Absence of a vestigial vapor pressure paradox

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The enigmatic but much accepted vapor pressure paradox for oriented lipid bilayer samples was recently justified theoretically. Subsequently, recent experiments have shown that there is no vapor pressure paradox. The first result of this paper is to consider another degree of freedom that reverses the previous theoretical conclusion, so that theory and experiment are now in agreement that there is no vapor pressure paradox. However, this analysis also suggests the possibility of a vestigial vapor pressure paradox that would rationalize why the vapor pressure paradox was historically so persistent and that would have led to an improved protocol for obtaining bilayer structure. This vestigial vapor pressure paradox would involve a phase transition as a function of applied osmotic pressure. We test this possibility experimentally using combined neutron and x-ray scattering data. The conclusion from these experiments is that there is not even a vestigial vapor pressure paradox. However, this negative result validates an improved method for calibrating osmotic pressure in x-ray studies of oriented samples. [S1063-651X(99)02506-4]

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I. INTRODUCTION

When placed in water, lipid molecules self-organize into bilayers that form the structural basis of biomembranes. In this simplest preparation of lipids and excess liquid water, the most common lipids, the electrically neutral dipolar lecithins, form multilamellar vesicles (MLVs). Locally, MLVs are smectic liquid crystals, with stacks of bilayers aligned perpendicularly to a director axis, with a lamellar repeat spacing d_m. Globally, MLVs are isotropic samples; in diffraction studies they are called powder samples, although they are thoroughly wet and the term ''fully hydrated'' is frequently applied to them.

For the purpose of elucidating structure, there are advantages to studying globally oriented samples made up of multibilayer stacks consisting of thousands of bilayers. One of the simplest preparations of oriented samples involves drying lipid from an organic solvent on a solid substrate and then hydrating the dry oriented stack by exposing it to water vapor. However, the repeat spacing d_o of such oriented bilayers has frequently been found to be smaller than the repeat spacing d_m for fully hydrated MLVs, even though the relative humidity (RH) of the water vapor was nominally 100% or even supersaturated [1]. A somewhat different preparation of oriented samples is the free standing film, for which the d_o spacing has also been reported to be smaller than d_m for MLVs [2].

The established name, vapor pressure paradox (VPP), emphasizes that these results are paradoxical [1]. In equilibrium, the chemical potential of water vapor at 100% RH is the same as that of bulk water. Why then, should a physical property, such as the repeat spacing d, be different in the two types of hydrated samples?

The first shift in how we think about the vapor pressure paradox emerged from studies of lipid bilayers in the $L_{\beta'}$ (gel) phase [3,4]. By using supersaturated water vapor and aligned stacks on a glass substrate, spacings of d_o as large as spacings d_m were achieved. However, the same experimental approach did not, at that time, achieve $d_o = d_m$ for lipid bilayers in the biologically relevant L_α phase bilayers aligned on either glass or silicon substrates; instead, such oriented stacks of bilayers simply floated off glass or silicon substrates at high humidity [4]. An important difference between gel $L_{\beta'}$ and fluid L_α bilayers is their intrinsic flexibility. The hydrocarbon tails of the lipid molecules in the gel phase are conformationally ordered and packed into more rigid structures than in the conformationally disordered L_α phase. Therefore, bilayers in the L_α phase have increased bending flexibility, resulting in substantial fluctuations; such fluctuations are a key ingredient in the recently proposed theory to explain the VPP [5,6].

The basic idea is that fluctuations (bending as well as relative displacement of adjacent bilayers) produce an extra repulsive force which is entropic in nature [7]. This accounts for the larger water spacings between bilayers in the L_α phase compared to the same lipid in chain ordered gel or subgel phases [8]. The new theoretical ingredient introduced to explain the VPP was that interfaces, such as the surface adjacent to the vapor or to a solid substrate surface, are important because they suppress bilayer fluctuations [5,6]. Fluctuation suppression results in a smaller repulsive fluctuation pressure, so there is less competition with the attractive van der Waals force, thus the bilayers stay closer together. Therefore, less water is taken up for oriented samples, resulting in $d_o < d_m$. The remarkable aspect of this recent theory is that the fluctuations are not just suppressed close to the surface, but throughout the interior of samples comprised of thousands of bilayers [5,6]. This theory is quite deep and details will not be repeated in this paper. However, it may be noted that the theoretical framework is in accordance with the well-known smectic liquid crystal theory that such systems should have long-range correlation functions that fall off as power laws rather than as exponentials [9–11]. It is the presence of these critical correlations, associated with having

