Multiple mechanisms for critical behavior in the biologically relevant phase of lecithin bilayers

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(Received 29 July 1998)

Lipid bilayer membranes manifest critical behavior in the lamellar D spacing observed by x-ray and neutron diffraction as the main phase transition is approached from the biologically relevant high temperature phase. The freezing out of conformational disorder of the hydrocarbon chains drives the main transition, but how this causes critical behavior of $D(T)$ has been an interesting puzzle and various models are under investigation. This paper presents x-ray scattering and NMR data to test the various models. One model involves the straightforward lengthening of hydrocarbon chains as T_M is approached, but it is shown that this accounts only for about half the anomalous increase in D. Another model of fluctuation induced expansion of the water region is shown to be inconsistent with two kinds of data. The first inconsistency is the lack of an increase in the Caillé fluctuation parameter η_1. The second inconsistency is with $D(T)$ data taken under osmotic pressure. Accurate simulations are employed to predict the theoretical values. A third model considers that the water spacing could expand because other interactions between bilayers may change as T_M is approached, but there is no quantitative support for this model at present. A fourth model involving expansion of the headgroup region is tested with NMR data; results are qualitatively consistent but quantitatively inconclusive. While the precise mixture of models is still unresolved, it is concluded that multiple mechanisms must be operating in this critical regime. [S1063-651X(98)12212-2]

PACS number(s): 87.22.Bt, 87.64.Bx, 87.64.Hd, 05.70.Jk

I. INTRODUCTION

Lipid bilayers are the structural basis of biomembranes, which define the spatial extent of cells and cellular organelles, as well as being the sites for many biochemical processes. Bilayers formed of single component lipids often exhibit several thermal phase transitions. The multiplicity of transitions reflects the fact that there are several competing interactions with a corresponding variety of fluctuations and order parameters. The most important transition, called the main transition with transition temperature T_M (T_M is in the physiological range for many lipids), is well understood on quantitative thermodynamic grounds to be driven by the conformational melting of the hydrocarbon chains of the lipids, in a manner analogous to the melting of solid polymers like polyethylene, though with important differences [1]. The high temperature phase above T_M, called the L_α phase, is the biologically relevant phase for biomembranes. It was originally suggested [2] that, as T is decreased to T_M within this phase, critical behavior begins to develop. However, the critical temperature T_c is not reached experimentally before being cut off by a first order transition (i.e., $T_c < T_M$); this can be understood when the lateral area fluctuations and the effective lateral pressure are included in the theory [3,4]. Because the critical point is not actually achieved, this behavior has been called ''pretransitional'' [5] or ''pseudocritical'' [6].

There are a number of quantities that have suggested pretransitional critical phenomena [7–11]. Attention has recently been focused on the lamellar D spacing in lecithin bilayers [5,6,12–15]; this is the repeat spacing for multilamellar, smectic liquid-crystalline samples that essentially consist of stacks of bilayers, each of average thickness D_B, that are locally flat when out of plane fluctuations are time averaged. There is also a layer of water, of thickness D_W, between adjacent bilayers, so $D = D_B + D_W$, as is shown schematically in Fig. 1.

Precritical behavior of $D(T)$ has been observed in DMPC [18] with 14 carbons in each hydrocarbon tail, in DPPC (16 carbons), and most recently in DLPC (12 carbons) [15]. Figure 2 shows D as a function of T for DMPC. The data above T_M are quite robust, especially regarding the T dependence [19]. Data for D below T_M are controversial, and several lines are drawn to indicate three different experimental results. Our group [5] has also reported data along line (2) and another group has reported data along line (1) [13] and also along line (3) [6]. The phase below T_M, called the P'_β phase, consists of static ripples with a long wavelength repeat distance in the plane of the bilayers [20]. Obtaining accurate experimental D values in this ripple phase would require resolving all the mixed (h denotes lamellar, k denotes ripple) reflections, which has only been achieved for partially hydrated samples [21]. More importantly, the critical point is cut off by a first order transition into a phase with different symmetry. Therefore the behavior of the D spacing below T_M and whether there is a maximum in D at T_M is irrelevant to the issue of the pretransitional critical behavior in the L_α phase. Also, while the experimental data in Fig. 2 strongly suggest precritical behavior, extraction of critical exponents or critical temperatures [6,12] should be viewed with caution

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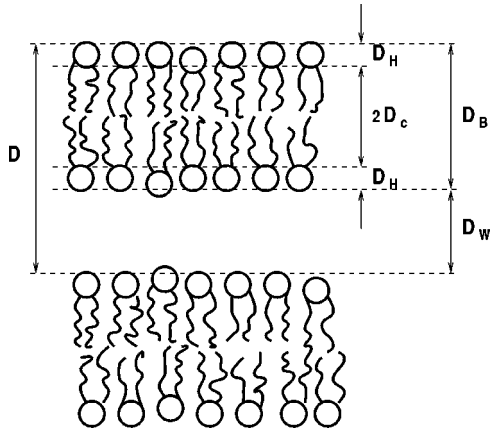

