Critical swelling in single phospholipid bilayers

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(Received 17 August 1999; revised manuscript received 19 November 1999)

We approach the controversial anomalous swelling problem in membrane systems using small angle neutron scattering to measure relative changes in the bilayer thickness of unilamellar vesicles of dimyristoylphosphatidylcholine lipid bilayers in the vicinity of the main transition. These measurements conclusively demonstrate that at least half of the anomalous swelling previously observed in multilamellar vesicles of this system can be accounted for by the critical thickening of the bilayer itself, in contrast to conclusions drawn from several recent studies.

PACS number(s): 87.16.Dg, 87.14.Cc, 87.64.Bx, 05.70.Jk

I. INTRODUCTION

Biomimetic membrane systems composed of certain single component phospholipid bilayers are known to exhibit increased fluctuations as the main transition temperature T_m, is approached [1–7]. Although the main transition is clearly first order, the build up of fluctuations is understood theoretically on the grounds that the main transition occurs in the vicinity of a critical point, T_C [8–10]. Since the main transition pre-empts the critical transition, the system cannot strictly be said to be exhibiting critical behavior and has therefore been alternatively described as displaying ''pretransitional critical'' [1,5] or ''pseudocritical'' [7,10] characteristics near T_m.

Recently, attention has focused on the nonlinear temperature dependence of the lamellar repeat distance d, in multilamellar vesicles (MLV's) of diacyl-phosphatidylcholines such as DPPC, DMPC, and DLPC [11] where d shows a marked, nonlinear increase, known as ''anomalous swelling'' [5,6], as the temperature is decreased towards T_m. Since membrane modulated biological function such as protein activity can depend strongly on small changes in the physical properties of the membrane, the anomalous swelling effect may have some biological relevance. While there is certainly a consensus that d increases in a nonlinear manner as T_m is approached, there remains disagreement as to the source of the swelling. The root of the controversy is in assigning the increase to the constituent parts of d. As demonstrated in Fig. 1, the lamellar repeat spacing d, observed in diffraction experiments using MLV's is the sum of the bilayer thickness d_B, and the thickness of the water layer d_W, between adjacent bilayers (i.e., $d=d_B+d_W$). The bilayer thickness can be further subdivided to take into account the size of the headgroup region d_H, and the effective length of the acyl chains d_C, such that $d_B=2d_H+2d_C$. Since fluid phase MLV's in the physiologically relevant ''excess water'' condition [12] generally do not display more than three orders of diffraction, it is impossible to directly determine an accurate structure of the unit cell and its subunits by constructing electron or neutron scattering length density profiles.

A number of models, which have come to be known as models I–IV [1,2], have been proposed to explain the anomalous increase in d. Model I [4,6] used paracrystalline theory to attribute the dominant contribution in the anomalous change in d to a thickening of the water layer d_W due to increased bilayer fluctuations. However, no evidence was found for the proposed fluctuations when a line-shape analysis was performed on high-resolution data using Modified Caillé Theory (MCT) [2,5,13] or by measurements of $d(T)$ under osmotic stress [2]. Model II [5] suggested an anomalous increase in the bilayer thickness d_B, with the implicit assumption of the hydrophobic region $2d_C$ as the source of the changes in d. Model III, like model I, suggests an increase in d_W but the proposed mechanism is an increased interbilayer hydration and van der Waals forces [1]. Evidence for model III is derived from experiments on systems to which sterols have been added [1] and depends on conclusions based on criticality applied to data outside the asymptotic critical regime very close to T_C. Analysis of partially hydrated aligned systems [3] attributes the thickening