only quasi-long-range order (QLRO) rather than crystalline long-range order, that gives rise to the experimentally documented power law tails in x-ray scattering peaks [2,12,13]. Since power law decays have no well-defined healing or decay length, the theoretical result, that surface perturbations could affect d_o over distances of 10 μm [5,6], seems quite plausible. Additional observations that d_o was systematically larger on rougher substrates, expected to provide less pinning to the surface, was interpreted as supporting the theory [14].

The second shift in how we think about the vapor pressure paradox has come from recent measurements that obtain $d_o = d_m$ even in L_α phase bilayers [15,16]. Using a new oven for neutron diffraction in which an aligned stack of bilayers was contained between two parallel silicon surfaces, all immersed in water, $d_o = d_m$ was obtained in all phases [15]. However, it was also noted that there existed an open possibility that the stack of membranes might become separated from each of the solid silicon surfaces. If such ''lift-off'' occurred, then there would be little surface suppression of fluctuations and $d_o = d_m$ is consistent with the recently proposed theory [5,6,15]. This ambiguity was resolved in a more recent paper [16] where it was reported that a membrane stack adsorbed to a single mica substrate and immersed in water does not float off and still $d_o = d_m$. Most importantly, it was reported that when the oriented bilayer stack was hydrated from the vapor, again $d_o = d_m$ [16]. From these results, it is now concluded that there is no VPP after all.

The simplest, though inelegant, explanation for dismissing the old VPP results is that the humidity was never close enough to 100% H_R. This dismissal is made more plausible by noting that a vapor with a temperature only 0.1 °C lower than the lipid sample would result in an osmotic pressure (P) of 10 atm, which is sufficient to decrease d by about 10 Å [1]. Specifically, if the windows in an x-ray sample chamber are cooler than the sample, then the vapor will condense on the windows (often observed), thereby lowering the vapor pressure at the sample. Compared to past x-ray diffraction studies, the particular advantage of neutron experiments [15,16] is that the sample chamber is encased entirely in aluminum. Since aluminum is a weak absorber of neutrons, no special windows are required, in contrast to x-ray sample chambers, thus allowing for better temperature and humidity control. Nevertheless, this explanation ignores some important questions. Why should a mica substrate matter? Is the theory wrong? (The theory cannot distinguish between the various substrates.) Can we reconcile theory, the old experiments, and the recent experiments?

In this paper we first show in Sec. II that there is a way to reconcile the theory and the recent experiments. This involves consideration of a degree of freedom that was not included in the previous theory, but that is compatible with it. The modified theory does not predict the VPP. However, it hints at the possibility of what we here name the vestigial VPP, that would help explain why the old VPP was historically so persistent. This possibility involves a kind of phase transition, as a function of applied osmotic pressure, between a state where a bilayer stack does not sense the substrate and one where it does. If shown to be true, the vestigial VPP possibility could also lead to an improved methodology for obtaining bilayer structure as we discuss in Sec. IV. To investigate the possibility of a vestigial VPP, we have measured by neutron diffraction the d_o spacing of an oriented stack of bilayers on a solid substrate as a function of controlled osmotic pressure and we have compared these d_o values to x-ray values of d_m for the analogous powder MLV samples. The results are described in Sec. III and their implications are discussed in Sec. IV.

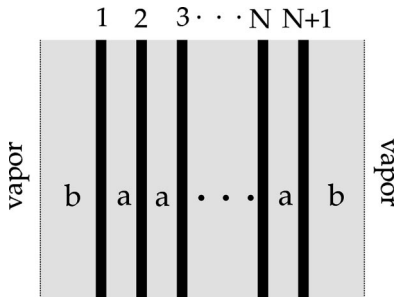

FIG. 1. Schematic of a stack of $N+1$ bilayers in a free standing film sample. Solid thick vertical lines show average position of the lipid bilayers. (For a realistic picture of fluctuations see [17].) The light gray portions show liquid water, with spacings a between adjacent bilayers and spacings b between the outer bilayers and the vapor phase.