FIG. 1. Sketch of two bilayers in the fluid L_α phase. Each circle represents the headgroup of a lipid molecule and the wavy lines represent the conformationally disordered hydrocarbon chains. Shown are the lamellar repeat spacing D, the overall bilayer thickness D_B, the pure water thickness D_W, the hydrocarbon chain thickness $2D_c$, and the headgroup thickness D_H. The D spacing is easily obtained from diffraction measurements; the other D's are much more difficult to obtain. The head region also contains significant numbers of water molecules [16,17].

because $T_M - T_c$ is so large that the data are limited to less than half a decade in $t=(T-T_c)/T_c$ [15,22].

The decomposition of the D spacing into a water spacing D_W and a bilayer spacing D_B is a major challenge even apart from the special issues that arise for the observed precritical phenomena [17,23–25]. Since there are many different kinds of changes that can take place as consequences of the primary hydrocarbon chain conformational freezing, interpretation of the details of the T dependence of the D spacing has not been easy. Two different models have been proposed to explain this phenomenon. Model I [6,13,14] suggests that the bilayer becomes softer so that the bending modulus K_c becomes smaller as T_c is approached. As Helfrich [26] showed, a decrease in K_c would increase undulational fluctuations, so model I would provide an increase in an effective repulsive force which would then lead to an increase in the water thickness D_W [27]. This model appeared to be quite plausible, especially since earlier results on unilamellar vesicles directly indicated such a decrease in K_c [28]. However, our data for the line shapes of the x-ray scattering did not support model I [5]. Model II was therefore advanced, that it is the bilayer thickness, $D_B=D-D_W$, that accounts for the increase in D. Model II is consistent with the usual picture that the end to end distance of polymers is smaller at higher temperatures, and the critical aspect follows from an older theory of hydrocarbon chain melting in bilayers [1].

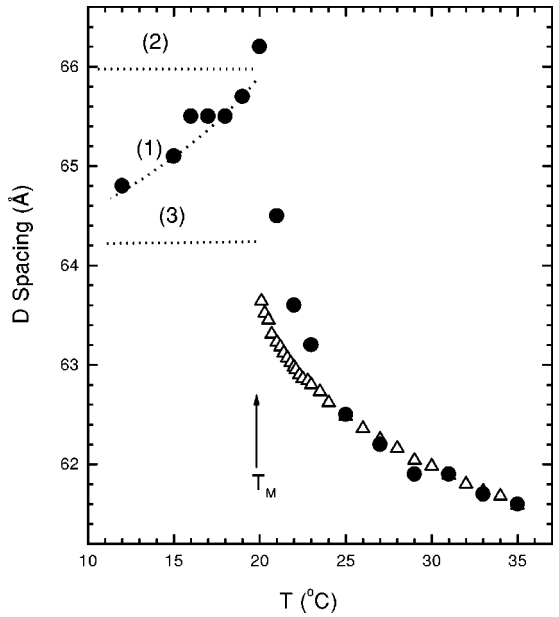

FIG. 2. Data (solid circles) show $D(T)$ for DMPC with deuterated hydrocarbon chains. Data for ordinary hydrocarbon chains are essentially identical except that $T_M=24.0\,^\circ$C [5]. The rapidly changing slope of $D(T)$ above T_M indicates precritical behavior. The open triangles show the temperature dependence of the thickness of the hydrocarbon chain region $2D_C$ as determined by NMR order parameters. Dotted lines (1)–(3) show the variety of apparent results for the ripple phase.

In this paper we first present in Sec. II NMR data that show that model II only accounts for about half the anomaly. We then turn in Sec. III to a reevaluation of model I. We present additional x-ray data that confirm that there is no significant change in the x-ray line shapes as T_M is approached. Furthermore, we have performed improved calculations (simulations) of the predicted effect which continue to predict an anomaly in the x-ray line shapes if model I is to account for the remaining anomaly in $D(T)$. We also add a different critical experiment, namely, $D(T)$ under osmotic pressure. These data and the simulations indicate that model I is inconsistent. We then turn in Sec. IV to consider two new models that might account for the data and present some new NMR data that address the more promising of these models. The remaining issues are summarized and discussed in Sec. V.

II. REEVALUATION OF MODEL II

In a previous paper [5] we showed that x-ray form factors imply an increase in D_B as T_M was decreased. However, for fully hydrated DMPC we could only obtain two orders of diffraction, so we could not state that all the increase in D was due to an increase in D_B. This issue is addressed in this section.