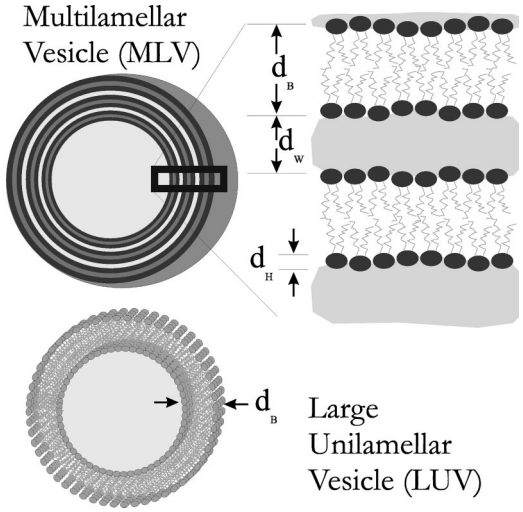

FIG. 1. Above: schematic of a multilamellar vesicle showing the repeating structural units and its composite subunits. Below: a large unilamellar vesicle.

to the water layer (models I and III) by extrapolating to the fully hydrated system, but the large discrepancy between the extrapolated bilayer repeat distance and that measured in fully hydrated MLV's is cause for concern. Finally, model IV [2], an extension of model II, contends that d_B does indeed swell, but that the hydrophobic region is responsible for only half of the anomalous increase in d. The headgroup region may also contribute to the swelling within model IV due to conformational changes, but a preliminary upper-bound on such a contribution from the headgroups still leaves some of the swelling unexplained [2].

As indicated above, the inherent complicating feature of the previous studies is the difficulty in interpreting the data from experiments using multilamellar vesicles. We circumvent the issue of separating the bilayer and water layer contributions to the lamellar repeat spacing by performing our experiments on large unilamellar vesicles (LUV's). Previous studies using LUV's have shown that neutron scattering techniques are sensitive to changes in the bilayer thickness d_B in such systems [14,15] and by studying a unilamellar system, the interpretation is greatly simplified by eliminating the water layer altogether (Fig. 1), allowing the thermal expansivity of the bilayer to be studied independently.

From a physical point of view, it is important to resolve this ongoing controversy in order to determine whether the behavior of these systems is consistent within the generally accepted theoretical framework [8] of the main transition. As well, changes in d due to ''hydration effects'' (a term that encompasses a rather broad range of significant and distinct properties) could necessitate the development of more complete theories of the role of water in membrane behavior. From a biological standpoint, this study using LUV's is important in determining if an effect seen in MLV's is also seen in LUV's, systems which are considered by many to be more biologically relevant. The contrast between phenomena occurring in LUV's and MLV's can also lend insight into the role of interbilayer forces in determining the properties of MLV's.

II. MATERIALS AND EXPERIMENTAL METHODS

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Northern Lipids, Inc. (Vancouver, BC) and used without any further purification. Approximately 80 mg of DMPC was suspended in 4.0 mL D_20/PIPES buffer (20 mM PIPES, 1mM EDTA, 150 mM NaCl in D_2O adjusted to a pH meter reading of 7.4). This buffer simulates physiological conditions and has the advantage of avoiding potential experimental artifacts caused by marked changes in pH due to the presence of small amounts of contaminants in an unbuffered solution.

The dispersion was freeze-thawed five times using alternating liquid nitrogen and warm water cycles to promote equilibrium transmembrane distributions of solutes [16]. It is important to avoid transmembrane osmolality variations as such conditions can lead to dramatic differences in vesicle size [17] and morphology [18]. LUV samples were then made by extruding the suspension ten times under nitrogen pressure using a stainless steel extrusion device (Lipex Biomembranes, Inc., Vancouver, B.C.). Each extrusion cycle was performed through two stacked 100 nm pore size polycarbonate filters (Nucleopore Corp., Pleasanton, CA), following the procedure outlined by Hope *et al.* [19]. LUVs prepared in this way are known to be narrowly distributed around a mean vesicle radius [17], almost exclusively unilamellar [19], and extremely stable over periods up to six months [20]. The experiments were performed using the N5 triple-axis spectrometer (used in double-axis diffraction mode) at the NRU reactor operated by Atomic Energy of Canada Limited with neutrons of wavelength $\lambda = 1.4$ Å obtained from the [002] reflection of a pyrolitic-graphite monochromator with a mosaic of 0.4°. With the collimation employed, the instrumental resolution of the spectrometer was determined to be 0.09 $Å^{-1}$ (full width, half-maximum). The temperature was controlled using a water bath with a stability of $\pm 0.05\,°C$. Differential scanning calorimetry (DSC) experiments using a Nano Differential Scanning Calorimeter (Calorimetry Sciences Corporation, Provo, UT) [21] with diluted specimens of the same sample indicate a consistent main transition temperature of $T_m = 24.8\,°C$, in good agreement with studies performed using MLV's [5].

