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ARTICLE

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The effect of cholesterol in small amounts on lipid-bilayer softness in the region of the main phase transition

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Abstract The temperature dependence of the small-angle neutron scattering from aqueous multilammellar DMPC lipid bilayers, containing small amounts of cholesterol, is analyzed near the main phase transition by means of a simple geometric model which yields the lamellar repeat distance, the hydrophobic thickness of the bilayer, the interlamellar aqueous spacing, as well as fluctuation parameters. The observation of anomalous swelling behavior in the transition region is interpreted as an indication of bilayer softening and thermally reduced bending rigidity. Our results indicate that the effect of small amounts of cholesterol, $\lesssim 3$ mole%, is a softening of the bilayers in the transition region, whereas cholesterol contents above this range lead to the well-known effect of rigidification. The possible biological relevance of this result is discussed.

Key words Cholesterol · DMPC · Small-angle neutron scattering · Bilayer rigidity · Fluctuations · Unbinding transition

Abbreviations $DC_{10}PC$ didecanoyl phosphatidylcholine · DMPC dimyristoyl phosphatidylcholine · DMPC-d_{54} DMPC with fully deuterated acyl chains · DPPC dipalmitoyl phosphatidylcholine · DPPC-d_{62} DPPC with fully deuterated acyl chains · DSC Differential Scanning Calorimetry · SANS small-angle neutron scattering

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Introduction

Cholesterol is a ubiquitous molecular component of eucaryotic organisms and it is universally present in the fluid-bilayer component of their plasma membranes (Bloom and Mouritsen 1995). The cholesterol content in the eucaryotic plasma membrane is usually rather high, e.g. about 20 wt.% in human erythrocytes and 19 wt.% in liver cells, whereas the internal membranes have much less, e.g. those of the Golgi apparatus have about 8 wt.%, the endoplasmatic reticulum (ER) has about 6 wt.%, whereas the mitochondria have only about 3 wt.% (Jamieson and Robinson 1977). It is generally accepted (Yeagle 1988) that the high levels of cholesterol in the plasma membrane are important for imparting the membrane with mechanical coherence, resistance to mechanical fatigue, and a high permeability barrier (Bloom et al. 1991; Finegold 1993), while still maintaining the fluid character of the bilayer. Hence, cholesterol on the one hand provides for a rigidification and stiffening of the lipid bilayer, while on the other hand it does not solidify the bilayer but maintains its liquid character which is of importance for protein function.

These two effects of high levels of cholesterol are seemingly in conflict. The nature of this paradox was only resolved when the phase diagram of the lecithin-cholesterol system was determined (Ipsen et al. 1987; Vist and Davis 1990). The generic structure of this phase diagram, which is shown schematically in Fig. 1 for the DMPC-cholesterol system, is expected to apply qualitatively to a large number of different lipid-cholesterol mixtures (Bloom and Mouritsen 1995). According to this phase diagram, cholesterol in large amounts, $x_C \gtrsim 20$–30 mole%, promotes a special lipid phase, the liquid-ordered phase, which is a liquid (fluid) from the point of view of lateral disorder and diffusion but at the same time the lipid-acyl chains in this phase have a high degree of conformational order. Whereas cholesterol in large amounts has a dramatic influence on the phase behavior of the lipid bilayer, the phase diagram in Fig. 1 shows that in small amounts, $0 < x_C \lesssim 6$–10 mole%, cholesterol has very little influence on the phase equilib-

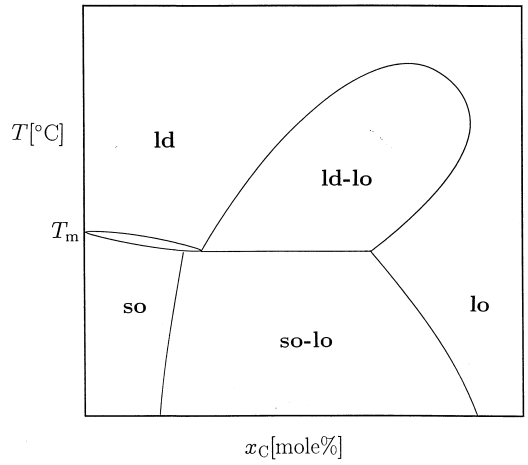

Fig. 1 Schematic phase diagram from mixtures of DMPC and cholesterol. T_m is the main transition temperature of pure DMPC. The different phases are labeled *so* (solid-ordered, the 'gel' phase), *ld* (liquid-disordered, the 'fluid' phase), and *lo* (liquid-ordered)

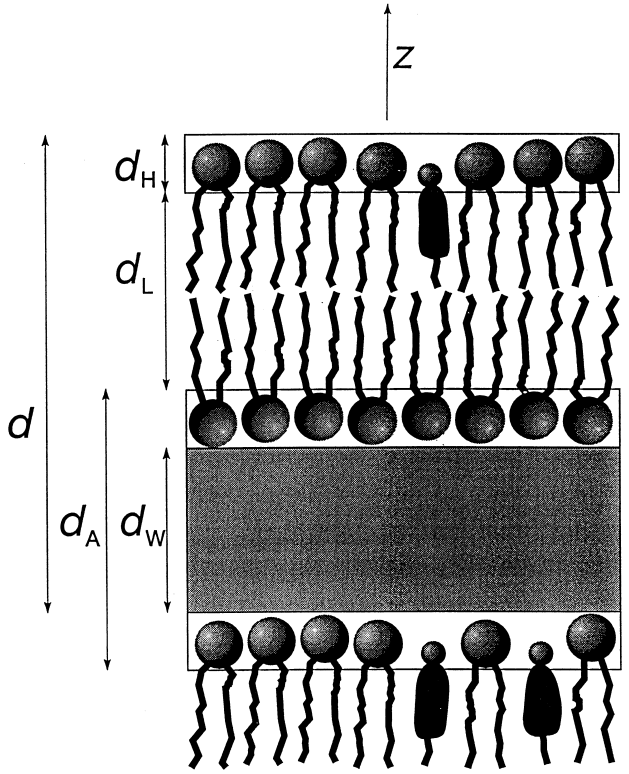

Fig. 2 Schematic representation of the different layers within a repeat unit of a multilamellar stack of lipid bilayers. Cholesterol molecules are drawn schematically as molecules with a small polar head group and a rigid hydrophobic body with a small flexible tail

ria in the sense that there is only a minute depression of the pure lipid-bilayer main phase transition temperature, T_m, and only a very narrow coexistence region can be detected. The physical reason for these peculiar features of the lecithin-cholesterol phase diagram is that cholesterol, as a hydrophobically smooth and rigid sterol molecule, prefers to have ordered lipid chains next to it (Zuckermann et al. 1993). However, ordered lipid chains in a bulk bilayer phase imply a solid (crystalline) lipid bilayer structure in which the cholesterol molecules do not pack very well. In small amounts, cholesterol solves this conflict by not preferring one lipid phase to the other, which results in the very narrow coexistence region at low cholesterol concentrations, cf. Fig. 1. Nevertheless, in larger amounts cholesterol is capable of breaking the solid and crystalline character of the lipid gel phase, giving rise to a new phase, the liquid-ordered phase. This phase is a liquid in the sense that the molecules diffuse laterally as in a fluid but at the same time the lipid-acyl chains have a high degree of conformational order.