NMR

We report NMR data for the orientational order parameters for the hydrocarbon chains in DMPC-d54 [18]. The values of the order parameters, measured at many closely spaced temperatures, agree reasonably well with older values of ^{2}H NMR first spectral moments [10]. These order parameters have been used for many years to obtain the effective length of the hydrocarbon chains along the bilayer normal [29]. This length is then conventionally doubled to obtain the thickness $2D_C$ of the hydrocarbon portion of the bilayer. However, because all the chains do not necessarily terminate in the center of the bilayer, as noted by de Gennes [30], another formula for $2D_C$ has been advanced [23]. The result of this latter method is shown in Fig. 2 (open triangles) where, to facilitate comparison with the D spacing, a con-

stant 36.3 Å has been added to account for the water spacing D_W and the thickness $2D_H$ of the lipid headgroups. Virtually the same result is obtained when the other NMR formula is used with a constant 39.3 Å. From this we conclude that the T dependence of $2D_C$ matches that of the D spacing for T greater than $T_M+3\,^\circ$C regardless of the method of analysis of the NMR data. This match is considerably better than the match between $2D_C$ and the hydrocarbon thickness as determined by interpretation of low resolution small angle neutron scattering (SANS) data [6,14]. Although it was intimated that the latter disagreement may have been due to the interpretation of the NMR data [6], we will discuss later why the SANS result is more likely to be suspect.

Figure 2 shows that there is a divergence between the T dependence of D and $2D_C$ between T_M and $T_M+3\,^\circ$C. This will now be called the model II anomalous region, because model II can only explain an increase in D of about 2 Å from $T_M+10\,^\circ$C and leaves unexplained an additional 2 Å increase in D that occurs within three degrees of T_M. While this means that model II is valid, it does not explain the whole phenomenon.

These results are similar to earlier results [12] which obtained rough water and bilayer thicknesses from just two orders of diffraction, which is usually considered too few to be reliable. The analysis indicated that both water and the bilayer thicknesses increased by 2 Å, although with a difference compared to our data that the increase in D_W occurred over a wider temperature range of 6 °C above T_M.

III. REEVALUATION OF MODEL I

Our original critique of this model [5] was based on the idea that a decrease in K_c would increase fluctuations in this smectic liquid-crystalline system. Such fluctuations are well known to affect the shapes of the lamellar scattering peaks that have maxima when $2D\sin\theta=h\lambda$. These shapes, especially the long power law tails, are governed by the Caillé η_1 parameter [31–33], given by

$$\eta_1=q_1^2kT/8\pi\sqrt{K_cB}=(\pi\sigma/D)^2, \tag{1}$$

where B is the bulk modulus for compression in the direction normal to the bilayers. The second part of Eq. (1) also shows that η_1 is proportional to the mean square spatial fluctuations σ^2 in the water spacing between adjacent bilayers [34,35]. If the entire increase in D of about 4 Å in Fig. 2 were due to an increase in D_W, then it was estimated [5] that η_1 should increase by at least a factor of 2. However, well resolved synchrotron x-ray scattering showed no discernible increase in η_1 [5]. In this section we add more η_1 data and recalculate the theoretical estimates.

A. X-ray line shape data

Our previous high resolution x-ray data [5] were taken for only three temperatures, $T_M+0.3\,^\circ$C, $T_M+3\,^\circ$C, and $T_M+9\,^\circ$C. Since only the lowest of these temperatures is in the model II anomalous region, we have added data in this region. Also, our earlier data had been taken on nondeuterated DMPC; new x-ray data were taken for both DMPC ($T_M=24.0\,^\circ$C) and DMPC-d54 ($T_M=20.0\,^\circ$C). The $h=1$ and $h=2$ peaks were fit simultaneously to obtain η_1 with the Caillé [31] harmonic constraint that $\eta_2=4\eta_1$. The fits to the data (not shown) are of comparable quality to our earlier published data [5,24,33,34]. The results shown in Fig. 3 do not support any anomalous increase in η_1 as T approaches T_M. It should be emphasized that we do see changes in η_1 for other conditions. For example, the data in Fig. 3 at higher T show an increase in η_1 consistent with an expected softening of the bilayers and this also occurs for EggPC bilayers [17]. Our data for several lipids, including DMPC, shows even larger decreases in η_1 as water is removed [24,25,33,34]; this latter result can be understood by an increase in the compression modulus B as D_W decreases.

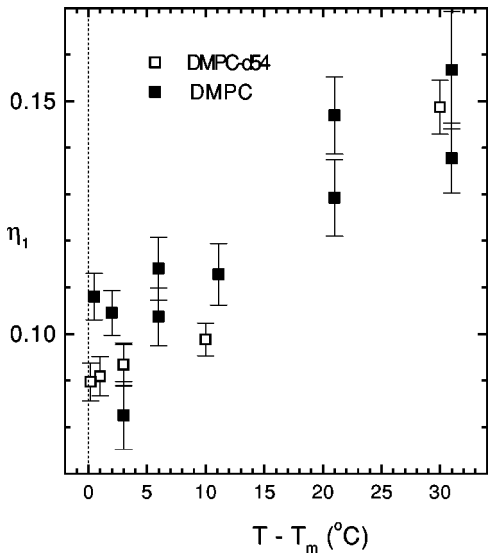

FIG. 3. η_1 for fully hydrated DMPC and chain deuterated DMPC-d54. η_1 was obtained by simultaneously fitting orders $h=1$ and $h=2$ with $\eta_2=4\eta_1$.

