Adsorbed to a Rigid Substrate, Dimyristoylphosphatidylcholine Multibilayers Attain Full Hydration in All Mesophases

John Katsaras
National Research Council, Steacie Institute for Molecular Sciences, Neutron Program for Materials Research, Chalk River Laboratories, Chalk River, Ontario K0J 1J0, Canada

ABSTRACT Whether hydrated from vapor or immersed in liquid water, aligned multibilayers of dimyristoylphosphatidylcholine adsorbed to a single mica "substrate" are shown by neutron diffraction to hydrate in all mesophases (e.g., $L_{\beta'}$, $P_{\beta'}$, and L_{α}) to the same extent as their liposomal counterparts suspended in liquid water. These data clearly demonstrate that the commonly accepted vapor pressure paradox does not exist.

INTRODUCTION

Even in lipid/water systems that have been extensively studied over the last three decades, the use of aligned lipid/water systems has allowed us in recent years to gain new insights into the structure of a variety of lipid phases (e.g., Smith et al., 1988; Katsaras et al., 1995; Raghunathan and Katsaras, 1995). Possibly the most common method of aligning lipid multibilayers is to deposit them from a concentrated lipid/solvent solution onto a solid support of either glass (Torbet and Wilkins, 1976; Raghunathan and Katsaras, 1995) or silicon (Katsaras and Jeffrey, 1997) and hydrate from a water-saturated atmosphere. The end result, compared to their liposomal counterparts in contact with liquid water, is multibilayers with a reduced level of hydration and, concomitantly, repeat spacings (*d*-spacings) (e.g., Torbet and Wilkins, 1976). Why this difference in *d*-spacings? Why is water at the same chemical potential behaving so differently when attempting to hydrate the same material? This is the widely recognized vapor pressure paradox (Rand and Parsegian, 1989).

Reports of aligned samples hydrated from vapor (100% relative humidity) exhibiting *d*-spacings much less than their equivalent liposomal dispersions in water have been extensive (e.g., Torbet and Wilkins, 1976; Franks and Lieb, 1979; Smith et al., 1987; Katsaras and Stinson, 1990; Katsaras et al., 1992; Katsaras, 1995). As such, the vapor pressure paradox appeared to be well established. Recent developments have inspired us to take a closer look at the vapor pressure paradox. One has been the theoretical treatment of the paradox by Podgornik and Parsegian (1997). In summary the theory states the following: Undulating bilayers experience an effective entropic force (Helfrich, 1978), causing the bilayers to repel each other, resulting in the uptake of water and an increase in *d*-spacing. However, when bounded by surfaces under tension (e.g., vapor/multilayer surface or adsorption to a solid substrate), the mechanical undulations in bilayers are suppressed, producing stabilizing attractive forces that are communicated from bilayer to bilayer over macroscopic distances. The outcome of these attractive forces is aligned bilayers with a reduced level of hydration (Podgornik and Parsegian, 1997). In a later paper expanded to include charged lipids, Podgornik and Parsegian state that reduced levels of hydration result from the combined perturbations of vapor/multibilayer surface tension and substrate adsorption (Parsegian and Podgornik, 1997).

Another development has been the production of fully hydrated, highly aligned lipid multibilayers exhibiting the same physical characteristics (e.g., *d*-spacing and transition temperatures), in all mesophases, as samples dispersed in water (Katsaras, 1997). As indicated by Nagle (personal communication), this result was seemingly in contradiction to the theory by Podgornik and Parsegian (1997). The result implied the possibility that the vapor pressure paradox did not exist. However, considering the fact that when immersed in water the aligned lipid multilayer stack can dissociate from the silicon substrate, it was suggested (Katsaras, 1997) that the silicon wafers may have acted only in confining the multibilayers and did not restrict them from undergoing mechanical undulations that result in the uptake of water. Finally, a more recent development by Fuller and Rand (personal communication) showed that in vapor, unoriented charged dioleoylphosphatidylserine bilayers separate indefinitely, and unoriented neutral layers separate maximally. This occurred only if the vapor volume is kept small and a water-saturated filter paper is used to provide a large evaporative surface. The obvious question then is, what are the hydration properties of aligned multibilayers adsorbed to a substrate, especially in the L_{α} phase?