As a consequence of its inability to prefer one lipid over the other at low concentrations, the cholesterol molecule would be expected to act effectively as an interfacially active agent that prefers to be located in the boundaries between gel and fluid domains of the bilayer (Cruzeiro-Hansson et al. 1989). In a series of computer-simulated calculations on a model for lipid-cholesterol interactions (Cruzeiro-Hansson et al. 1989; Corvera et al. 1992) it was indeed found that cholesterol tends to accumulate in the boundaries between the bulk lipid phase and the lipid domains of either fluid- or gel-like lipids that are formed in the transition region owing to the strong thermal density fluctuations which characterize the main phase transition (Mouritsen and Jørgensen 1994). It was predicted that cholesterol in the low-concentration regime leads to an enhancement of the fluctuations in the region close to the phase transition region, which in turn implies that response functions such as the area compressibility and the specific heat would be increased in the 'wings' of the transition. As a particular consequence it was predicted that the passive bilayer permeability, e.g. to small cations, would be enhanced. This was subsequently confirmed by experiments on Na^+-permeability in DPPC bilayers (Corvera et al. 1992) which showed that, whereas the well-known effect of suppressing the permeability is present for high contents of cholesterol (40%), 5% cholesterol leads to a significant enhancement of the permeability. These findings are in accord with previous work on K^+-permeation (de Gier et al. 1979). Furthermore, a few scattered studies of various membrane fluctuation phenomena (Blume and Hillmann 1986; Genz et al. 1986; Michels et al. 1989) also indicated that low levels of cholesterol may promote membrane fluctuations. Presumably, owing to biochemists' and biophysicists' preoccupation with the high-cholesterol-containing membrane state as the relevant case for eucaryotic plasma membranes, only very few quantitative biophysical studies have been reported on the effect of small levels of cholesterol on the physical properties of lipid bilayers.

Motivated by the idea of possibly significant effects of small amounts of cholesterol on lipid bilayers we shall in this paper report on the results of a small-angle neutron-scattering (SANS) study carried out on multilamellar DMPC lipid bilayers containing small amounts of cholesterol, $x_C = 0$, 0.5, 1, 2 and 4 mole%. The experiments are carried out with the specific purpose of systematically in-

vestigating the direct effect of small amounts of cholesterol primarily on the lamellar repeat distance, $d(T, x_C)$, and subsequently on the hydrophobic and the hydrophilic bilayer thicknesses, $d_L(T, x_C)$ and $d_A(T, x_C)$, separately (cf. Fig. 2). This is achieved by analyzing the scattering data in terms of a one-dimensional paracrystalline theory (Lemmich et al. 1996a). The analysis also provides estimates for the fluctuations in the e.g. the repeat distance, which in turn provides qualitative information about the effect of cholesterol on the elastic properties of the bilayers in the transition region. Recent SANS work on DMPC multilamellar bilayers has revealed (Hønger et al. 1994; Lemmich et al. 1995) that $d(T)$ displays a peak at the phase transition, indicating anomalous swelling behavior. The reason for this phenomenon is that the main phase transition in pure phospholipid bilayers is pseudo-critical (Ipsen et al. 1990a), i.e. close to a critical point, and is therefore accompanied by very strong in-plane density fluctuations, which lead to a substantial decrease in the area compressibility modulus, K_A (Needham and Evans 1988). The in-plane density fluctuations, the membrane conformational fluctuations, and the coupling between their degrees of freedom for a single bilayer can in the fluid phase be described in terms of a simple Gaussian model (Hønger et al. 1994). Within the Gaussian approximation, the difference in the density fluctuations of the two monolayers leads to an effective reduction (thermal renormalization) of the bending rigidity, κ, according to

$$\kappa = \kappa_0 - \frac{r^2}{K_A}, \tag{1}$$

where κ_0 is the bare membrane bending elasticity modulus and r is a phenomenological constant describing the coupling between the in-plane density fluctuations and the bilayer curvature. Recent experimental measurements of κ in the transition region for DMPC and DPPC, using flicker-noise analysis (Fernandez-Puente et al. 1994), have shown that κ for DMPC can be dramatically reduced when lowering the temperature in the fluid phase from above the transition to the transition temperature.

The reduction in κ leads to an effective steric repulsion between stacked membranes. In the simplest case where the membranes are considered unbound, this effect can be explained in terms of Helfrich's theory for undulating membranes (Helfrich 1978) as caused by a repulsive force or interaction free energy per unit area, f_S, which acts between the bilayers in a multilamellar stack and which scales with the repeat distance as

$$f_S \sim \frac{(k_B T)^2}{\kappa d^2}. \tag{2}$$

Thus the repulsion and the repeat distance increase as κ is reduced. Anomalous swelling behavior at the phase transition can therefore be interpreted as a manifestations of a thermal renormalization of the bending rigidity and hence as being due to a softening of the lipid bilayer (Hønger et al. 1994). The derivation of Eq. (2) requires that no other forces act between the bilayers, which is of course a simplification, especially in the present case, where the membranes are bound. A more precise description of the swelling effect in terms of critical unbinding can be obtained from a renormalization-group treatment of the full effective free energy functional for two interacting membranes (Leibler and Lipowsky 1987; Lipowsky and Leibler 1986; Lipowsky 1988). Here the direct interplay between the microscopic forces (e.g. van der Waals forces and hydration forces) and the steric forces, as described above, is taken into account. Within this theory it can be argued that a critical unbinding transition can take place which brings the system from a bound state to a completely unbound state. At this transition, $d(T)$ will diverge as

$$d(T) \sim (T - T_c)^{-\psi}, \tag{3}$$

with $\psi = 1$. In this expression, T_c is the critical unbinding temperature. Since, in the present case, a first-order transition taking the bilayers from the fluid phase to the gel phase intervenes before the singular point is reached, the unbinding transition does not fully develop. Nevertheless, the characteristic fluctuations of the unbinding transition manifest themselves as a pseudocritical (pretransitional) phenomenon over a wide range of temperatures in the fluid phase. This was demonstrated (Lemmich et al. 1995) for pure DMPC-d_{54} and DPPC-d_{62} bilayers by fitting the lamellar repeat distance to the expression

$$d(T) - d_0 \sim (T - T^*)^{-1},\ T \to T^*_+. \tag{4}$$

Here T^* is the pseudocritical temperature, and d_0 is the repeat distance in the fluid phase far from the phase transition.

Using a line of reasoning similar to that presented above we shall exploit the observed influence of small amounts of cholesterol on the measured thermal variation in lamellar repeat distance to provide indirect information on the softness of DMPC bilayers in the transition region. Our results indicate that the effect of small amounts of cholesterol, $\lesssim 3$ mole%, is to soften the bilayers in the transition region, whereas cholesterol contents above this range lead to the well-known effect of rigidification.