B. Theory of $\eta_1(T)$

We reconsider our earlier analysis [5] that predicts a twofold increase in η_1 if model I is correct. For systems in the regime of strong fluctuations, i.e., hard confinement, theory [26,35,36] and experiment [37] predict that η_1 would not change much for the changes in D shown in Fig. 2. However, our x-ray data [34] show that the quantity $\mu=(\sigma/D_W)^2$ is smaller than the hard confinement value, which has been evaluated to be in the range 0.16–0.21 [26,35,40]. Furthermore, μ varies as D_W decreases upon application of osmotic pressure [34]. These results mean that our system is in the soft confinement regime [35] where the hydration force, the van der Waals force and fluctuations all play a role. Previously, we calculated changes in the modulus B, necessary to calculate changes in η_1 in Eq. (1), from the second derivative of the total free energy [5]. This analysis is circular, as noted by Sornette and Ostrowsky [38] (who nevertheless employed it) because the free energy includes a fluctuation repulsion term which in turn is derived from a theory that includes B. We have recently compared the various B moduli and have conclusively shown that the B that should be used in Eq. (1) is not the second derivative of the total free energy [34].

We next considered the best analytic theory of soft confinement, due to Podgornik and Parsegian [35], which mani-

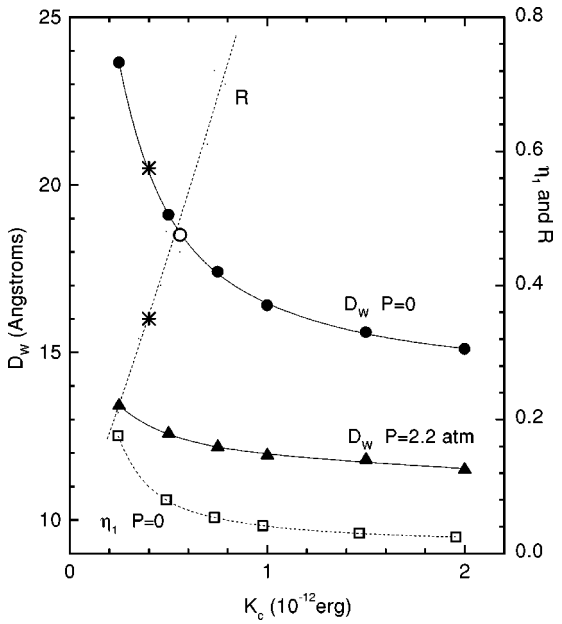

FIG. 4. Simulations for η_1 (open squares and dashed line) and fractional increase R (dashed line) in η_1 (both on right vertical scale). Also shown are simulated results for water spacing D_W (left vertical scale, solid circles for $P=0$ and solid triangles for $P=2.2$ atm) versus K_c. Bare interaction parameters for DMPC are $\lambda=1.91$ Å, $A_{\rm hyd}=1.32\times10^9$ ergs/cm^3 and Hamaker $H=7.13\times10^{-14}$ ergs. The asterisks show the best values of D_W and R for T_M given the measured value [17] of D_W (open circle) at $T=30\,^\circ$C.

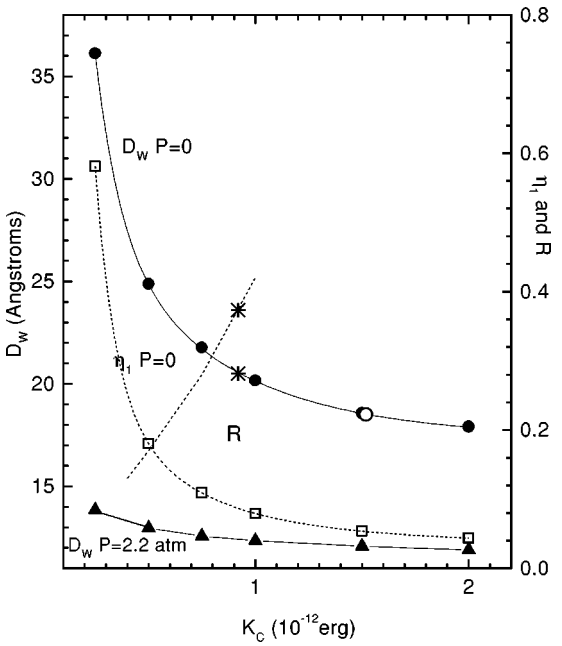

FIG. 5. Same as Fig. 4 except that the bare interaction parameters are $\lambda=2$ Å, $A_{\rm hyd}=1\times10^9$ ergs/cm^3, and Hamaker $H=5\times10^{-14}$ erg.

festly obtains B self-consistently. This theory also predicts that η_1 should increase with increasing D_W, but the better theory reduces the increase from 100% to about 50%. Because the anomalous increase in D that is unexplained by model II is now only 2 Å instead of 4 Å, this reduces the predicted increase in η_1 by another factor of 2, so the overall predicted increase in η_1, assuming a mixture of model I and model II, is now only 25%. However, recent simulations [39–41] show that the analytic theory [35] is also not quantitatively accurate, so more accurate simulation results will now be presented.

