V/V_G$, and $\Delta H/H_G$ calculated from electron density profiles. Measurements of the gel-state wide-angle spacing, $\Delta V/V_G$, and head-group separations in electron density profiles are all made with relatively small experimental uncertainty (see Figure 2 and Results). Potentially, the most imprecise part of the calculation is obtaining a value for bilayer hydrocarbon thickness from the electron density profile. That is, there is a certain arbitrariness in defining the location of the boundary between the hydrocarbon and head group regions of the bilayer. However, on the basis of the crystallographic data of Hitchcock et al. (1974) as well as space-filling models for DLPE, a distance of 5 Å between head group peak and the edge of the hydrocarbon region appears to be a reasonable estimate. An error of ± 1 Å in this estimate, combined with the experimental uncertainty in $\Delta V/V_G$ (Chang & Epand, 1983) and in A_G, results in a difference of only ±1.2 Å^2 in our calculated value of A_{LC}.

The electron density profiles of DLPE (Figure 2) clearly show that the fluid space between adjacent bilayers is quite small, in agreement with previously published X-ray and neutron diffraction studies of BPE (Simon et al., 1982; McIntosh & Simon, 1986; Simon & McIntosh, 1985). To determine an approximate bilayer thickness, L, we have assumed that the conformation of the amine group is the same in the Lβ and Lα phases as it is in the DLPE crystal. Using this assumption, we obtain 5.3 and 5.1 Å for the fluid layer thicknesses for gel and liquid-crystalline bilayers, respectively. If in fact the amine group extends further out from the bilayer surface in the Lβ or Lα phases than in the crystal, then these fluid spaces would be even smaller. Thus, these estimates of fluid layer thicknesses are upper estimates. Even so, our values for the width of the fluid space in fully hydrated gel and liquid-crystalline state DLPE are 7–10 Å smaller than the fluid spaces in gel- and liquid-crystalline-state phosphatidylcholines, respectively (McIntosh & Simon, 1986). Marra and Israelachvili (1985) and Lis et al. (1982) have also reported that the fluid layer thickness is smaller for PEs than for PCs. However, our value of about 5 Å for the fluid layer thickness for both gel and liquid-crystalline DLPE is considerably smaller than the fluid layer thickness of 20.5 Å calculated for egg PE (Lis et al., 1982). In fact, the fluid spaces for DLPE are small enough that at most two water molecules can span the space between adjacent bilayers (the "diameter" of a water molecule is about 2.8 Å). Or, put another way, our calculations on the distribution of water show that a total of only about 4 waters per lipid must be removed from between liquid-crystalline DLPE bilayers before adjacent bilayers come into physical contact.

Since fluid PE bilayers are only separated by about 5 Å and PCs by about 15 Å, it is evident that bilayers containing large amounts of PE will be closer together than those containing large amounts of PC. Relevant to this point, Düzgünes et al. (1985) have recently shown that the steps involved in liposome fusion are aggregation, followed by destabilization and and fusion. Of the three steps only the aggregation step is reversible. Düzgünes et al. (1981) previously showed that when Ca^{2+} is added to vesicles of PC/PS, only aggregation is observed, whereas when Ca^{2+} is added to PE/PS vesicles, fusion occurs. Düzgünes et al. (1985) also showed that vesicles composed of oleic acid (OA) and PE fuse when the fixed charges on OA are titrated but do not fuse when PC is substituted for PE under identical conditions. These latter experiments show that Ca^{2+} bridging between the bilayers is not a necessary condition for fusion for charged vesicles. It appears that Ca^{2+}, like H^+, acts to neutralize the fixed charges, permitting the vesicles to attain their equilibrium spacing, which for PE-containing bilayers means they are practically in contact. Destabilization could then occur as a result of the different surface energies (due to different surface charge densities) of the inner and outer monolayers of the bilayer (Hall & Simon, 1976) or from a phase separation of PE from the other lipid components of the liposome, which could lead to the formation of hexagonal II phases (Rand et al., 1985) or other instabilities (Bentz et al., 1985) in the contact region. With PC in the bilayers, the neutralized vesicles are too far from each other for fusion to occur.

The question naturally arises to why the equilibrium fluid spacing between PE bilayers is significantly smaller than for PC bilayers. The interaction between neutral bilayers at small fluid spacing distances is thought to arise from a balance between a repulsive hydration pressure, P_r, which decays exponentially from the surface, and an attractive pressure, P_A, which arises from the van der Waals interactions of the bilayers acting through the aqueous phase (LeNeveu et al., 1976). Lis et al. (1982) considered the smaller equilibrium spacing between PE compared to PC vesicles to arise from a larger P_A. However, as these investigators found much larger fluid spaces for PE than Marra and Israelachvili (1985) and we do, they did not consider the possibility that, at these very small fluid spacings, other attractive interactions between PE bilayers may occur. Of the possible interactions, two of the most likely (which may not be mutually exclusive) are (1) electrostatic interactions and (2) hydrogen bond formation between the phosphate (PO_4^-) moiety in one bilayer and the amine (NH_3^+) moiety in the apposing bilayer. In the electrostatic interaction, the interbilayer water would behave as a structureless liquid characterized by a dielectric constant, whereas in the hydrogen bond formation the water molecules could be directly involved in the interaction, possibly forming water bridges between opposing bilayers. As was first noted by Marcelja and Radic (1976), the energy gained in forming hydrogen bonds between bilayers is large enough to overcome the repulsive hydration force.

The idea of electrostatic or hydrogen bonding between adjacent bilayers is supported by the recent observation that monomethyl-PE has about the same fluid space as PC (Rand et al., 1985). The addition of a methyl to the PE head group would tend to decrease electrostatic or hydrogen bond formation between molecules. It might be argued that these

interactions are formed preferentially in the plane of the bilayer, between PE molecules in the same monolayer, rather than between adjacent bilayers across the fluid space. However, two experiments provide evidence that these interactions are not solely in the plane of the bilayer. First, the addition of cholesterol to BPE bilayers, at a 1:1 molar ratio, does not change the fluid space between bilayers (Simon et al., 1982), and second, the fluid space between DLPE bilayers does not significantly increase upon going through the thermal phase transition (Figure 2). Both the addition of cholesterol and the melting of the hydrocarbon chains increase the distance between head groups in the plane of the bilayer. This increase in distance would tend to reduce the magnitude of specific hydrogen bonds in the plane of the bilayer (Pascher et al., 1981). Thus, when the magnitude of electrostatic or specific hydrogen bonds between PE molecules is reduced (by the addition of a methyl to the PE head group), the fluid space increases (Rand et al., 1985), whereas when the magnitude of interactions in the plane of the bilayer is reduced (by the addition of cholesterol or hydrocarbon chain melting), the fluid space remains constant. Therefore, electrostatic interactions and/or bond formation between adjacent PE bilayers seems to be a likely possibility.

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