III. RESULTS AND DISCUSSION

From a low-resolution scattering standpoint, appropriate for the present set of measurements, dilute LUV samples can be modeled as hollow, noninteracting spheres for which the scattering function is simply the difference between the Fourier transforms of concentric spheres. This can be written analytically as:

$$S(Q) = A\left\{ (R+d_B)^3 \times \frac{\sin[Q(R+d_B)] - Q(R+d_B)\cos[Q(R+d_B)]}{[Q(R+d_B)]^3} - R^3 \frac{\sin(QR) - QR\cos(QR)}{(QR)^3} \right\}^2, \quad (1)$$

where A is an overall amplitude of the scattering, R is the radius of the LUV, d_B is the thickness of the bilayer, and $Q = 4\pi/\lambda \sin(\theta)$ (2θ is the scattering angle). Our experiments measure $S(Q)$ convolved with an appropriate instrumental resolution function:

$$I(Q) = \int_{Q'} S(Q') \cdot \mathrm{Res}(Q - Q')\, dQ', \quad (2)$$

where $S(Q')$ is the scattering function given in Eq. (1) and $\mathrm{Res}(Q-Q')$ is the instrumental resolution function, here well approximated by a Gaussian. It is important to appreciate that rapid variations of $S(Q)$ with R will be smeared out by the instrumental resolution, while the slow variation of $S(Q)$ with d_B will be relatively unaffected. *This is not a limitation*, as it can easily be shown that instrumental integration over the rapid oscillation of $S(Q)$ with R effectively removes R as an important parameter in the scattering function for values of $R \gtrsim 400$ Å. In addition, any polydispersity present in the sample and thermal fluctuations of the vesicles will have a similar averaging effect as the instrumental resolution. As a result, we cannot extract accurate values for the

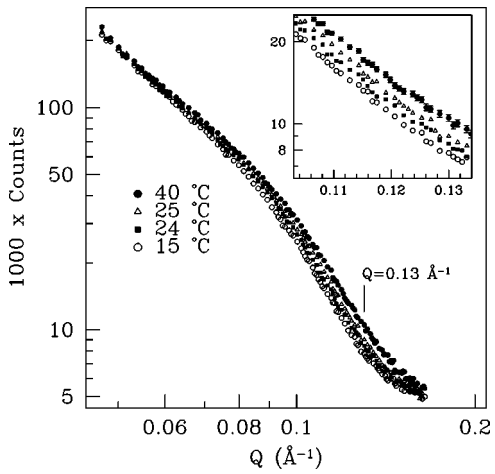

FIG. 2. Plot of scattering intensity vs Q at four different temperatures. The inset shows the high-Q region where the monotonic temperature dependence of the scattering intensity is clear. Representative errorbars are included for the $T=40\,^{\circ}\mathrm{C}$ data in the inset.

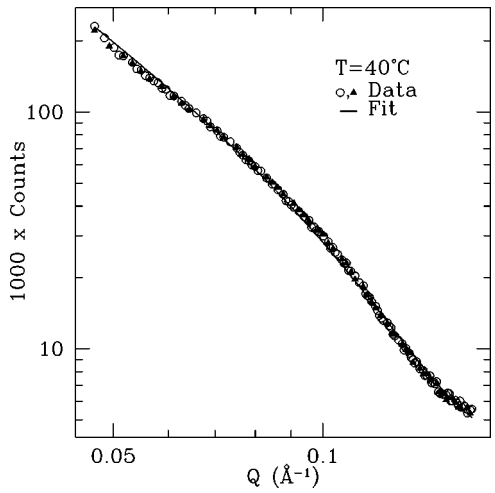

FIG. 3. Fit of Eq. (2) to the scattering profile of DMPC LUV's in the fluid L_α phase. Reproducibility of the data is demonstrated by the overlap of the two data sets collected two weeks apart.

vesicle radius from our data and treat it as an external parameter, as will be described below.