The simulation method employs an efficient Fourier Monte Carlo method [39,40]. Interaction parameters, H for the van der Waals interaction and $A_{\rm hyd}$ and λ for the hydration force $A_{\rm hyd}\exp(-D_W/\lambda)$, are given by our previous analysis of DMPC hydration data [34]. Figure 4 shows the D_W water spacing and η_1 as a function of K_c obtained from a simulation performed in the constant osmotic pressure ensemble at $P=0$. As expected, D_W increases with decreasing K_c; in model I this decrease in K_c would occur as T decreases towards T_M. The most useful quantity for comparison to the data in Fig. 3 is the fractional increase R in η_1. Since the absolute values of K_c are not yet settled, it is useful to consider R as a function of the value of K_c at T_M, and this is also plotted in Fig. 4. To be precise, $R+1$ is defined to be the ratio of η_1 at $T_M=24\,^\circ$C to η_1 at $T=30\,^\circ$C with the constraint that the increase in K_c used in the calculation causes a decrease in water spacing D_W equal to 2 Å. For example, the value of $R=0.48$ at $K_c=0.5\times10^{-12}$ erg was obtained by assuming that $K_c=0.5\times10^{-12}$ erg at $T=T_M$ and then a value of $K_c=0.80\times10^{-12}$ erg was required at $T=30\,^\circ$C in order that D_W decrease by 2 Å. We note that the simulation gives σ in Eq. (1); the conversion to η_1 in Fig. 4 was made using $D=62$ Å. However, in computing R, we have included the measured changes in D as a function of T.

The bilayer interaction parameters used in the simulations shown in Fig. 4 are our current best values as determined by fitting simulation results to our hydration data [34]. Figure 4 shows that R decreases rapidly as K_c decreases. This is expected because D_W increases rapidly and the system is approaching the hard confinement limit where η_1 is nearly independent of K_c. Our structural result $D_W=18.5$ Å at $T=30\,^\circ$C [17] (indicated by the open circle in Fig. 4) locates K_c to be 0.56×10^{-12} erg, which is close to our best value from simulations [41]. It may be noted that a value of $K_c=0.56\pm0.06$ has been reported for DMPC at 29 °C [42], which lends support to this analysis. For model I, this then would require K_c at T_M to be about 0.4×10^{-12} erg (indicated by asterisks in Fig. 4). This, in turn, requires a fractional increase in η_1 of $R=0.35$ (also shown as an asterisk in Fig. 4). A value of R this large is inconsistent with the data in Fig. 3.

There are, however, other experimental values for K_c for giant unilamellar vesicles. Meleard and co-workers [28] report $K_c=0.8\times10^{-12}$ erg at $T=25\,^\circ$C and $K_c=1.3\times10^{-12}$ erg at $T=30\,^\circ$C [28]. These large values of K_c are inconsistent with our combined simulations and η_1 data. However, if we ignore this inconsistency, we can adjust the interaction parameters to give appropriate values of D_W in simulations. The results of such simulations are shown in Fig. 5. For K_c near 0.5×10^{-12} erg, the value of R is now small enough that the noise in the data in Fig. 3 could hide the effect. However, for the simulation in Fig. 5 the appropriate value of K_c at T_M is in the range $(0.8–1.0)\times10^{-12}$ erg where R is still near 0.35, just as for the simulations in Fig. 4.

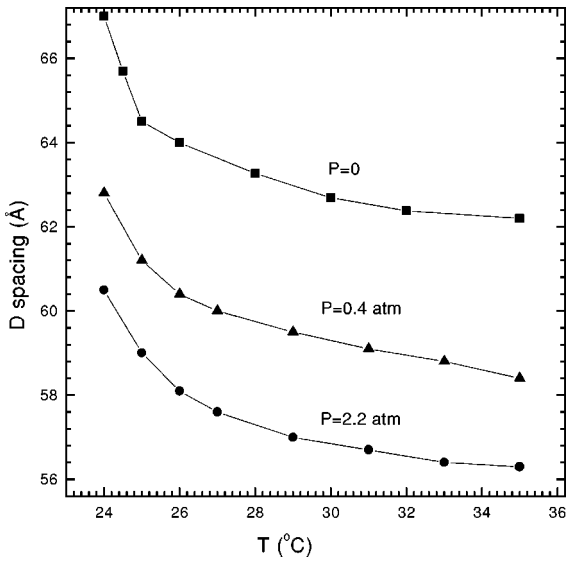

FIG. 6. Comparison of $D(T)$ for DMPC with two values of osmotic pressure P to fully hydrated $P=0$.