To lower the humidity from 100% to ~99.9%, the "oven" containing the sample has only to contain a temperature gradient of 0.01°C. This change in humidity results in ~5-Å decrease in the *d*-spacing of egg phosphatidylcholine (Rand and Parsegian, 1989). However, changes in humidity can be avoided by immersing, in water, aligned lipid multilayers

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Address reprint requests to Dr. John Katsaras, National Research Council, Steacie Institute for Molecular Sciences, Neutron Program for Materials Research, Chalk River Laboratories, Bldg. 459, Stn. 18, Chalk River, Ontario, K0J 1J0, Canada. Tel.: 613-584-3311, ext. 3984; Fax: 613-584-4040; E-mail: john.katsaras@nrc.ca.

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adsorbed to a solid support. Because the chemical potentials of liquid water and water vapor in equilibrium with its mother liquid are the same, we would be gaining insight into the following questions: Are the universally observed reduced *d*-spacings in aligned multibilayers hydrated from vapor the result of the solid support or, possibly, as inconceivable as it may be, the result of the inability of ovens used in diffraction to achieve humidities better than ~99.0%? For the present studies the substrate that was found to be suitable was mica.

Mica has been used extensively to align a variety of biologically relevant macromolecules (e.g., Henderson, 1975; Fang et al., 1997) and various cationic surfactants and lipids (e.g., Fang and Yang, 1997; Sharma et al., 1997), especially when monolayers are imaged by atomic force microscopy (Fang et al., 1997; Fang and Yang, 1997; Sharma et al., 1996). In the present study we discovered that even when immersed in water, aligned stacks comprising hundreds or even a few thousand dimyristoylphosphatidylcholine (DMPC) bilayers could be adsorbed to a single mica surface and remain stable over a period of days. This is in contrast to multibilayers deposited on glass (Hartung et al., 1994) and silicon substrates.

In this paper we report experimental data in which a stack of aligned DMPC multibilayers adsorbed to a mica substrate display the same *d*-spacings as liposomal preparations of DMPC in contact with an excess of liquid water (e.g., Janiak et al., 1976; Lis et al., 1982; Stümpel et al., 1983; Zhang et al., 1995), regardless of whether the multibilayers were hydrated from vapor or from liquid water.

MATERIALS AND METHODS

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids (Birmingham, AL) and used as supplied. Deuterated water (D_2O) of purity 99.95% and with a pH 6.4 was kindly supplied by Atomic Energy of Canada (Chalk River, ON, Canada).

Aligned multibilayers of DMPC were prepared as follows: 2 ml of a concentrated lipid/methanol solution (10 mg/ml) was pipetted onto the 5 cm × 6 cm surface of an oxidized mica crystal. After evaporation of the methanol, the sample was placed under vacuum for a period of 12 h to remove any traces of solvent. Subsequent to this, the sample was transferred to an atmosphere saturated with D_2O vapor and annealed for ~12 h at a temperature of ~35°C. At this temperature and humidity the DMPC multibilayers are in the L_α phase. Twenty milligrams of sample spread over an area of 30 cm^2 should result in ~1800 DMPC bilayers. The mica/lipid sample was then placed in a sample holder (Fig. 1) comparable to the one described by Katsaras (1997). The main difference between the two sample holders was that the multibilayer stack in the present sample holder was in contact with only one rigid surface (mica) and bulk water (Fig. 1). There was nothing preventing the multibilayers from detaching themselves from the mica substrate except the inherent "adhesive" properties of the mica. A sample made up of ~600 bilayers was also prepared.

The sample holder or "oven" used to hydrate aligned samples from water vapor is presented in Fig. 2. To minimize temperature gradients the sample holder was surrounded by an aluminum vacuum chamber (not shown). Aligned samples were hydrated from water vapor emanating from a saturated sponge of dimensions equivalent to those of the mica substrate. The sponge and the mica substrate were placed parallel to each other, separated by a gap of ~2 mm. The degree to which the sponge was saturated with water determined whether the sample attained full hydration