Information about the other vesicle parameter in Eq. (1), d_B, can be obtained from a low-resolution experiment. For DMPC in a D_2O buffer, known scattering length density profiles [22] suggest that the effective d_B measured will correspond approximately to the distance between opposing carbonyl or glycerol backbone groups in the bilayer, so the bilayer thicknesses we report will include (but exceed) the hydrophobic thickness $2d_C$ and exclude most of the headgroup region. Therefore, the measured values will be less than the full bilayer thickness $2(d_C+d_H)$. It should be noted that the nature of these small-angle, low-resolution experiments precludes accurate measurement of the absolute bilayer thickness, but can reliably measure relative *changes* in d_B provided that there have been no dramatic changes in the form of the scattering length density profile of the bilayer.

The scattering intensity $I(Q)$ was measured for a series of temperatures in the L_α phase as the temperature of the DMPC/water system was stepped down from 40 °C to 25 °C. Additional measurements were done below T_m. The clear variation of $I(Q)$ with temperature is seen in Fig. 2, particularly for values of $Q>0.08$ Å^{-1} where $S(Q)$ is sensitive to small changes in d_B. A fit to the data at 40 °C using Eq. (2) is shown in Fig. 3. The fit shown contains two free parameters, A and d_B in Eq. (1), a fixed value for the vesicle radius $R=560$ Å (set to agree with Ref. [23]) and a constant background term. This model can be extended to fit the scattering over more than a decade in Q [15]. Furthermore, the data are highly reproducible as shown in Fig. 3 where the two data sets shown were collected more than two weeks apart with several warming and cooling cycles in between. This test of reproducibility of the 40 °C data confirms that the variations in $I(Q)$ are the result of the bilayers undergoing conformational change and not some time-dependent variation in the condition of the sample (e.g., degradation of the lipids, aggregation, formation of MLV's).

Extracting changes in bilayer thickness

Given the temperature dependence of $I(Q)$ seen in Fig. 2, we can now extract values for the apparent bilayer thickness $d_B(T)$ from the data by using a variety of methods which we will outline below. The three approaches that we use to model the data give consistent results for $d_B(T)$, showing that the bilayer swells nonlinearly by 2.3 Å from 40 °C to 25.1 °C, just above the main transition temperature T_m. This result demonstrates that a substantial portion of the approximately 4 Å increase in the lamellar repeat spacing d in MLV samples is due to thickening of the bilayer, in contradiction to models which attribute the anomalous increase in d to thickening of the water layer.

In the first case, we extract $d_B(T)$ with fits to the data using Eq. (2). However, the uncertainty in the fitted effective d_B values is estimated to be approximately ± 0.5 Å, restricting the conclusions we could make about the behavior of $d_B(T)$. It is important to note that these estimated uncertainties are for the uncertainty in the fit to the model; the uncertainty for the absolute bilayer thickness is model dependent and would be an order of magnitude higher. To ensure that the fits were not being limited by the simple representation of the scattering length density of the LUV upon which Eq. (1) is based, a simple extension of the model was implemented. Figure 4 shows the form of the scattering length density of a typical diacyl-phosphatidylcholine bilayer (for an accurate measurement, see Ref. [22]). The form of the scattering function given in Eq. (1) assumes that the material within a spherical shell is characterized by some average scattering length density and that both inside and outside the shell is characterized by a different scattering length density. This first-order approximation to the true scattering length density is shown in Fig. 4. Also shown in the figure is the second-order approximation, in which the average scattering length densities of the polar headgroups and hydrophobic acyl chains are considered separately, effectively modeling the LUV's as two concentric shells of scattering material. The results of these fits, along with the apparent $d_B(T)$ calculated using the two remaining approaches to be described below, are shown in Fig. 5. As seen in the figure the refinement to the scattering length density model described above merely shifted the fitted $d_B(T)$ by a constant value, indicating that the first-order approximation given by Eq. (1) is sufficiently accurate for measuring relative changes