II. THEORY

A. Free standing films

It is convenient to begin the discussion of the theory for the system of free standing films [2]. Although the actual film must terminate on a support where the film configuration becomes complex, in the middle of the film the geometry consists of a stack of multibilayers all oriented in the same direction with the two outermost layers bounded by a vapor/water interface shown schematically in Fig. 1. Such films have sharp and well-defined lamellar spacings d, so the bulk of the lamellae have the same average spacing. The corresponding water spacing between bilayers will be denoted a. Because the headgroups are hydrophilic, there should also be water layers between the outermost bilayers and the vapor; the thickness of these water layers will be denoted b. At this point, a possible caveat should be mentioned. Instead of each bare water/vapor interface, there might also be a monolayer of lipid with chains protruding into the vapor. Indeed, such a monolayer would be expected in true equilibrium because it would lower the vapor/liquid surface tension, but it might not be present in experiments because of sample preparation history and slow kinetics for dissociation of lipid molecules from bilayers. The presence of a monolayer will have no qualitative effect upon the discussion as long as the surface tension remains greater than zero [18].

We will now consider the free energy $F(a,b)$ as a function of both variables. As is well known, there is a bare interaction between bilayers that consists of an attractive van der Waals interaction and a repulsive hydration interaction [1,19]; the sum is shown as F_B in Fig. 2 with minimal F_B at water spacing a_B. For lipid bilayers that do not have significant fluctuations, such as the gel and subgel phases [8], the bare interactions can be measured directly for $a < a_B$ by ap-

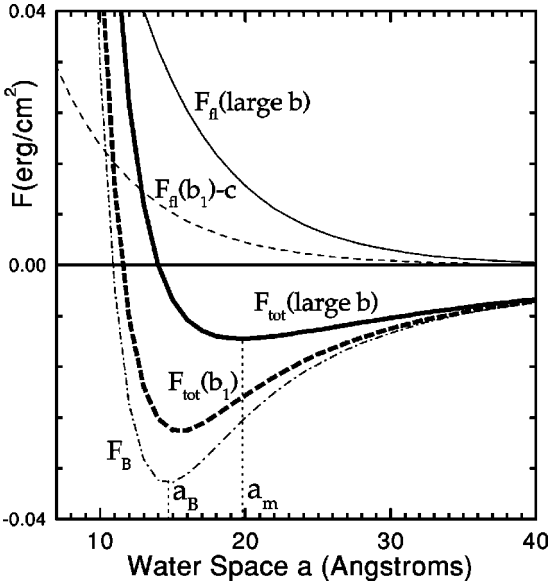

FIG. 2. Free energies from [19] versus interlamellar water spacing a. Bold solid or dashed lines show $F_{\rm tot}$, which is the sum of the bare potential F_B (thin dot-dashed line) and the fluctuating potential $F_{\rm fl}$ (thin solid or dashed lines). The solid lines are for a large value of b while the dashed lines are for a smaller value b_1. For convenience of comparison, $F_{\rm fl}(b_1)$ is shown with a positive constant subtracted. a_B is the stable spacing for the bare B potential between bilayers and a_m is the stable spacing for a freely fluctuating stack with $b=\infty$.

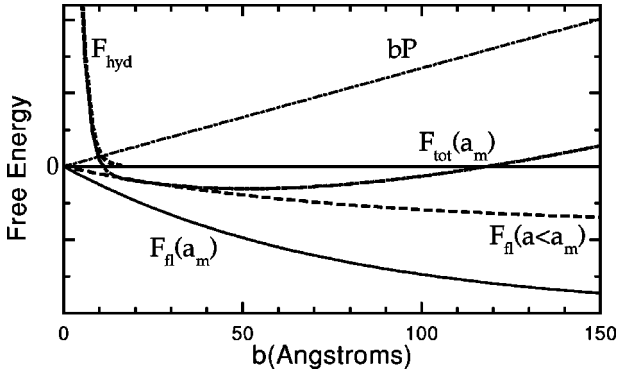

FIG. 3. Free energy as a function of b. The hydration free energy $F_{\rm hyd}$ is shown by the dotted curve and the fluctuation free energy $F_{\rm fl}$ is shown for two values of a (solid and dashed curves). For $a=a_m$, $F_{\rm tot}=F_{\rm fl}+F_{\rm hyd}+bP$ is shown (dot-dashed curve) for a nonzero P with free energy bP (dot-dashed straight line). Free energy scale is unknown.