Materials and methods

DMPC with fully perdeuterated acyl chains (DMPC-d_{54}) and cholesterol were obtained from Avanti Polar Lipids Inc. (Birmingham, AL). The materials were used without further purification. D_2O (99.9 atom % D) and the rest of the chemicals used were of reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Sample preparation

Samples of multilamellar bilayers of deuterated phospholipid, specifically DMPC-d_{54}, with 0, 0.5, 1, 2, and 4 mole% cholesterol were prepared under excess water conditions in order to observe equilibrium swelling behav-

ior of the lamellar stack of bilayers. Stock solutions of DMPC-d_{54} and cholesterol in $CHCl_3$ were mixed and the organic solvent was subsequently evaporated under dry nitrogen atmosphere. Residual $CHCl_3$ was removed by high vacuum for 8 hours. The dry lipid films were hydrated in a D_2O buffer (50 mM Hepes ~ pD 7.2, 10 mM NaCl, 1 mM NaN_3 and 60 μM EDTA) to a total lipid concentration of 300 mM for use in the SANS experiments. The samples were temperature cycled through T_m and vortex-mixed numerous times throughout a two-hour period. A further dilution of the SANS samples to 6 mM lipid was performed for use in the DSC experiments.

Differential scanning calorimetry (DSC)

The phase behavior of the samples was examined by DSC using an MC-2 calorimeter from Microcal Inc. (Northampton, MA). All samples were scanned three times from 5 to 35 °C with a scan rate of 20 °C/h (12 °C/h for the pure DMPC-d_{54} sample). In all cases, the second and third scans were identical. With a solid-sample insert in the calorimeter, we established that the SANS samples displayed equilibrium hydration, i.e. no difference was observed between DSC scans of the concentrated and diluted lipid suspensions.

Small-angle neutron scattering (SANS)

The SANS experiments were performed using the Risø SANS facility. The samples were investigated under three different experimental conditions. 2.8 Å neutrons were used with sample-to-detector distances of 1 m and 3 m, corresponding to scattering vectors, q, in the high-q range of 0.07–0.58 $Å^{-1}$ and the intermediate-q range of 0.02–0.2 $Å^{-1}$, respectively. 9 Å neutrons were used with a sample-to-detector distance of 3 m in order to cover the low-q range of 0.007–0.06 $Å^{-1}$. The scattering data were corrected for background, and the experimental smearing was taken into account in the analysis. The resulting intensity function, $I(q)$, which is isotropic because the samples are non-oriented, was monitored in a wide temperature range around the main phase transition temperature, $T_m = 20.7$ °C, of pure DMPC-d_{54}. In all cases, the samples were heated stepwise allowing for an equilibration time of 30 min at each temperature step.

Data analysis and results

DSC analysis

In Fig. 3 are shown the specific-heat, C_p, vs. temperature for DMPC-d_{54} containing small amounts of cholesterol, $x_C = 0$, 0.5, 1, 2, and 4 mole%. The transition temperature of pure DMPC-d_{54} is $T_m = 20.7$ °C which is below that of protiated DMPC owing to the deuteration. The addition of small amounts of cholesterol introduces a small, but

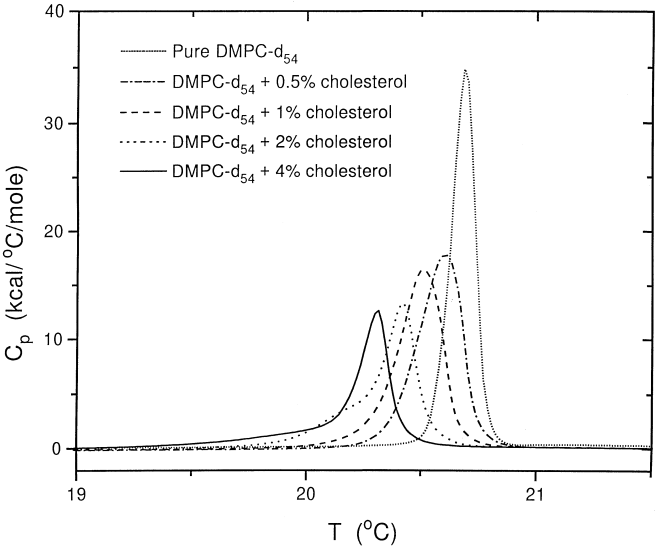

Fig. 3 Specific-heat, $C_P(T)$, as a function of temperature for multilamellar DMPC-d_{54} bilayers with cholesterol in different concentrations, $x_C = 0$, 0.5, 1, 2, and 4 mole%. Scan rates were 20 °C/h, except for the pure DMPC-d_{54} sample, were the scan rate was 12 °C/h

systematic freezing-point depression, $\Delta T_m(x_C) = -0.1$ °C, –0.2 °C, –0.3 °C, and –0.4 °C, for $x_C = 0.5$, 1, 2, and 4 mole%, respectively, compared to the pure system. $T_m(x_C)$, the apparent transition temperature, is evaluated as the temperature at which C_p displays its maximum value. The width of the specific-heat peak is increased for $x_C = 0.5$ and 1 mole%. For $x_C = 2$ and 4 mole%, the top parts of the peaks narrow again, but here we observe a considerable broadening at the bottom part of the curves, especially for $x_C = 2$ mole%, indicating that the transition effectively takes place over quite a broad temperature region. The calorimetric enthalpy of transition, ΔH, systematically decreases with increasing cholesterol content. This decrease, as well as our values for the freezing-point depression, introduced by the small amounts of cholesterol, are in good quantitative agreement with recent DSC studies performed on multilamellar bilayers of DMPC, incorporated with small amounts of cholesterol similar to the levels we are studying (Spink et al. 1996). The shapes of the specific-heat curves obtained by Spink et al. (1996) do, however, differ somewhat from the shapes we observe in Fig. 3. In their study, the specific-heat peaks are systematically much broader, even though their scan rates are lower than ours, and the shape does not change significantly upon addition of up to 3 mole% cholesterol. The broadness of the peaks could possibly be attributed to residuals amounts of organic solvent in the samples.

Analysis of SANS data

Examples of scattering data obtained for DMPC-d_{54} with $x_C = 1$ and 4 mole% cholesterol are shown in Figs. 4 and 5. The data shown in the figures cover the intermediate-q

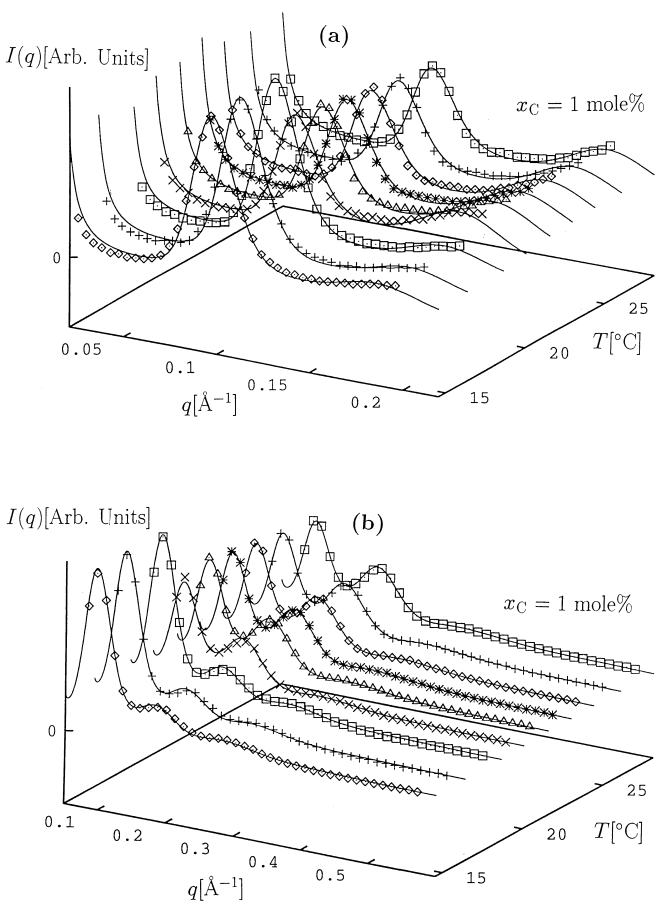

Fig. 4a, b Examples of experimentally obtained scattering functions, $I(q)$ (smeared data) for DMPC-d_{54} with $x_C = 1$ mole% cholesterol. Different symbols denote different temperatures in the increasing series: $T = 14.6$, 16.4, 18.6, 20.1, 21.6, 23.0, 24.4, 26.1, and 28.1 °C. Also shown are the fits to the theoretical model (*solid lines*). **a** Intermediate-q range. **b** High-q range. Note that $I(q)$ is displayed in arbitrary units