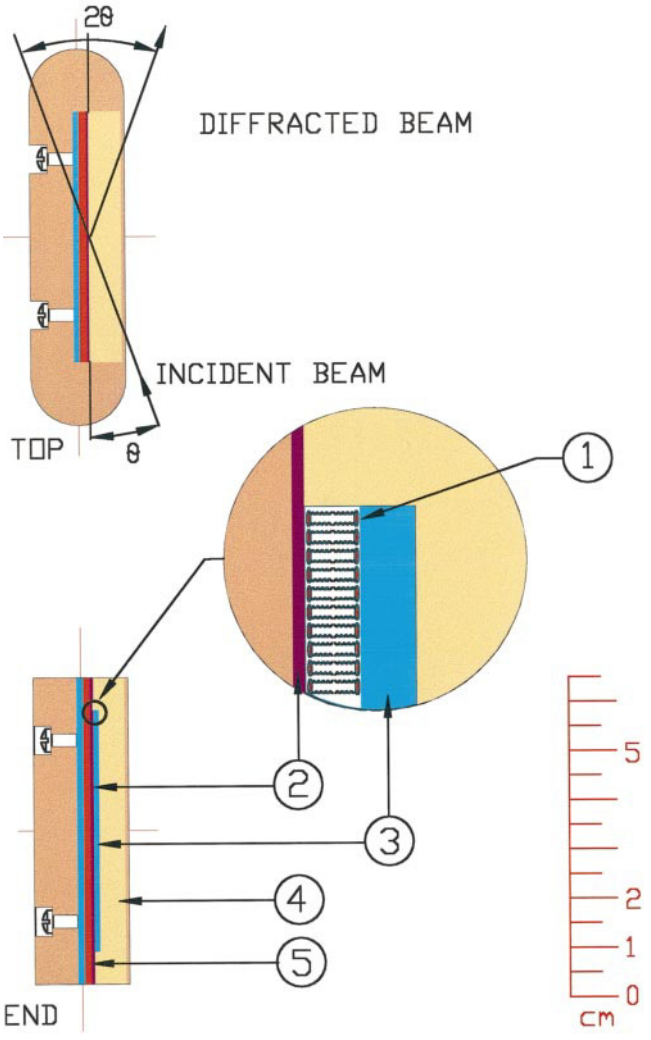

FIGURE 1 Aluminum sample holder and diffraction geometry used for the experiments of DMPC bilayers aligned on a mica substrate and hydrated from liquid water. (*1*) Lipid multibilayer stack made up of either 1800 or 600 DMPC bilayers. (*2*) Mica substrate on which the lipid bilayers are aligned. (*3*) Water layer in direct contact with the aligned lipid sample. (*4*) U-shaped aluminum block used to prevent the lipid from coming into contact with anything but water. (*5*) Aluminum pressure plate used to anchor the mica substrate and the u-shaped aluminum block. Except for the mica substrate, aluminum was used throughout for the construction of the sample holder. D_2O was used in place of H_2O (please refer to Katsaras, 1997).

and the time interval between full hydration and the first signs of condensate forming on the sample. For this sample preparation, 5 mg of DMPC was spread over a mica surface of 10 cm^2 in the manner described above. The temperature for both sample holders (Figs. 1 and 2) was controlled with a water bath, the water of which was circulated through the base of the sample holder.

The experiments were carried out at the NRU reactor located at AECL's Chalk River Laboratories (Ontario, Canada), using the N5 triple-axis spectrometer. At the monochromator position the neutron flux is ~5.4 ± 0.3 × 10^9 neutrons cm^{-2} s^{-1}. Neutrons (2.37 Å) were selected with the [002] reflection of a pyrolytic-graphite monochromator with a mosaic of 0.4°, and higher order neutrons were suppressed via the use of a graphite filter. With the collimation employed, the instrumental resolution of the spectrometer was determined to be 0.012 $Å^{-1}$ (full width half-maximum,