plying known osmotic pressure P [1]. For vapor systems, P is given by

$$P=-(k_BT/V)\ln(R_H), \tag{1}$$

where V is the volume of a water molecule and R_H is the relative humidity defined as the ratio of the partial pressure of the water vapor to its value at saturation where the vapor is in equilibrium with bulk water.

For flexible L_α phase bilayers there is also a significant repulsive fluctuational free energy $F_{\rm fl}(a,b)$. Although the pressure due to fluctuations is larger than the attractive van der Waals interaction when a is very large, and this could lead to an unbinding transition [7,21], for many (but not all) bilayer systems there is a stable bound state with a finite average value of a, as indicated by a_m in Fig. 2. So far, this is the usual theory that applies to fully hydrated MLVs immersed in water when b is also very large. According to the recent theory [5,6], what makes free standing films different is that because b is small the surface tension of the vapor/water interface prevents fluctuations of the outermost bilayers; this reduces the dependence of $F_{\rm fl}$ upon a, as shown by the curve labeled $F_{\rm fl}(b_1)-c$ in Fig. 2, where b_1 is small. The ensuing shift to a smaller equilibrium water spacing a that is close to a_B in Fig. 2 would then account for the VPP.

What was missing in the previous analysis [5,6] is the dependence of $F_{\rm fl}$ on the external water spacing b, which we now analyze. First, let us define b to be zero for a completely dry system. Next, let us consider the free energy $F_{\rm hyd}$ for hydration of the headgroups. The smallest external water layer that would exist in saturated water vapor, denoted b_1, would consist of enough water to solvate the lipid headgroups of the outermost bilayers to the same level as the inner bilayers; namely, about half of the water spacing a_m for fully hydrated MLVs. Studies of fully hydrated lipids in the L_α phase give a_m about 20 Å [20], but the fluctuation force plays the major role in this distance. As such, for the present purpose, using an a_m of about 13 Å obtained from the L'_β gel phase [20], whose $F_{\rm tot}$ is dominated by F_B, is a better choice. Therefore, at $b_1\approx 7$ Å, the chemical potential of the adsorbed water is the same as the chemical potential of the vapor. (If there is a monolayer at the vapor/water interface, then $b_1\approx a_m\approx 13$ Å would be more appropriate.) This leads to a $F_{\rm hyd}$ that decays within b_1 as shown by the dotted line in Fig. 3. Now, for such a small external water layer, the fluctuations in the bilayer proximal to the interface would indeed be severely constrained because the surface tension of water favors a flat vapor/water interface and local fluctuations from b_1 in the thickness would involve large dehydration energies for some lipid headgroups. This leads to an additional decay in $F_{\rm fl}$ as b is increased beyond b_1, as shown in Fig. 3.

Consider first the case in which the relative humidity of the water vapor is 100% and a is fixed at some value a_1 that is less than a_m. As b is increased beyond b_1, the proximal bilayer has more room to fluctuate, which decreases its free energy. According to [5,6] this leads to a decrease of $F_{\rm fl}$ for the whole stack. While we do not know the functional form for $F_{\rm fl}(a_1,b)$ for $b>b_1$, it is certainly monotonically decreasing. We estimate that the effective decay length of $F_{\rm fl}(b)$ will be close to the value b_f that is the root mean square fluctuation σ_1 of a single bilayer immersed in water. (Although interbilayer interactions would be expected to reduce undulation fluctuations in the outermost bilayer immersed in bulk water relative to its single neighboring bilayer, that neighboring bilayer and the whole stack are also fluctuating, so the single bilayer calculation seems a reasonable approximation for the decay length of fluctuation suppression.) For values of the bending modulus K_c near 10^{-12} erg and an in-plane coherence length $L=1$ μm, using the formula [22]

$$\sigma_1^2=L^2kT/4\pi^3K_c \tag{2}$$

gives a decay length $b_f \approx 100$ Å.