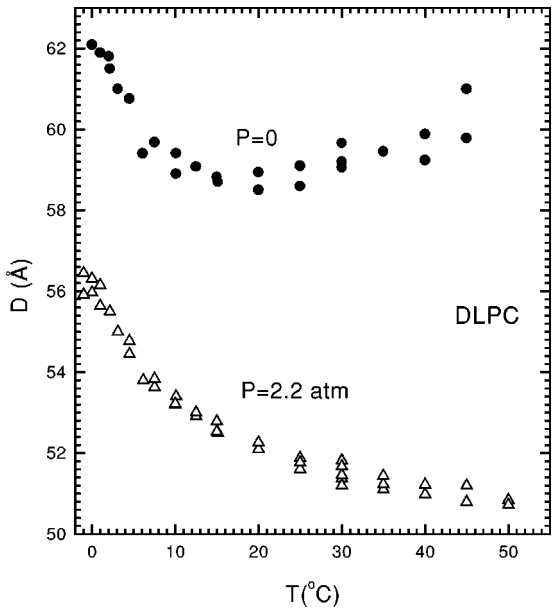

FIG. 7. $D(T)$ for DLPC for $P=0$ (solid circles) and for $P=2.2$ atm (open triangles).

C. A new test of Model I: $D(T)$ under osmotic pressure

We have also obtained $D(T)$ data under osmotic stress. As shown in Fig. 6, above T_M the $D(T)$ curves under osmotic pressure P lie below the $P=0$ $D(T)$ curve. However, the $D(T)$ under osmotic pressure still has the same magnitude of anomalous behavior. This is inconsistent with model I because osmotic stress suppresses undulational fluctuations [24,35]. Indeed, for $P=2.2$ atm our experimental value of η_1 [34] is smaller by a factor of 3 compared to Fig. 3. If model I were correct, then one would expect that this suppression would result in a smaller swelling in $D(T)$ because the effects of the same decrease in K_c would be competing against stronger restraining forces.

The preceding qualitative expectation is supported by simulation results. The simulated D_W as a function of K_c for an osmotic pressure $P=2.2$ atm is shown in Figs. 4 and 5. Any assumed change in K_c due to a change in T should be the same at $P=0$ and at $P=2.2$ atm. Therefore much smaller changes in D_W with decreasing T are predicted by the $P=2.2$ atm curves in Figs. 4 and 5 than by the $P=0$ curves. This disagrees strongly with the data in Fig. 6.

A recent paper reported that DLPC (12 carbons/chain) has an even larger precritical effect [15]. That study examined oriented samples on a solid substrate under osmotic pressures as high as 400 atm. After extrapolating to zero osmotic pressure, it was concluded that it is the water spacing that swells anomalously as T is lowered. We decided to complement this study by obtaining $D(T)$ for unoriented samples at full hydration $P=0$ as well as $P=2.2$ atm. Our results are shown in Fig. 7. Clearly, DLPC also has precritical swelling above T_M (which is about $-1\,^\circ$C). From 0 to 10 °C, the extent of the anomaly is about the same for DLPC in water as for DLPC osmotically stressed at 2.2 atm. At higher T, $D(T)$ continues to decrease for the osmotically stressed sample, as expected, because the bilayer thickness decreases with increasing conformational disorder in the hydrocarbon chains. However, for the sample with no osmotic stress, one expects the water spacing to increase because the bending modulus decreases with increasing T. This latter increase is evidently larger than the decrease in bilayer thickness because $D(T)$ in Fig. 7 increases with T above $T=20\,^\circ$C when $P=0$. The reason that the water spacing does not increase in the osmotically stressed sample, even though the bending modulus is decreasing, is that the osmotic pressure reduces the water spacing into the regime where the dominant repulsive force is the hydration force, which, of course, does not depend upon the bending modulus. Finally, our D values at $P=0$ in Fig. 7 are considerably larger (about 8 Å) than the extrapolated D values in the previous study [15]. It is unclear whether this difference is caused by inaccurate extrapolations or whether it may be due to differences between oriented and unoriented samples.