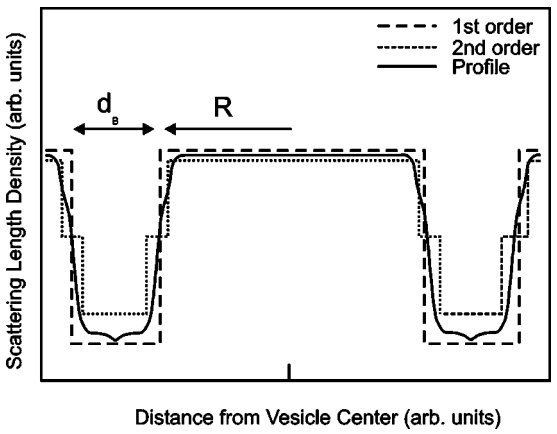

FIG. 4. Sketch of the scattering length density of a diacylphosphatidylcholine LUV (not to scale). The dashed lines show the shapes of the first and second order approximations to the scattering length density profile.

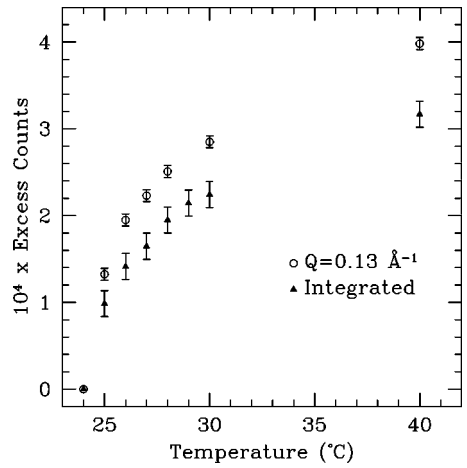

FIG. 6. Plot of excess intensity (relative to at 24 °C) versus temperature for the integrated intensity and the intensity measured at $Q=0.13$ Å^{-1} showing its nonlinear variation with temperature.

in d_B in these low-resolution measurements, which is not surprising since the scattering profile is adequately described by a one-step function [22] and the effect of convolving the scattering function with a broad resolution function is to ''wash out'' the exact details of the scattering length density profile. For all fits, the vesicle radius parameter R was constrained to keep the bilayer volume constant. Such a constraint is supported both theoretically [24] and experimentally [24,25].

More reliable values of the apparent bilayer thickness were obtained at $T=24\,^{\circ}$C and $T=40\,^{\circ}$C by averaging the fitting results of several independent runs at these temperatures, resulting in values of $d_B=34.6\pm0.5$ Å at $T=24\,^{\circ}$C (below T_m) and $d_B=31.3\pm0.5$ Å at $T=40\,^{\circ}$C in the L_α phase. As expected, these values of d_B are systematically lower than those reported in x-ray studies of MLV's, which are measured on an absolute scale and include contributions from the headgroups [26,27]. However, the 3.3 Å difference in d_B between the fluid and the solid phases is consistent with the best measurements on MLV systems [2,4].