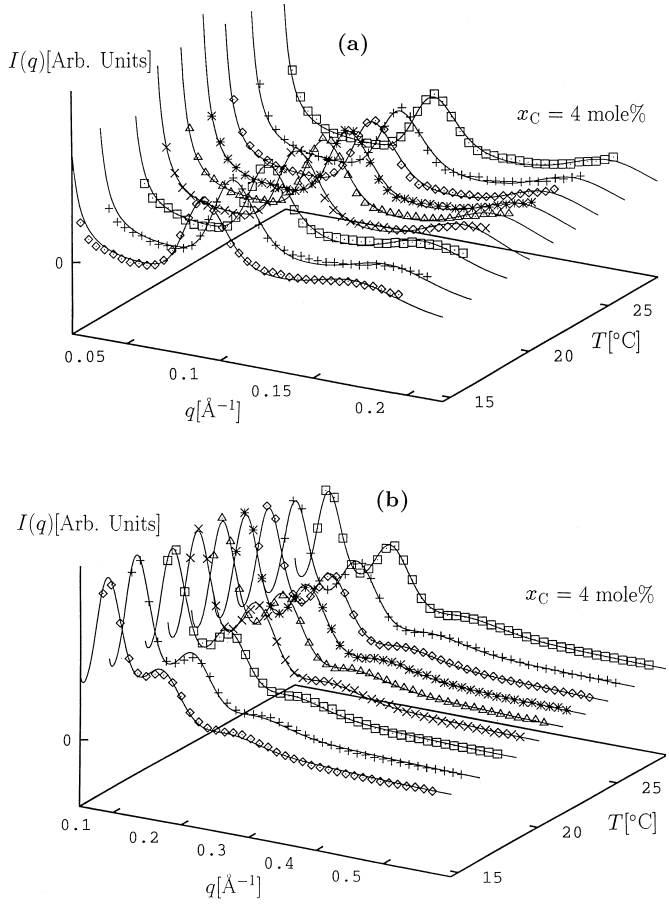

Fig. 5a, b Same as Fig. 4 for $x_C = 4$ mole% cholesterol. In this case the temperatures series is: $T = 14.5$, 16.3, 18.6, 20.0, 21.5, 23.0, 24.4, 26.1, and 28.1 °C

range and the high-q range (see above). Three orders of Bragg reflections, (1, 0), (2, 0), and (3, 0), can be discerned. The data sets have been selected with a view to illuminate the structural changes which take place at the phase transition. Clearly, the visually observable changes in the form of the scattering function at the phase transition are more pronounced for $x_C = 1$ mole% than for $x_C = 4$ mole%. This reflects not only the broader temperature region over which the transition takes place for $x_C = 4$ mole%. It also reflects that for $x_C = 4$ mole% the structural properties of the two lipid phases are more similar than is the case for $x_C = 1$ mole%. The very sharp transition, observed for pure DMPC-d_{54}, enabled us to calibrate the temperatures, measured during the SANS measurements. Thus all temperatures have been corrected by a small but finite off-set.

We have analyzed the scattering intensity function, $I(q)$, in terms of a one-dimensional paracrystalline theory. A detailed account of the theory (and the motivation for the choice of this particular theory) as well as details regarding the data analysis have been given elsewhere (Lemmich et al. 1996a). Thus, we shall here restrict ourselves to giving a less detailed description.

The one-dimensional approach relies on the assumption that the radii of curvature of the multilamellar vesicles are large compared to the thickness of the individual bilayers, i.e. the system is made up of stacks consisting of N nearly flat bilayers. Within this description, the scattering intensity function, $I(q)$, is given by

$$I(q) \propto \frac{I_1(q)}{q^2}, \tag{5}$$

where q^{-2} is the Lorentz factor for nonoriented powder samples and $I_1(q)$ is the one-dimensional intensity function, calculated along the z direction normal to plane of the bilayer (cf. Fig. 2). $I_1(q)$ is described in terms of a paracrystalline theory (Hosemann and Bagchi 1962), where a repeat unit is taken to consist of a sandwich of four layers (cf. Fig. 2). Each of the layers are allowed to vary in thickness according to a Gaussian distribution with the mean values d_H (the two head-group regions), d_L (the hydropho-

bic layers, which in our definition include the acyl-oxy groups of the fatty acids), and d_W (the water layer). The corresponding Gaussian spreads are denoted σ_H, σ_L, and σ_W, respectively. The deuteration of the acyl-chains and the use of D_2O as solvent lead to a maximum contrast in scattering length density along the bilayer profile as well as a minimization of the incoherent background scattering, mainly due to protons. The model invoked for the layered system, described above, ensures that the expectation value of the one-dimensional intensity function, $I_1(q)$, can be evaluated without decoupling the fluctuations in the membrane structure (as described by the bilayer form factor) from the fluctuations in the intermembrane distances (as described by the structure factor). The preservation of this coupling is particularly important in the strong fluctuation regime close to the main phase transition (Lemmich 1996; Lemmich et al. 1996a).

The procedure of fitting the experimental data to the theoretical expression for $I(q)$ (Eq. [18] in Lemmich et al. 1996a) involves determining eight free parameters. Besides the mean values and the Gaussian spreads of the layer distribution functions, these parameters include the lamellarity of the stacks, N, and a parameter $\tilde{b}_r$, determining the relative scattering length density between head group and hydrophobic layers. In practice, the data analysis involves performing simultaneous fits to the intermediate-q and the high-q spectra, recorded at a given temperature, T, and composition, x_C, with an initially very high fixed value of N, corresponding to an infinite number of layers in the stack. Subsequent fits to the corresponding low-q spectra are then performed in an iterative manner, in order to adjust the value of N. Typically, the values of N obtained by this procedure is around 25. Examples of the fits to the intermediate-q and the high-q spectra are shown in Figs. 4 and 5 for $x_C = 1$ and 4 mole%, respectively.

It should be noted that the gel phase which is present below T_m is a rippled ($P_{\beta'}$) phase. The ripples give rise to additional scattering peaks (Mortensen et al. 1988; Matuoka et al. 1990, 1994) which are not taken into account in the one-dimensional analysis of the scattering data. The broad low-amplitude (0, 1) peaks, characterizing the periodicity of the ripples, are partly visible in the low-q spectra (not shown). As for the higher order reflections, they are not directly visible from the spectra owing to the limited resolution, but they will still contribute to the scattering, thus affecting the fits. At first, this means that an n'th order Bragg peak (n, 0) is artificially broadened and shifted up in q-value, since it will also contain the (n, 1) peak.