IV. ADDITIONAL MODELS

Since there are still inconsistencies that result from the use of model I to explain the remaining 2 Å anomalous increase in D, let us consider other models. Model III is defined to be the possibility that the parameters involved in the hydration force or the van der Waals force might change, thereby changing D_W while only changing η_1 slightly within experimental error. As T approaches T_M the membrane thickness D_B increases. Due to the form of the van der Waals interaction [43],

$$U_{\mathrm{vdW}}(z)=\left(\frac{1}{z^2}-\frac{2}{(z+D_B)^2}+\frac{1}{(z+2D_B)^2}\right), \qquad (2)$$

this results in an increase in the van der Waals interaction, which would decrease D_W rather than increase it. The membrane also becomes denser, but given the small difference in polarizability of solid and liquid hydrocarbons, this density change would seem to have only a negligible effect on the Hamaker parameter H [44]. A possible change in the right direction is that interfacial surface area per lipid decreases; this would make the interface more like the gel phase and some data [27] suggest that the hydration force is larger for

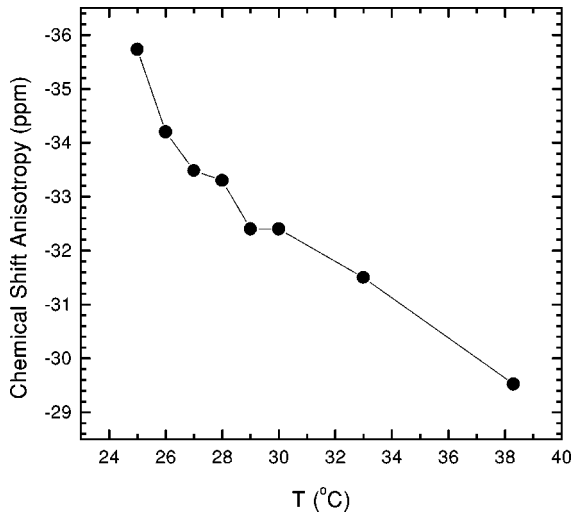

FIG. 8. Chemical shift anisotropy of the ($^{1}3C$ labeled, $T_M = 24.5\,^{\circ}\mathrm{C}$) carbonyl on the sn-1 chain of DMPC versus temperature.

the gel phase. However, for fully hydrated $P=0$ the hydration force is not the dominant force; rather, it is the balance between the van der Waals interaction and the fluctuation pressure that is primarily responsible for determining the water spacing. It seems, therefore, that the various forms of model III are not likely explanations.

Another possible model is revealed by realizing that the D spacing includes another piece besides the hydrocarbon chains, which was the original focus of model II, and water, which was the focus of model I. A third piece accounts for the thickness $2D_H$ of the two regions in which the lipid headgroups lie (see Fig. 1), so

$$D = D_W + 2D_C + 2D_H . \tag{3}$$

From neutron diffraction [45] D_H (the definition of which includes the glycerol backbone and the fatty acid carbonyls) is about 8–10 Å, so a 2 Å increase in $2D_H$ could be envisioned. We shall use the name ''model IV'' for the suggestion that the anomalous increase in D is due to an increase in D_H. We have performed two additional NMR measurements to test model IV. First, the deuterium order parameters were determined for DMPC with the two methylenes in the headgroup region deuterated to examine whether the orientation of the headgroup changed anomalously near T_M. These data indicate no anomalous change. Second, the anisotropy of the chemical shift of the *sn-1* carbonyl was measured. The data in Fig. 8 qualitatively support model IV; there is an increase with upward curvature in the magnitude of the chemical shift near T_M. Unfortunately, quantitative interpretation of these chemical shift data is difficult because of (i) the possibility of different temporal regimes for the motion of the carbonyl relative to the lipid molecular axis and for the motion of the lipid molecular axis relative to the normal to the bilayer and (ii) the possibility of breakdown of the independence of these two motions that could lead to anisotropy within the plane of the bilayer for the faster motion. Ignoring (ii) and considering models for (i) gives a change in $2D_H$ of the correct sign, but our best model gives an increase that is at most 0.6 Å. Analysis that allows for (ii) is unwieldy and involves too many parameters for useful conclusions. We therefore suggest that model IV may be possible, but we cannot conclude that it is proven.