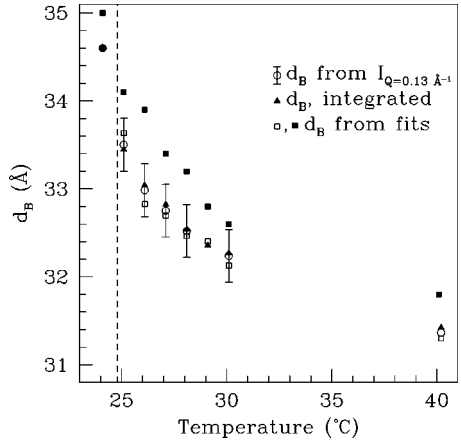

FIG. 5. Plot of d_B versus temperature for each of the three methods described in the text. The dashed line indicates $T_m=24.8\,^{\circ}$C. The open squares are from fits using Eq. (2) while the solid squares are taken from fits using the second-order fitting approximation described in the text. Solid triangles show the results of comparing the simulated and measured integrated intensities, while the open circles represent the results of the linear mapping of the intensity at high Q to simulated intensities. Error bars of ±0.25 Å are included for the open circles (method 3) only.

The second method employed for calculating changes in $d_B(T)$ evaluates the integrated intensities of $I(Q)$ for $Q>0.1$ Å^{-1}, shown in Fig. 6, and compares them to simulated $I(Q)$. This approach requires that a simulated integrated intensity and d_B be known *a priori* at one temperature, here 24 °C, and used as a reference to calculate the remaining intensities and corresponding bilayer thicknesses. Very good agreement between the bilayer thicknesses calculated using this method and $d_B(T)$ from the fits were found for all temperatures (Fig. 5). The agreement at 40 °C is particularly encouraging as this temperature represents the extreme range in temperature when measured from $T=24\,^{\circ}$C where the two methods are pegged to give the same result. Any systematic deviations of the model from the actual scattering function would be expected to propagate with the temperature difference. This method, however, also relies on the form of $S(Q)$ given in Eq. (1) so it cannot be said to be an independent confirmation of the form of $d_B(T)$.

The final method used to calculate the apparent d_B does not depend on the exact form of the scattering function used to describe the data. Improved counting statistics were obtained with the spectrometer positioned at a scattering angle corresponding to $Q=0.13$ Å^{-1} (cf. Fig. 2), and are shown in Fig. 6 as a function of temperature. Both of the models of the bilayer scattering length density discussed above were tested, along with some simple extensions to these models, and it was found that, in each case, small changes in the bilayer thickness d_B implied linear changes in intensity for wavevectors greater than $Q\sim0.11$ Å^{-1}. For completeness, we also considered the effects of polydispersity on the order reported by Hunter and Frisken [23] in our simulations and found that it did not affect linearity. The relationship between the intensity at $Q=0.13$ Å^{-1} and the apparent bilayer thickness for one of the simulations is shown in Fig. 7. Such a linear relationship between d_B and the intensity at $Q=0.13$ Å^{-1}, independent of the details of the LUV scattering length density, is not surprising as for sufficiently large vesicle radius, the convolved structure function de-

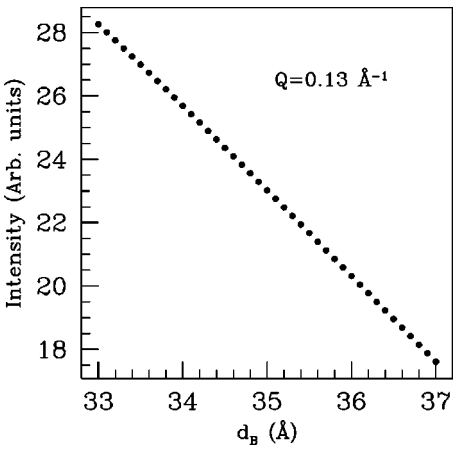

FIG. 7. Simulated intensity at $Q=0.13$ Å^{-1} versus apparent bilayer thickness showing the approximately linear relationship between the two. The simulations here use the model given in the text and include resolution and polydispersity effects.