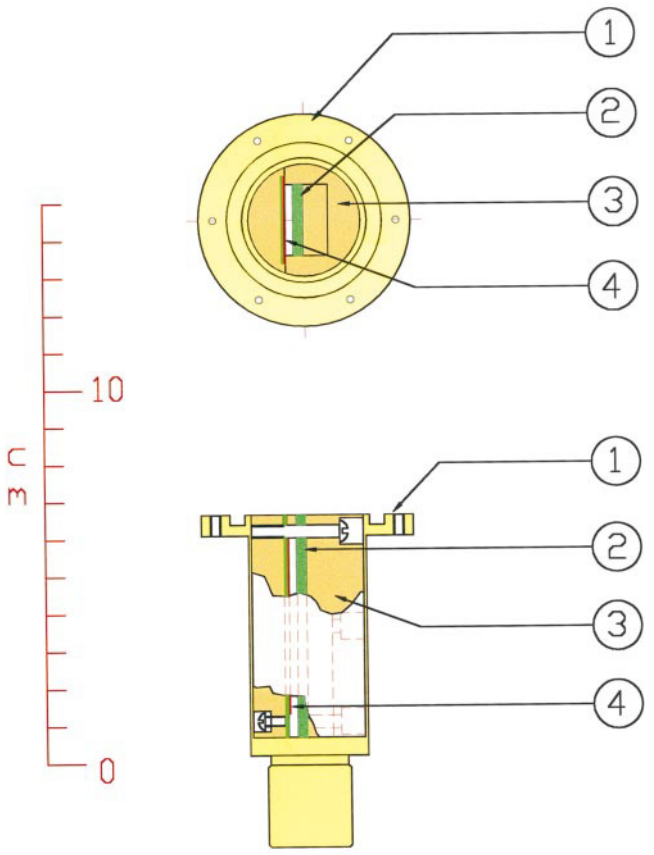

FIGURE 2 Sample holder for hydrating an aligned multibilayer stack from water vapor in equilibrium with liquid water. (*1*) Sealed aluminum body. (*2*) Water-saturated sponge. (*3*) Removable inner assembly. (*4*) Mica substrate to which the sample is adsorbed.

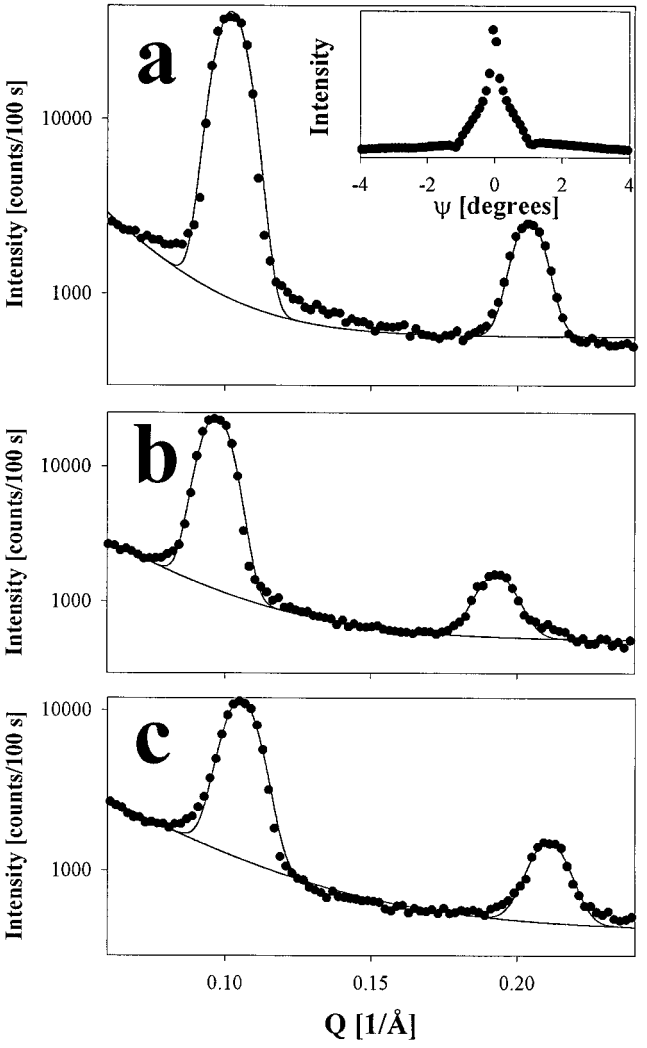

FIGURE 3 θ-2θ scans of ~1800 aligned DMPC bilayers adsorbed to a mica substrate and immersed in liquid water. (*a*) 61.6-Å L_α bilayers at a temperature of 32°C. (*b*) 65.5-Å $P_{\beta'}$ multibilayers at 20°C. (*c*) $L_{\beta'}$ multibilayers at 10°C exhibiting a *d*-spacing of 59.7 Å. Bragg reflections were fitted using Gaussians for the sole purpose of determining peak position. The estimated error in *d*-spacing is ±0.3 Å. The inset in *a* contains the rocking curve of L_α DMPC multibilayers (~0.5° (FWHM) mosaic). The specimen was rotated through a series of angles ψ (ψ denotes the rotation of the sample with respect to the incident beam), and the detector was fixed at a Q value corresponding to the first-order Bragg maximum. Q is equal to $4\pi \sin \theta_B/\lambda$.