The average values of a and of b are those that minimize the total free energy, so

$$(\partial F/\partial a)_b = 0 = (\partial F/\partial b)_a . \quad (3)$$

Providing that the relative humidity of the surrounding vapor is maintained at 100%, Fig. 3 indicates that the equilibrium value of b should become very large. Stated more colloquially, the stack of membranes can lower its total free energy by attracting enough liquid water to its external surfaces that it can fluctuate as freely as in bulk water. Therefore, the stack of membranes should have the fully hydrated water spacing a_m.

It is especially important to appreciate that the above analysis includes and does not contradict the mathematical analysis of the previous theory [5,6]. It does point out that there is an extra degree of freedom, namely, the size of b, that was not previously considered. Therefore, even though it was concluded from the previous theory that a should be smaller than a_m due to the suppression of fluctuations at the vapor interface, it is now realized that the system can lower its free energy by growing b until the fluctuations are not suppressed. Therefore, the recent theory [5,6] does not really predict that a should be smaller for a free standing film than a_m for MLVs immersed in water, provided that the relative humidity is 100% (osmotic pressure $P=0$). Thus, the theory now predicts that there is no VPP in the sense that it was first understood.

Next, let us consider the case when the relative humidity is less than 100%. Because of the fluctuation free energy, the interesting regime for small P is still $b > b_1$; then the external water is essentially bulk water which is at a higher chemical potential than the vapor. This requires an additional osmotic pressure term in the free energy per unit area,

$$F_P = P(Na + 2b), \quad (4)$$

where P is given by Eq. (1) and $N+1$ is the number of bilayers. Adding $2Pb$ to F in Fig. 3 shows that b will now be constrained to be finite. For large values of P, equilibrium values of a and b will be determined mostly by the competition between the osmotic pressure and the bare repulsive hydration force. This is indicated in Fig. 3 for $b < b_1$ by the steeply rising F_{hyd}.

However, one must also remember that application of osmotic pressure decreases a as can be easily seen by adding PNa to the total free energy in Fig. 2; this will reduce the amplitude of decay of $F_{\mathrm{fl}}(b)$ as shown in Fig. 3, which, in turn, will further decrease b. A decrease in b further decreases F_{fl} for each value of a, so there is positive feedback in the reductions of a and b induced by P. Of course, without a detailed functional form for $F_{\mathrm{fl}}(a,b)$ as a function of both variables, the only quantitative conclusion is that the final a and b must satisfy Eq. (3). Nevertheless, there is the possibility that, as P is increased from zero, there may be a critical value P_c at which a and b undergo a transition, either discontinuously or higher order. We will call such a possibility the vestigial VPP.

Finally, it should be noted that the number of bilayers N should play a role. The fluctuational free energy F_{fl} should be roughly proportional to N whereas the surface part of the osmotic free energy, namely, $2Pb$, is not. Generally, the larger N is, the larger the driving force for growing the b water layers relative to the osmotic energy required to constrain the growth of b. Therefore, if there is a critical P_c, it should increase as N increases.

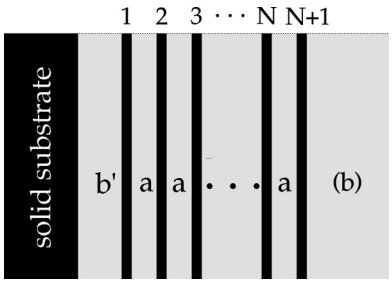

FIG. 4. Schematic of a stack of $N+1$ bilayers on a solid substrate. Mean spacing between bilayers is a and the space between the stack and the substrate is b'. The water (gray area) extends far to the right of the stack when the stack is immersed in liquid water and extends a distance b when the stack is in vapor.