From the study of Matuoka et al. (1990) on pure DMPC-d_{54} multilayers we expect this effect to be negligible for the (1, 0) peak, since the intensity of the (1, 1) peak relative to that of the (1, 0) peak is very small, whereas the effect is stronger, regarding the (2, 0) peak. Here the two peaks seem to be comparable in size. The overall effect of this can be revealed by a close inspection of Figs. 4b and 5b. One sees that the fits to the (2, 0) peaks are shifted down in q-value with respect to the experimental data in the gel phase, whereas this is not the case in the fluid phase. No such effect is observed for the (1, 0) peaks. Thus we conclude that the presence of the ripples in the gel phase does not affect the position of the fits to the Bragg peaks, i.e. the effect on the value of the repeat distance is negligible, but the artificial broadening of the peaks will, all other things being equal, mean that the fluctuations in the system are somewhat overestimated. This is reasonable, since in terms of our simple one-dimensional geometrical model, the ripples could be perceived as excess fluctuations in the bilayer thickness.

It should be noted that in general the reduced χ^2 sums, obtained from the fits, are quite large, especially in the gel phase. This is mainly due to the fact that certain localized regions of the spectra contain some scattering intensity that cannot be accounted for by our simple one-dimensional model. In the fluid phase, the only q-region which is unsatisfactorily fitted is between the (1, 0) and (2, 0) peaks, where some additional scattering intensity can be discerned (see Figs. 4a and 5a). In the gel phase, the additional scattering is mainly due to the ripple structure, as discussed above, but Figs. 4a and 5a also reveal that the scattering intensity is somewhat overestimated just below the (1, 0) peak.

Results for $d(T, x_C)$, $d_A(T, x_C)$, $d_L(T, x_C)$, and $\sigma_D(T, x_C)$

In Fig. 6 we have shown a selection of data, obtained from an analysis of the SANS data as described above. The data for the lamellar repeat distance, $d(T, x_C)$, in Fig. 6a show that $d(T, x_C)$ displays a pronounced peak at $T_m(x_C)$ for $x_C = 0$, 0.5, 1, and 2 mole% cholesterol, indicating anomalous swelling behaviour (as described in the Introduction). Evidently, the peak gets broader with increasing x_C, i.e. the anomalous swelling takes place over a larger temperature region. This broadening makes it harder to detect the true height of the peaks owing to the finite temperature resolution. The peak observed for $x_C = 2$ mole% is however significantly higher (~2.5 Å) than the others (~1.5 Å), but it should be emphasized that this conclusion is based on only a single point. At $x_C = 4$ mole% the anomalous peak in $d(T, x_C)$ is completely removed. In general the $d(T, x_C)$ vs. T plots in Fig. 6a reveal that the transitions effectively take place over broader temperature regions and start at lower temperatures than is immediately expected from the shape of the C_p curves in Fig. 3 as the amount of cholesterol is increased.

Although the data analysis provides separate information on d_H and d_W, we have chosen to focus on the total hydrophilic layer thickness, $d_A = 2d_H + d_W$ (cf. Fig. 2). This choice is to some extent motivated by the fact that the hydration of the head group, which is temperature dependent, makes it conceptually difficult to separate the head group regions from the water layers (Lemmich et al. 1996a). In fact, less scatter in the data is observed for $d_A(T, x_C)$ than for $d_H(T, x_C)$ and $d_W(T, x_C)$ separately. It is however also clear from Fig. 6b that it is the anomalous behavior of the total hydrophilic layer thickness, d_A (T, x_C), at the phase transition, for $x_C = 0$, 0.5, 1, and 2 mole%, which is the main source for the anomaly in $d(T, x_C)$, since $d_A(T, x_C)$ shows