FWHM). The instrument was configured to give a slightly higher resolution (FWHM = 0.008 $Å^{-1}$) for the samples hydrated from water vapor. Radial (θ-2θ) scans and rocking curves (ψ scans) were performed in the manner described by Katsaras (1997).

RESULTS AND DISCUSSION

Fig. 3 shows three different mesophases of aligned DMPC multibilayers adsorbed to a single mica substrate. With the use of the first-order Bragg maximum, the rocking curve of L_α bilayers is presented in the inset of Fig. 3 *a*. The two-component lineshape composed of a narrow peak and diffuse scattering is typical for a rocking scan of lipid multibilayers aligned on a substrate (e.g., Büldt et al., 1979; Franks and Lieb, 1979). The coexistence of a resolution-limited Bragg peak and diffuse scattering is well known in disordered crystalline solids, where the former implies that the lipid multibilayers are on average flat over a distance of $2\pi/\Delta Q$ (long-range order), whereas the latter arises from distortions and lattice defects (short-range correlations) as a result of bilayers having an orientation that deviates from the average structure (Robinson, 1986; Sinha et al., 1988; Gibaud et al., 1993). From the sharp feature we can measure the alignment of the present samples to be better than 0.5° (FWHM) and in good agreement with recent experimental data from fully hydrated aligned bilayers (Katsaras, 1997).

DMPC/water is a well-characterized system and, under excess water conditions, is marked by three stable mesophases. Repeat spacings of L_α (30°C), $P_{\beta'}$ (20°C), and $L_{\beta'}$ (10°C) multibilayers have been measured by x-ray diffraction to be ~62 Å, ~65 Å, and ~60 Å, respectively (Janiak et al., 1976; Lis et al., 1982; Stümpel et al., 1983; Zhang et al., 1995). In Fig. 3 we present diffraction patterns collected from L_α (32°C), $P_{\beta'}$ (20°C), and $L_{\beta'}$ (10°C) bilayers adsorbed to a single mica substrate exhibiting *d*-spacings of 61.6 Å (Fig. 3 *a*), 65.5 Å (Fig. 3 *b*), and 59.7 Å (Fig. 3 *c*),

respectively, in agreement with values from liposomal preparations (Janiak et al., 1976; Lis et al., 1982; Stümpel et al., 1983; Zhang et al., 1995). Furthermore, *d*-spacings for samples containing ~600 bilayers were not appreciably different (L_α [61.7 Å], $P_{\beta'}$ [66.6 Å], and $L_{\beta'}$ [60.2 Å]) compared to their thicker counterparts of ~1800 bilayers. It therefore seems that the substrate is not exerting any appreciable influence upon the *d*-spacing of the bilayers, as the data presented here are in agreement with previously published liposomal results. Before attempting to claim that there is no vapor pressure paradox, the possibility that liquid water and water vapor hydrate aligned bilayers differentially must be ruled out.

Most diffraction measurements of aligned lipid bilayers have been carried out using x-rays (e.g., Torbet and Wilkins, 1976; Franks and Lieb, 1979; Smith et al., 1987; Katsaras and Stinson, 1990; Katsaras et al., 1992; Katsaras, 1995). Because x-rays are easily absorbed, "x-ray ovens" contain appropriate "windows" (e.g., kapton or mylar) whose thermal properties are in all likelihood much different from those of the materials used to construct the rest of the oven (e.g., aluminum, brass, etc.). This mixture of oven materials allows for the possibility of substantial thermal gradients, which result in the condensation of vapor. Depending on the rate at which water condenses, the relative humidity will range anywhere from slightly less than 100% to much less than 100%. To further complicate matters, x-ray and neutron ovens offer substantial volumes to accommodate the required instrumentation (e.g., Smith et al., 1987; Katsaras et al., 1992; Katsaras and Jeffrey, 1997). In comparison, the sample holder shown in Fig. 2 has a large thermal mass; a very small (~2 cm^3) "vapor volume," translating into short equilibration times; and no windows, as aluminum is transparent to neutrons.