B. Films on a solid substrate

Figure 4 shows a stack of membranes next to a solid substrate at distance b' and in contact on the other side with liquid water. If b' is small, then the substrate will suppress fluctuations, much like the vapor interface does for free standing films [5,6] and this will increase F_{fl} compared to a stack of lipid bilayers detached from the substrate with large b'. Therefore, since water easily permeates lipid bilayers in the L_α phase, and provided that there are negligible nonsteric interactions of the stack with the substrate, then the stack will detach and fluctuate freely with the same a_m as fully hydrated bilayers. As with free standing films, there should then be no VPP. If there is a strong and specific short-range adhesion between a lipid bilayer and the wall, then the first bilayer, or maybe even a few bilayers, may remain pinned closely to the wall. But, when the number of bilayers in the stack becomes large, then the total free energy can always be lowered by having a larger water layer with spacing b' at a distance beyond which the direct short-range interactions with the substrate have decayed. Thus, Fig. 4 should be interpreted as allowing for a small number of bilayers remaining firmly adhered to the substrate, but with the bulk of the film fluctuating with the fully hydrated value of a_m.

A different case arises if there are long-range forces between the substrate and the film, such as van der Waals or induced electrostatic forces. The observation that stacks float off glass substrates but do not float off mica substrates could be explained by the greater effectiveness of long-range forces with mica substrates. Figure 5 illustrates qualitatively the substrate energies that could bring this about. As shown, there is a finite equilibrium value of b' (designated b'_u in Fig. 5) that keeps the stack loosely attached to the substrate, with large enough b'_u that the fluctuations are not much suppressed. In this case the oriented spacings are approximately equal to the MLV spacings, $a_o \approx a_m$ and $d_o \approx d_m$.

We next consider the case in which the liquid water on the nonsubstrate side of the film is replaced by an aqueous solution that exerts an osmotic pressure on the water between the bilayers. This is accomplished experimentally by the es-

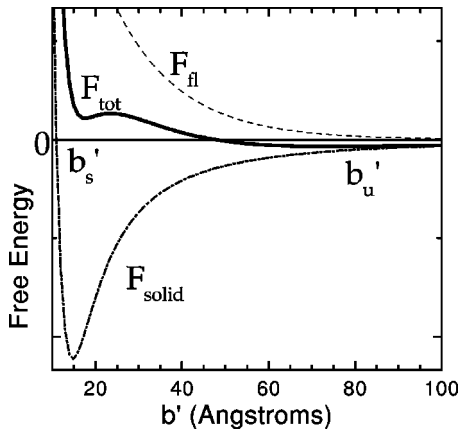

FIG. 5. Fluctuation free energy F_{fl} and interaction with solid substrate F_{solid} versus b' with the sum F_{tot} indicated by a solid line. This loosely unbound stack has $b'=b'_u$, which undergoes a transition to b'_s with application of osmotic pressure. Free energy scale is unknown.

tablished procedure of mixing into the water large polymers that do not mix with the lipid but which compete with the bilayers for the water [1]. Since the bilayers are impermeable to these polymers, any water spacing b' near the substrate (as well as the interbilayer water spacings a) must consist of pure water which is now under osmotic pressure P. The extra free energy of this water is then $P(b'+Na)$, of which Pb' is added to F_{tot} in Fig. 5. Let us ignore, for the moment, any feedback involving reductions in a_m due to P. If F_{tot} has a secondary minimum as shown in Fig. 5 (or even a nonconvex portion suffices), then there will be a critical value P_c at which b' will jump from a large value for lower P to a smaller one at higher P. Since this suppresses fluctuations in the stack, a will also jump from a value near a_m to a lower value. This is the second reason that a vestigial VPP might exist for a film on a solid substrate immersed in a polymer/water solution. The first reason is the same feedback mechanism discussed for free standing films.

Finally, we consider hydrating the bilayers with water vapor. This system is conceptually similar to the two previous ones. Compared to the free standing film, the difference is the interaction with the solid substrate. Compared to the polymer/water solution, the difference is the liquid water layer between the outermost bilayer and the vapor. The fluctuational free energy becomes a function of three variables, $F_{\mathrm{fl}}(a,b,b')$, and the osmotic contribution to the free energy is $P(Na+b+b')$. This complicates the free energy graphs and one would expect a shift in critical pressure P_c at which a putative jump in a might occur, but no fundamentally new features are involved.