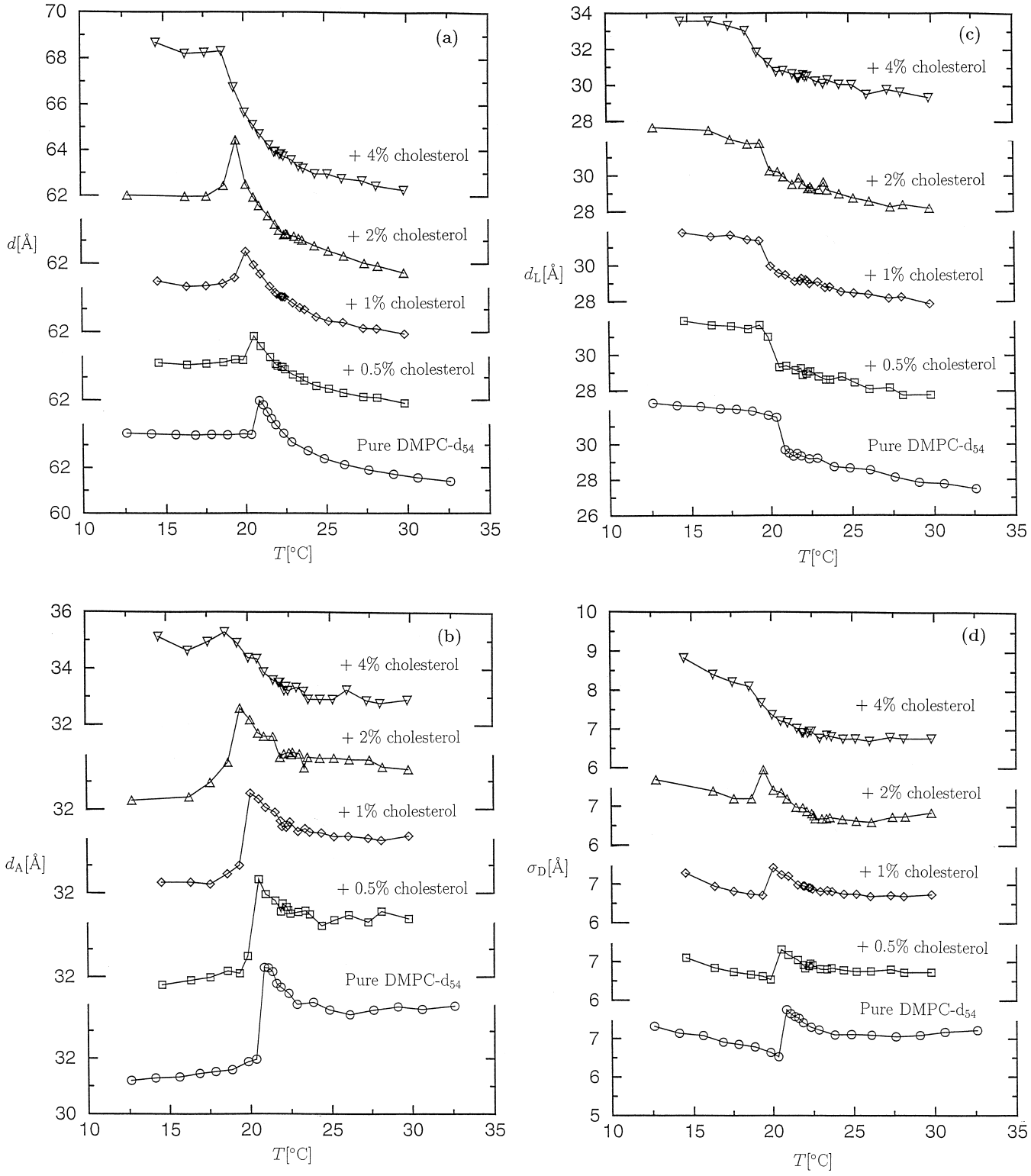

Fig. 6 Lamellar repeat distance, $d(T, x_C)$ (**a**), hydrophilic layer thickness, $d_A(T, x_C) = d_W(T, x_C) + 2d_H(T, x_C)$ (**b**), hydrophobic lipid bilayer thickness, $d_L(T, x_C)$ (**c**), and the Gaussian spread on the repeat distance, $\sigma_D(T, x_C)$ (**d**), as functions of temperature for multilamellar bilayers of DMPC-d_{54} incorporated with cholesterol in concentrations $x_C = 0$ (○), 0.5 (□), 1 (◇), 2 (△), and 4 (▽) mole% the same peak behavior as $d(T, x_C)$. Again, we observe that the peak in $d_A(T, x_C)$ is higher for $x_C = 2$ mole%, whereas no peak is observed for $x_C = 4$ mole%. These features are difficult to discern when $d_H(T, x_C)$ and $d_W(T, x_C)$ are plotted individually, since $d_H(T, x_C)$ decreases at the phase transition, whereas $d_W(T, x_C)$ increases, except for $x_C = 4$ mole% (not shown).

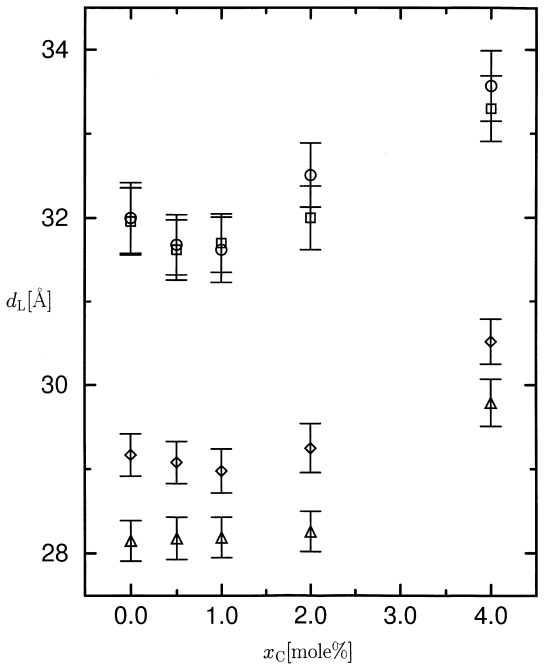

Fig. 7 Hydrophobic layer thickness, $d_L(T, x_C)$, as a function of the cholesterol concentration in multilamellar bilayers of DMPC-d_{54}, displayed for four different values of the temperature, $T = 16.4\,°C$ (○) and $T = 17.6\,°C$ (□) representing the gel phase and $T = 22.4\,°C$ (◇) and $T = 27.4\,°C$ (△) representing the fluid phase. The error bars indicate the statistical errors, as obtained from fits to the scattering data in terms of the paracrystalline theory described in the text

The hydrophobic layer thickness, $d_L(T, x_C)$, in Fig. 6c on the other hand varies monotonically through the transition region. There are no significant differences in the values of $d_L(T, x_C)$ for $x_C = 0$, 0.5, 1, and 2 mole%, except for the fact that the decrease in $d_L(T, x_C)$ at the phase transition due to the chain melting takes place over a broader temperature range when x_C is increased. Also, there are some indications at low temperatures in the gel phase that $d_L(T, x_C)$ is slightly higher for $x_C = 2$ mole%, than for $x_C = 0$, 0.5, and 1 mole%. However for $x_C = 4$ mole%, $d_L(T, x_C)$ is significantly larger (~1.5 Å) in both the gel and the fluid phase. In order to better illustrate these features, we have plotted $d_L(T, x_C)$ vs. x_C in Fig. 7 for four selected temperatures. Two of these temperatures represent the gel phase, whereas the other two represent the fluid phase. It should be remarked that the temperatures given in Fig. 7 each are averages of five fairly close individual temperatures, corresponding to actual temperature values recorded at measurements for the different cholesterol concentrations, $x_C = 0$, 0.5, 1, 2 and 4 mole%.

Figure 6d displays the Gaussian spread on the repeat distance, $\sigma_D(T, x_C)$. By definition, we have: $\sigma_D = \sqrt{2\sigma_H^2 + \sigma_L^2 + \sigma_W^2}$. When interpreting the values for the Gaussian spread, one should bear in mind that these parameters only to some extent represent the real thermal fluctuations in the system. As stated above, the ripples in the ($P_{\beta'}$) gel phase cause an increased broadening of the Bragg peaks and therefore an increased level in the fluctuation parameters. This obscures the physical interpretation of $\sigma_D(T, x_C)$ in the gel phase. Thus, we shall restrict ourselves to comparing the values of $\sigma_D(T, x_C)$ for $T > T_m(x_C)$, i.e. in the fluid phase. In all cases, we observe a dramatic increase in $\sigma_D(T, x_C)$ as the phase transition is approached from above ($T \to T_m(x_c)_+$). Evidently, the largest value of $\left|\frac{\partial \sigma_D(T, x_D)}{\partial T}\right|_{T \to T_m(x_C)+}$ is observed for $x_C = 2$ mole%.

Discussion

Evidence of anomalous swelling behaviour in terms of a peak in $d(T, x_C)$ at the phase transition has previously been observed in a synchrotron X-ray study of multilamellar lipid bilayers of DMPC, incorporated with small amounts of cholesterol, $x_C = 2$, 3, and 4.6 mole% (Matuoka et al. 1994). These authors were however mainly interested in studying the ripple structure in the gel ($P_{\beta'}$) phase and did not comment on the unusual swelling behavior. From inspection of their data, a peak of about 1.5 Å in $d(T, x_C)$ is seen for $x_C = 2$ mole%. A smaller, but significant peak is seen for $x_C = 3$ mole%, whereas no peak can be discerned in the data for $x_C = 4.6$ mole%. Although we observe a somewhat higher peak in $d(T, x_C)$ for $x_C = 2$ mole% in our SANS data in Fig. 6a, the X-ray data of Matuoka et al. (1994) are in good qualitative agreement with our results. The X-ray values for $d(T, x_C)$ are however systematically higher than ours. As a point of reference we can compare the values obtained in the gel phase, just below the phase transition, i.e. as $T \to (T_m)_-$. In this limit, Matuoka et al. (1994) observe: $d(T \to (T_m)_-$, 2 mole%) = 67.8 Å and $d(T \to (T_m)_-$, 4.6 mole%) = 71.8 Å, whereas we observe $d(T \to (T_m)_-$, 2 mole%) = 64.9 Å and $d(T \to (T_m)_-$, 4 mole%) = 68.5 Å, i.e. their values are about 3 Å larger than ours. This discrepancy may be due to the fact that Matuoka et al. (1994) cool their samples rather fast (0.25 °C/min) during the measurements. During our experiments we have observed that too rapid changes, i.e. when thermal equilibrium presumably is not reached, usually lead to larger repeat distances. In another X-ray study of DMPC/cholesterol mixtures (Hui and He 1983), a value of $d(T \to (T_m)_-$, 4 mole%) = 69.5 Å was reported, which is reasonable close to our value, quoted above.