It has previously been shown that, aligned on a glass surface, $L_{\beta'}$ dipalmitoylphosphatidylcholine multibilayers attain full hydration when exposed to a supersaturated water vapor atmosphere (Katsaras et al., 1992; Tristram-Nagle et al., 1993). Despite that, applying this same method resulted in L_α bilayers with *d*-spacings nearly 10 Å less than those of liposomal preparations (Tristram-Nagle et al., 1998). This suggests that the hydration properties of so-called rigid bilayers (e.g., $L_{\beta'}$ and $P_{\beta'}$ phases) are not dependent to any great extent upon bilayer fluctuations, as both their fluctuations and motional properties (e.g., *trans-gauche* isomerizations, headgroup motions, lateral diffusion, etc.), compared to L_α bilayers, are inherently damped (Yeagle, 1992). As such, under appropriate conditions aligned $L_{\beta'}$ multibilayers exhibit the same repeat spacing as liposomal preparations dispersed in an excess of liquid water (Katsaras et al., 1992; Tristram-Nagle et al., 1993; Katsaras, 1997). On the other hand, "fluid-like" L_α bilayers depend on fluctuations to achieve full hydration (Helfrich, 1978). The presence of any surface tension such as that produced at a vapor/multibilayer or substrate/multibilayer interface suppresses the fluctuations in that bilayer stack (Helfrich and Servuss, 1984). Further proof along this line of evidence was provided using L_α multibilayers at relative humidities approaching 100%, whereby bilayers aligned on "rough" substrates exhibited *d*-spacings greater than those bilayers aligned on "smooth" surfaces (Tristram-Nagle et al., 1998). Supposedly, the rough surface destabilizes the bilayer stack, permitting the bilayers to undulate and take up increased amounts of water. The experimental data to date have been overwhelmingly consistent in support of the vapor pressure paradox.

The diffraction pattern of 62.4 Å L_α DMPC bilayers hydrated from vapor and having a mosaic spread of ~1.0° (FWHM) (Fig. 4, *inset*) is shown in Fig. 4. This is the first time that aligned L_α bilayers hydrated from vapor have achieved repeat spacings equivalent to their liposomal counterparts. Previous to this experiment, the *d*-spacing values of L_α DMPC bilayers hydrated from vapor (RH ~100%) have been 54 Å (Smith et al., 1987) and 53.7 Å (Katsaras, unpublished data), consistent with the data reported by Tristram-Nagle et al. (1998), in which their L_α bilayers hydrated from a supersaturated vapor displayed repeat spacings almost 10 Å less than bilayers dispersed in water.

Using this sample holder (Fig. 2), the DMPC bilayers went from a *d*-spacing of ~57.8 Å to ~62.4 Å in ~2 h. This