III. EXPERIMENT

The purpose of the experiments was to determine if there is a vestigial VPP as defined in the preceding section. This requires measuring d_o as a function of osmotic pressure P to determine whether there is a critical value P_c where d_o changes rapidly or jumps, when compared to d_m for unoriented MLV samples. The historical VPP suggests that P_c should be small. However, it is very difficult to measure and control small osmotic pressures (relative humidities near

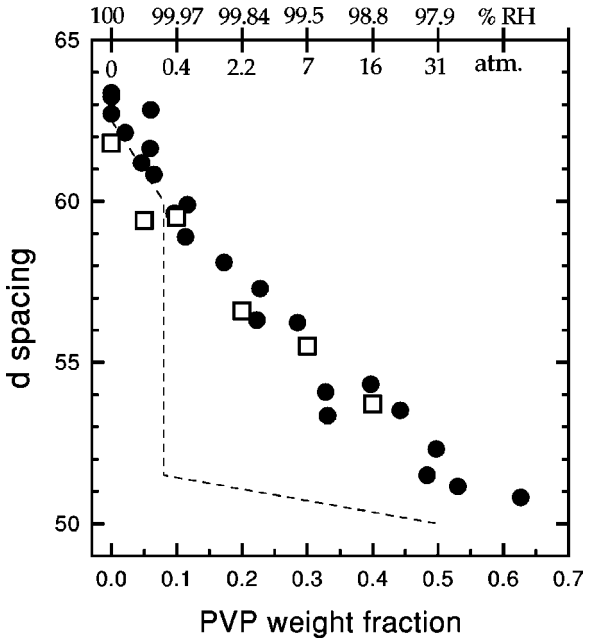

FIG. 6. Lamellar repeat spacing d for DMPC at $T=303$ K versus polymer (PVP) concentration (weight fraction) on the lower horizontal scale. The upper horizontal scale shows the percent relative humidity ($\%R_H$) and the corresponding osmotic pressure in atmospheres. Neutron data for d_o from oriented samples are depicted by open squares and x-ray data for d_m for unoriented MLV samples are shown by solid circles. The dashed curve schematically indicates d_o behavior if there were a vestigial VPP with $P_c=0.3$ atm.

100%) in a vapor. It is much easier to control osmotic pressure using a polymer/water solution [1]. Therefore, for the experiments we chose the system of a stack of bilayers on a single mica substrate which was then immersed in solutions of water and the polymer PVP (polyvinylpyrrolidone — MW 40 000). As discussed in the preceding section, this system is also somewhat simpler theoretically, while conceptually similar to hydrating from the vapor.

The lipid chosen was DMPC (L-α-dimyristoylphosphatidylcholine from Avanti Polar Lipids) which is in its L_α phase above 297 K. For oriented samples the lipid was deposited on a mica substrate in organic solvent which was then allowed to evaporate. The aluminum sample chamber and sample preparation were the same as described previously [16]. The dry stack was then immersed in water/PVP solution. The aligned multibilayer experiments were carried out at the NRU reactor (Chalk River Laboratories, Ontario, Canada) using the N5 triple-axis spectrometer which has a thermal flux of 5×10^9 cm^{-2} s^{-1} at the monochromator position. Neutron wavelength 2.37 Å was selected using the (002) reflection from a pyrolitic-graphite monochromator and a graphite filter was employed to eliminate higher-order λ/n neutrons. The first two orders of lamellar diffraction were measured and used to obtain $d_o(P)$. As in previous work [15,16], the stack remained well oriented.

Results for d_o as a function of weight fraction of PVP in water are shown in Fig. 6. The upper horizontal axis shows the corresponding osmotic pressure P (obtained following [23]) and the relative humidity [using Eq. (1)]. Although d_o decreases rapidly with P, it does so smoothly and continu-

ously, so there is no apparent critical P_c. Also shown in Fig. 6 are the lamellar spacings d_m for MLVs, obtained by x-ray diffraction [24]. The result that $d_o = d_m$ for $P = 0$ agrees with the previous results that there is no VPP [16]. If there were a vestigial VPP, then synergistic or cooperative effects would reduce d_o below the value d_m obtained for powder MLV samples as indicated by the dashed line in Fig. 6. From the experimental result it must be concluded that there is no vestigial VPP for $P < 16$ atm.