SANS data have also been used to address changes in the structure of the layers within the D spacing. Results were only reported [6,14] for $2D_C$ and for the sum $D_A = D_W + 2D_H$. An anomalous increase in D_A was interpreted as evidence for model I, but this could equally well be taken as evidence for model IV, since the anomalous increase in D_A could be due to an anomalous increase in either D_W or D_H. Indeed, a preprint [46] reports the individual spacings and it is D_H and not D_W that has most of the anomalous increase. This is consistent with model IV, but not model I. However, we have reservations regarding the interpretation of the SANS data. The fits to the SANS data required at least eight free structural parameters to fit three scattering peaks. Without peak shape information, such data only give two pieces of information, namely, the relative intensities of the three peaks, so peak shapes must be necessary to determine all the parameters in the structural model. The SANS resolution was at best 0.18 in $\delta\lambda/\lambda$, nearly 1000 times poorer than the resolution of our x-ray scattering data. This makes it impossible to resolve intrinsic line shapes, which our x-ray scattering shows have narrow central widths $\delta q/q_1 \approx 0.001$. Interpretation of the SANS data involved convolving a broad resolution function with a theoretical line shape derived from paracrystalline theory. It was argued [6,14] that extending paracrystalline theory to allow for three different fluctuating thicknesses within a single D spacing is more important than allowing for undulational fluctuations, which all paracrystalline theories omit. However, without fluctuations no paracrystalline theory can produce long power law tails in the scattering peaks. Earlier work [32] showed quite clearly that scattering from lipid bilayers has long power law tails and our recent work [33] specifically confirmed that paracrystalline theory does not give the correct line shape to fit fully resolved peak shapes. The argument for employing paracrystalline theory [6,14] is that it does not require the common assumption that fluctuations affecting the form factors (i.e., fluctuations in the structure of single bilayers) are statistically independent of fluctuations affecting the structure factor (i.e., fluctuations in the positions of the bilayers relative to each other). However, as was noted earlier (see the Appendix to [47]), the common assumption is a good one because the spatial fluctuations that affect the structure factor are long range, extending over many D spacings, so that the corresponding forces are too small to cause fluctuations in bilayer structure. Even nearest neighbor fluctuations in D_W are unlikely to affect fluctuations in D_B because interbilayer forces are weak compared to intrabilayer forces. Direct evidence comes from the result that bilayers do not change shape appreciably even when enough osmotic pressure is applied [24] to reduce D_W by even more than a factor of 2, which is considerably larger than the root mean square fluctuations σ [33]. Despite these reservations, it is noteworthy that the SANS results are also consistent with model IV because the anomalous increase in D_A could be due to an increase in $2D_H$ instead of the increase in D_W required for model I.

V. DISCUSSION

The most direct result supporting model I is from measurements of K_c as a function of T on single walled vesicles of DMPC [28]. Since K_c should only depend upon single bilayers and not upon whether bilayers interact, this should be a valid system for obtaining K_c for the smectic multilamellar vesicles studied here. The results given for DMPC [28] are $K_c = 0.8\times10^{-12}$, 1.5×10^{-12}, and 1.2×10^{-12} erg at $T = 25\,^{\circ}\mathrm{C}$, $27\,^{\circ}\mathrm{C}$, and $30^{\circ}\mathrm{C}$, respectively. The vesicles exhibit hysteresis when taken through the transition. Also, such results require considerable analysis and values from different laboratories have varied considerably [48]. The analysis assumes that the volume of the vesicle is constant, but this constraint would be weakened by water permeability which allows the membrane to fluctuate away from spherical without any change in area. If there is an anomalous increase in water permeability similar to the increase in ion permeability near T_M [7], this would allow for increased fluctuations that would, using this analysis, lead to an artificial reduction in the apparent K_c near T_M. It is intriguing that a smaller experimental result, $K_c = 0.56 \pm 0.06$ for DMPC at $29\,^{\circ}\mathrm{C}$, is obtained by a completely different analysis of force versus area of unilamellar vesicles under tension [42] that does not use the constant volume assumption. It would be valuable to test the temperature dependence of K_c for DMPC using this latter method. In any case, we are unable to reconcile a decrease in K_c by a factor of 2 with our combined simulation and x-ray results.

In conclusion, the change in D above T_M is only half explained by model II where the hydrocarbon chain region thickens as T approaches T_M, so there must be a mixed model with another mechanism also playing a role. We continue to find no evidence for model I that the precritical behavior is signalling an unbinding transition caused by a decrease in the bending modulus. The expected increase in η_1 is now smaller because only half the anomaly has to be explained by it and because improved theoretical analysis indicates a smaller effect, but the predicted increase of 35% seems outside the error limits in our η_1 data. The failure of osmotic pressure to reduce the anomaly also strongly mitigates against model I. An additional possibility is that the water spacing D_W might increase due to changes in the hydration and/or van der Waals forces (model III), but this does not seem to be a likely explanation. Model IV, involving thickening of the headgroup region, has some experimental support but it is difficult to quantify. Incidentally, our earlier analysis [5] did not distinguish between models II and IV and only asserted that the bilayer thickness changed, but we implicitly had only the hydrocarbon chain region in mind.

While the picture is still not completely clear, it does appear that there are at least two different precritical structural responses of lecithin bilayers to temperature near T_M. Further understanding the details of these responses may increase our understanding of the variety of ways that membranes can accommodate the environmental requirements of the different integral membrane proteins that perform biochemical functions.

ACKNOWLEDGMENTS

We wish to thank Adrian Parsegian for information regarding van der Waals interactions. This research was supported by the U.S. National Institutes of Health, including Grant No. GM44976-07 (J.F.N.). Synchrotron beam time was provided under CHESS Proposal No. P619 and the CHESS facility is supported by NSF Grant No. DMR-9311772.

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