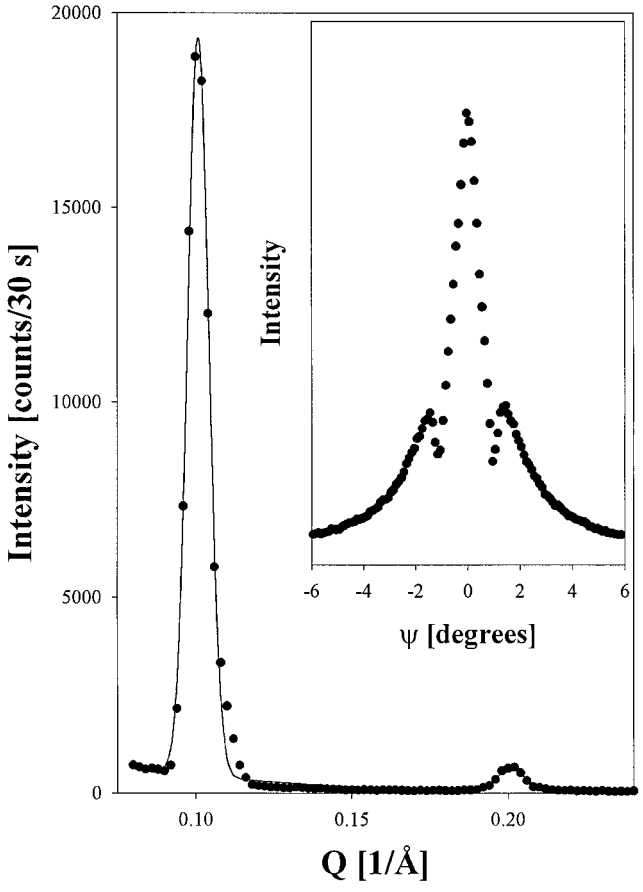

FIGURE 4 Resolution-limited Bragg maxima obtained from an aligned L_α DMPC multibilayer stack attached to a mica substrate and hydrated from water vapor. At 30°C the repeat spacing of these aligned bilayers (~1.0° mosaic (FWHM), *inset*) was 62.4 Å.

period of equilibration is highly variable and depends on the amount of water used to wet the sponge. The relative humidity when the sample attained full hydration was not measured and could not be measured with any degree of accuracy. Furthermore, we do not pretend that condensation of water on the sample cannot occur; however, this can be monitored through the intensity of the Bragg reflections. As the sample was hydrating, the intensity of the first-order Bragg maximum increased until the repeat spacing reached its limiting value, at which point the sample holder was disassembled and the aligned multibilayers were visually inspected for signs of water condensation. No condensate was observed. Once the full repeat spacing was achieved, decreased Bragg reflection intensity was indicative, as verified visually, of water condensing on the sample. Condensation occurred after 12 h at humidities approaching 100%. It seems that for the described conditions we have, with some degree of confidence, an indicator of when condensate forms.

Preliminary data from aligned palmitoyl-oleoylphosphatidylcholine (POPC) bilayers shows that fully hydrated POPC bilayers (65 Å *d*-spacing) at 25°C (about room temperature) are even easier to prepare, as the thermal gradients are much reduced. In these samples no condensation was observed, even after a few days at ~100% relative humidity. However, if there were a layer of water (tens of angstroms in thickness) adhering to the surface of the multibilayer stack, we would not have been able to know of its presence. Of greater significance has been the observation, after a few days of equilibration at 23°C, of 59-Å POPC bilayers hydrated using a damp but not water saturated sponge. The spacing of these aligned bilayers, although less than that of fully hydrated samples (65 Å *d*-spacing), is greater than previous observations of aligned POPC multibilayers supposedly at 100% relative humidity (51.2 Å *d*-spacing) and at similar temperatures (Katsaras et al., 1993). This observation further makes the point that reduced levels of humidity, as a result of temperature gradients present in ovens, were responsible for aligned lipid multibilayers exhibiting smaller *d*-spacings.

It therefore seems that because of the inherent deficiencies of previous ovens, humidities much better than 99% were never achieved. It may also be that the only accurate gauge of humidity is the bilayer itself, because no electronic devices are capable of measuring humidity to an accuracy of better than ±2%.

SUMMARY AND CONCLUDING REMARKS

Using neutron diffraction we have demonstrated that whether immersed in water or hydrated from water vapor, DMPC multibilayers adsorbed to a mica substrate achieve repeat spacings similar to those of DMPC liposomal preparations. We can thus state that there is no vapor pressure paradox. Data similar to those presented here have also been obtained by Fuller and Rand (personal communication) by using bilayers aligned on a glass substrate.

The vapor pressure paradox was the result of an overwhelming number of studies in which aligned multibilayers were supposedly investigated under conditions of 100% humidity. What made the paradox a necessity was the consistency of the data. In a paper written almost 20 years ago, Franks and Lieb (1979) curiously observed that their samples in excess water exhibited lamellar spacings that were always substantially larger than those observed with aligned lipids hydrated from water vapor at 100% relative humidity. According to Franks and Lieb (1979), "the reason for this is unclear." We have shown that this is no longer the case.

Finally, we have accepted that L_α bilayers fully hydrate as a result of an effective entropic force causing the bilayers to repel each other. We now have in place the systems that will enable us to measure differences, if any, in the mechanical undulations between samples aligned on a rigid substrate and liposomes dispersed in an excess of water.

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