IV. DISCUSSION

The main theoretical result in this paper is that theory does not predict a VPP. This result does not contradict the essential analysis of the previous theory [5,6], namely, that long-range correlations exist in stacks of flexible bilayers and that a stack pinned to a solid substrate or with a nearly bare vapor/bilayer interface will have reduced d_o. Instead, our result builds upon the previous theory by critically examining the assumption that a lipid multibilayer stack will remain pinned to a solid substrate or that the vapor/bilayer interface remains nearly bare. Our analysis shows that the system should reduce its total free energy by exploiting the breakdown of this assumption and increasing the thickness of the water layers bounding the bulk of the stack. Such thicker water layers then permit the fluctuations that avoid the VPP.

As is well known for MLV samples, suppression of the fluctuations is accomplished by application of osmotic pressure [13,20]. A question raised in Sec. II is whether the surface effects in oriented samples could synergistically trigger a transition to the fluctuation suppressed regime at some nonzero osmotic pressure P_c. If P_c is close to 0 (RH close to 100%), then the difficulty in obtaining and controlling P in the range $0-P_c$ could help explain why the VPP was historically so persistent.

Such a vestigial VPP could also have been useful to the biophysical goal of obtaining better structures for a variety of lipid bilayers. One of the most basic measures of the structure of lipid bilayers is the electron density profile $\rho(z)$ along the bilayer normal z. As is well known, the hth term in the Fourier expansion of $\rho(z)$ is related to the intensity of the hth order of low angle lamellar diffraction. To obtain the best possible spatial resolution in $\rho(z)$ requires measurement of as many orders h as possible. However, the combined undulations in flexible membranes and fluctuations in the water spacing that give rise to the repulsive fluctuation pressure also have a profound effect on the shapes of the lamellar diffraction peaks. Instead of simple Bragg peaks (essentially δ functions), these fluctuations remove intensity from the central peaks and put it into power law tails of quasidiffuse scattering. This effect increases dramatically with q to the point where all the scattering becomes diffuse and higher-order diffraction peaks cannot be seen. This is a pernicious artifact from the viewpoint of obtaining a good bilayer structure. One way to avoid this problem is to suppress the fluctuations with application of osmotic pressure, but this stresses the bilayers which cause them to thicken [1,24,25]. A vestigial VPP offered hope to minimize the fluctuation artifact by suppressing fluctuations at a lower pressure P_c due to a synergistic effect of the surfaces. This would involve a kind of phase transition in which the small osmotic pressure would flip the stack from being loosely bound to a substrate to one where it was strongly pinned and therefore suppressed fluctuations. However, such a vestigial VPP was clearly absent in our experiments, at least up to $P = 16$ atm. Because the hydration forces dominate the fluctuation forces at higher P [19], one does not expect a higher value for P_c.

On the other hand, our result should be helpful in a different way for future studies of lipid bilayer structure. One can usually measure more orders (larger q and therefore better spatial structural resolution) for oriented bilayers because the diffraction peaks are not spread into rings as is the case for powder samples (this saves a factor of q in the Lorentz factor). Moreover, high-flux x-ray sources make it easier to obtain intrinsically weaker high q data, but layers of solvent and solid substrates absorb x rays. Therefore, the best sample preparation for x-ray studies is a stack of bilayers hydrated from water vapor, but it is difficult to measure the osmotic pressure in such experiments. The data in Fig. 6 suggest that one needs only measure d for oriented x-ray samples in vapor and then use a d versus P curve obtained from unoriented MLV samples to assign the correct value of P to the oriented samples.

Our experimental result that there is neither a VPP nor a vestigial VPP is consistent with our new theoretical analysis. This analysis involves too many undetermined parameters to do more than make the suggestion of a vestigial VPP. That suggestion has now been refuted by our experiments, at least for DMPC bilayers on smooth mica substrates. It is, of course, possible that roughening the substrate or using different substrates might give different behavior. However, it seems that mica was more likely to exhibit a vestigial VPP than other conventional substrates and DMPC is a characteristic lipid, so the VPP appears to be absent, even vestigially.

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