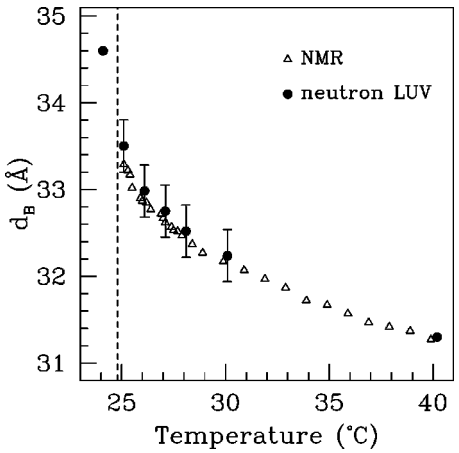

FIG. 8. Comparison of our best estimate of the anomalous swelling of the bilayer to NMR data taken from Ref. [2]. The NMR data from chain-deuterated DMPC has been shifted in temperature to match the main transition temperature of our undeuterated samples and shifted by a constant along the ordinate to agree with our results at $T=40\,°$C.

pends only on d_B, which itself undergoes small relative changes. The result of such a linear mapping between intensity and d_B is shown as the open circles in Fig. 5. This procedure requires two known values of the effective d_B, which we choose to be the values at $T=24\,°$C and an average of the d_Bs found by the preceding two methods at $T=40\,°$C. As mentioned previously, the difference in d_B between these two temperature end points is consistent with previous results reported from work on MLV's [2,4]. Of the three methods used to analyze the data this method is the only one of the three where we can assign error bars with confidence as it does not rely on a particular model of $S(Q)$. As can be seen in Fig. 5, all three methods agree as to the form of $d_B(T)$, indicating a nonlinear bilayer thickening of approximately 2.3 Å from 40 °C to 25.1 °C.

The results shown in Fig. 5 clearly show nonlinear behavior of the bilayer thickness from a direct measurement. We should stress again that it is only *relative* changes in d_B that we are able to measure with accuracy. The absolute values of the bilayer thickness suggested here, while reasonable, could only be reported accurately if the scattering were modeled using scattering length densities calculated from a more precise model of the bilayer component contributions [27,28] in a high-resolution experiment covering a broad range in Q space. As discussed previously, the scattering length density contrast in this DMPC/D_2O system will make the effective d_B a measurement of the distance between the region around carbonyl groups of the DMPC molecules, so the changes in d_B we report here are due mainly to swelling of the hydrocarbon chain regions. As shown in Fig. 8, this result, including the form and magnitude of the swelling, is consistent with chain-lengthening results measured by NMR on MLV samples which gave rise to model IV, as well as with mixed models concluded in the same work. It should be noted that a change in the lamellar repeat d, due to increased hydration of the headgroup as function of temperature as T_m is approached, as suggested in model III, is not ruled out by our measurements. However, our results do put an upper bound of approximately 2 Å on such a contribution, especially when considering that the effect of increased hydration would be to shift the scattering length contrast boundary away from the headgroup-solvent interface towards the acyl chains, with the net result, if any, being a *reduction* in the effective d_B that we measure.

In summary, we have provided diffraction based evidence of anomalous swelling of the bilayer from measurements of a unilamellar system that allows the calculation of relative changes in the bilayer thickness directly, without relying on a particular model for fluctuations in the system or other methods which seek to decouple the bilayer and water layer thicknesses. The 2.3 Å increase in the apparent d_B over the temperature regime studied within the L_α phase strongly supports model IV of the anomalous swelling in which critical lengthening of the hydrocarbon chains due to the ''freezing out'' of conformational degrees of freedom are shown to account for approximately half of the anomalous increase in the lamellar repeat spacing observed in MLV systems [2]. Our result disagrees with previous results attributing the anomaly to an increase in the water layer thickness [1,3,4]. In addition, our results put an upper bound on the remaining contribution to the anomaly, which may be due to factors contained within model IV and the other models. This work, conducted on LUV's, which are a closer analogue to biological membranes than are MLV's, could be biologically relevant given that small changes in bilayer thickness can have a measurable affect on the function of integral membrane proteins [29,30]. As well, the observance of anomalous swelling in LUV's shows that interbilayer coupling is not a necessary factor in the swelling of the bilayer.

We wish to acknowledge useful discussions with J. F. Nagle. This work was supported in part by NSERC of Canada and the MRC of Canada under Grant No. MT-7654.

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