It is possible to make a qualitative comparison between our results for $d_L(T, x_C)$ and measurements of the first moment, M_1, of the quadrupolar magnetic resonance spectrum from ^{2}H-NMR studies of DPPC-d_{62}/cholesterol mixtures (Vist 1984; Vist and Davis 1990). The reason for this is that M_1 is proportional to $\langle|S_{CD}|\rangle$, the average absolute value of the order parameter, S_{CD}, with respect to the CD-bonds on the acyl chains (Davis 1983), which to a good approximation can be taken to be proportional to the thickness of the acyl-chain layer under the assumption that the conformational and rotational motions of the acyl chains

are of axial symmetry around the bilayer normal (Seelig and Seelig 1974; Schindler and Seelig 1975; Ipsen et al. 1990b). In the fluid phase, Vist and Davis (1990) observe little or no change in the values of $M_1(T, x_C)$ for $x_C = 1.25$ mole%, compared to pure DPPC-d_{62}. For $x_C \geq 2.5$ mole%, $M_1(T, x_C)$ increases monotonically with increasing x_C. Taking $d_L(T, x_C)$ to be proportional to $M_1(T, x_C)$, this is in good qualitative agreement with our results. In the gel phase, the assumption of axial symmetric motion can not be fulfilled owing to the tilt of the acyl chains. It is however, still interesting to compare the values of M_1 to our results for $d_L(T, x_C)$. In the gel phase, $M_1(T, x_C)$ at first increases with increasing x_C up to around 5 mole% cholesterol, whereupon it starts to decrease monotonically. Thus it seems reasonable that we observe an increase in $d_L(T, x_C)$ upon addition of 4 mole% cholesterol in the gel phase, whereas a decrease in $d_L(T, x_C)$ is to be expected for high cholesterol contents (cf. the Introduction). The increase in $d_L(T, x_C)$ for small values of x_C in the gel phase can be attributed to the fact that apart from breaking the gel-phase crystalline lattice and disordering the chains in the gel phase, which lead to a decrease in $d_L(T, x_C)$, cholesterol also has the effect of decreasing the tilt angle (Needham et al. 1988) which leads to an increase in $d_L(T, x_C)$. Apparently the latter effect is the dominant one for small amounts of cholesterol.

Our results for $d(T, x_C)$ and $d_A(T, x_C)$ in the phase transition region show that not only in the anomalous swelling behavior preserved when small amounts of cholesterol, $x_C = 0.5$, 1, and 2 mole%, are incorporated into multilamellar DMPC-d_{54} bilayers; we also observe an enhancement of the height of the peaks in $d(T, x_C)$ and $d_A(T, x_C)$ for $x_C = 2$ mole% as well as a systematic broadening of the peaks with increasing cholesterol content. Taken together with the fact that no peaks in $d(T, x_C)$ and $d_A(T, x_C)$ are observed for $x_C = 4$ mole%, this indicates that adding small amounts of cholesterol, up to 2–3 mole%, maintains or even increases ($x_C = 2$ mole%) the in-plane density fluctuations in the phase transition region, compared to the unperturbed system. According to Eqs. (1)–(2), an increase in the in-plane density fluctuations in the phase transition regions leads to a stronger reduction in the bending rigidity, i.e. a softening of the bilayers. Addition of more than 3–4 mole% cholesterol, on the other hand, has the opposite effect, i.e. the fluctuations are damped, and the bilayers become more rigid. With reference to the theory of phase transitions this implies that small amounts of cholesterol, up to around 3 mole%, bring the phase transition in DMPC-d_{54} closer to a critical point, whereas larger amounts ($\gtrsim 3$ mole%) drives it more first-order again. This terminology, of course, assumes that one can speak of a single transition point, i.e. one neglects the existence of the narrow coexistence-region between gel phase and fluid phase, cf. Fig. 1. Our observations are in good agreement with theoretical studies, based on computer simulations (Cruzeiro-Hansson et al. 1989; Corvera et al. 1992), which suggest that cholesterol acts as an interfacially active molecule which positions itself in the boundaries between the gel and fluid domains which are dynamically created in the phase transition region (Mouritsen and Jørgensen 1994). Thereby, cholesterol leads to an enhancement of the fluctuations and as a consequence bilayer softening and increased passive permeability. From studies of the anomalous swelling behavior of multilamellar DMPC-d_{54} lipid bilayers, incorporated with small amounts ($\sim x = 1$ mole%) of other amphiphilic solutes, such as short-chain lipids ($DC_{10}PC$) and bola lipids, there are indications that these lipids have the same effect as cholesterol in small concentrations, i.e. they also cause a softening of the bilayers in the transition region (Lemmich et al. 1996b).

Further evidence of the increased level of fluctuations at low cholesterol contents can be discerned in the specific-heat curves, cf. Fig. 3, as an increased intensity in the 'wings' of the transition, indicating an increased level of fluctuations in the transition region. This effect is particularly pronounced at $x_C = 2$ mole%. There are, however, also distinct 'wings' for $x_C = 4$ mole%, indicating that even though no anomalous swelling can be discerned at this concentration, the level of the fluctuations in the phase transition is still considerable. It should however also be taken into account that the narrow coexistence-region between gel phase and fluid phase, cf. Fig. 1, at low cholesterol contents causes a broadening of the peaks. At a better resolution one should be able to discern two distinct peaks in this regime.

It is also interesting to analyze the behavior of $\sigma_D(T, x_C)$. Within the paracrystalline theory applied here, the squared periodicity fluctuations grow linearly with the system size, i.e. for a stack of n bilayers, we have

$$\sigma^2_{nD} = n \cdot \sigma^2_D. \tag{6}$$

Within another scattering theory, the Caillé theory (Caillé 1972), a structure factor can be derived which accounts for the elastic deformations of the layer positions for a stack of unbound fluid membranes, i.e. membranes which are held together by an outer (osmotic) pressure only. According to the Caillé theory, the free energy density, g, of the membrane stack is given by a harmonic expression

$$g = \frac{1}{2}\frac{\kappa}{d}\left(\frac{\partial^2 u}{\partial x^2} + \frac{\partial^2 u}{\partial y^2}\right)^2 + \frac{1}{2}B\left(\frac{\partial u}{\partial z}\right)^2, \tag{7}$$

where $u(x, y, z)$ is the local displacement of the membranes along the direction z normal to the layers (Helfrich 1973). B is the bulk modulus of layer compression. Within this approach, it can be shown (Nallet et al. 1993; Zhang et al. 1994) that the squared periodicity fluctuations depends on n as

$$\sigma^2_{nD,\text{Caillé}} \simeq \frac{k_B T}{4\pi\sqrt{\kappa B/d}}[\gamma + \log(\pi n)], \tag{8}$$

where γ is Eulers constant. Thus, in the limit of large n, $\sigma^2_{nD,\text{Caillé}}$ grows effectively logarithmically with the number of bilayers, n, in the stack. From Eqs. (6) and (8) it is clear that the fluctuations grow less rapidly with n in Caillé theory than in paracrystalline theory. Caillé theory includes a quantitative description of the dynamics of the system, which cannot be accounted for in the purely geometric paracrystalline theory. However, a priori, Caillé theory is

not expected to work for a system of bound membranes, especially not in the strong fluctuation regime close to T_m, since the form- and structure factors are completely decoupled within this theory. In that sense, we can think of Caillé theory as a kind of mean-field theory. In another work (Lemmich 1996) it has been shown that whereas the paracrystalline theory applied here gives the best overall description of the scattering data for the type of systems considered here, Caillé theory adequately describes the system in the fluid phase. That is, when the structure factor derived from Caillé theory is combined with a form similar to the type applied here, Caillé theory and paracrystalline theory fit the scattering data equally well, and the structural parameters, d_H, d_L, and d_W are identical within the uncertainty of the fits. In a recent X-ray study of DPPC at 50 °C Caillé theory was compared to a special version of paracrystalline theory in which the form and structure factors are decoupled. In this special case, where both theories treat the fluctuations in a mean-field way, it was shown that Caillé theory provided a better description of the scattering data (Zhang et al. 1996).

Even though the description of the fluctuations are different in paracrystalline theory and Caillé theory, it is interesting to qualitatively relate fluctuation parameters from the two theories. The fluctuations in Caillé theory derive solely from elastic deformations of the membrane stack (cf. Eq. [7]) and can therefore be described in terms of the elastic constants, κ and B. By setting n equal to 1 in Eq. (8) and rearranging, we get

$$\kappa B \sim \frac{(k_B T)^2 d}{\sigma_{D,\text{Caillé}}^4} \tag{9}$$

so within Caillé theory, an increase in $\sigma_{D,\text{Caillé}}$ is interpreted as a decrease in κB, i.e. as a softening of the lamellar system. Since the elastic theory, Eq. (7), is formally related to Helfrich's theory for steric repulsion between unbound undulating membranes, as given by Eq. (2) (Helfrich 1978; Leibler and Lipowsky 1987), this must in turn lead to swelling of the membrane stack. It is to be expected that a substantial contribution to σ_D, as obtained within the paracrystalline theory, is related to membrane undulations. In this light. our observations of an increase in $\sigma_D(T, x_C)$ as $T \to T_m(x_C)_+$ could be interpreted as a decrease in the product of the elastic constants, κB. It therefore seems reasonable that we observe the largest value of $\left|\frac{\partial \sigma_D(T, x_D)}{\partial T}\right|_{T \to T_m(x_C)+}$ for $x_C = 2$ mole%, where the maximum height of the peak in $d(T, x_C)$ is observed. Thus, the variation in $\sigma_D(T, x_C)$ complements $d(T, x_C)$ and $d_A(T, x_C)$ in the description of the anomalous swelling behavior.

A more reasonable description of the membrane conformational fluctuations can be obtained from the theory of critical unbinding (cf. the Introduction). Within this theory the elastic modes are highly correlated, and it is predicted that enthalpically bound membranes can undergo a finite temperature critical unbinding transition, where $d(T, x_C)$ and $\sigma_D(T, x_C)$ diverge (Leibler and Lipowsky 1987). Thus, a more quantitative description of the anomalous swelling behavior can be obtained by analyzing $d(T, x_C)$ in terms of critical unbinding for $T > T_m(x_C)$ according to Eq. (4). In all cases good fits were obtained when $d(T, x_C)$ was fitted to Eq. (4) with d_0 and T^* as free parameters in the temperature interval $[T_m(x_C), T_m(x_C) + 5\,°C]$. We shall in what follows define $T_m(x_C)$ as the temperature, at which the maximum value of $d(T, x_C)$ was observed. In the case of $x_C = 4$ mole%, $T_m(x_C)$ is chosen as the first point where $d(T, x_C)$ starts to decrease. In this way, we get a more realistic picture, since the values of $T_m(x_C)$, derived from the maximum value of $C_P(T, x_C)$ on the DSC curves, do not necessarily represent the temperature, where the major structural changes take place, as discussed earlier. Using this definition of $T_m(x_C)$ from the behavior of $d(T, x_C)$ together with the values of $T^*(x_C)$ obtained from the fits to Eq. (4),

we obtain $t = \left(\frac{T_m(x_C) - T^*(x_C)}{T_m(x_C)}\right) = 0.010,\ 0.011,\ 0.011,$ 0.005, and 0.011 for $x_C = 0$, 0.5, 1, 2, and 4 mole%, respectively. Even though the values of t are quite sensitive to changes in the definition of $T_m(x_C)$, it seems safe to conclude from this analysis that the system is closer to a critical point in terms of unbinding at $x_C = 2$ mole% than in the other cases. One could speculate whether it is possible to find a specific cholesterol concentration, x_C^*, such that the system would undergo a complete critical unbinding transition at $T_m(x_C^*)$. One could also speculate whether the existence of such a transition may have any biological relevance.

Eucaryotic plasma membranes contain relatively large amounts of cholesterol, which may be related to cholesterol's ability to act as a permeability barrier for the membrane by introducing conformational ordering of the lipid chains and provide for mechanical stiffness and at the same time preserving the fluidity of the membrane (Bloom and Mouritsen 1995). However, for some of the processes taking place in the internal membrane systems, such as the endoplasmatic reticulum (ER), where proteins are synthesized and folded, and in the Golgi apparatus, where the sorting of newly synthesized proteins and lipids takes place, it is of paramount importance that the membranes are permeable to relatively large molecules (such as protein fragments) and that the membranes are softer and more deformable in order to facilitate the continuous vesicle-budding processes that provide for the transport of material between different parts of the cell. Possibly for this reason, these internal membrane systems contain a much lower amount of cholesterol (cf. the Introduction). On the other hand, cholesterol is synthesized in the ER and since the amount of cholesterol here is much lower than in the plasma membrane, it is quite plausible that there exists a cholesterol gradients in the Golgi apparatus, through which the cholesterol is distributed to the plasma membrane (Bretscher and Munro 1993). In fact, it has been suggested that this cholesterol gradient could act so as to facilitate the sorting of the proteins in the secretory pathway (Bretscher and Munro 1983, Pelham and Munro 1993). Golgi

consists of several compartments, between which there is a continuous and selective transport of material through vesicle-budding processes. A cholesterol gradient could ensure that there would be a gradual increase in the membrane thickness from the 'cis' compartments, close to ER to the 'trans' compartments in the other end, owing to cholesterol's ability to order (stretch) the lipid chains. Evidently, it has been found that the average length of the trans-membrane domains (TMDs) of the proteins retained in the Golgi apparatus are shorter (~15 residues) than the average length of the TMDs of the proteins found in the plasma membranes (~20 residues). Furthermore, the TMDs of the Golgi apparatus protein contain more than twice the amount of the residue phenylalanine (Munro 1995). Phenylalanine prefers disordered lipid chains around it, due to its bulky phenyl group. Since it is energetically unfavorable for the proteins with short TMDs and a large amount of phenylalanine to move to membranes which are thicker and more ordered, and vice versa, the cholesterol concentrations gradient can act as an effective sorting mechanism in the Golgi apparatus. In light of the results presented here, it seems possible that the variation of small cholesterol contents also could provide for an effective control of the membrane softness and morphology, thereby controlling the vesicle-budding processes as well as the intermembrane distances in the ER and the Golgi apparatus.

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