Changes of the physical properties of the liquid-ordered phase with temperature in binary mixtures of DPPC with cholesterol

A ^{2}H-NMR, FT-IR, DSC, and neutron scattering study

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ABSTRACT The structure of the so-called liquid-ordered (lo) phase of binary mixtures of DPPC-d_{62} with cholesterol was studied between 20–50 mol% cholesterol using ^{2}H-NMR, FT-IR, DSC, and neutron specular reflection. Different model systems such as multilamellar vesicles, spherical supported vesicles, and oriented multilayers were used. We observed significant changes of the lo phase structure in the physiological relevant temperature region between 30–45°C. ^{2}H-NMR in combination with lineshape simulations provides evidence for a drastic effect of cholesterol on the shape of multilamellar vesicles due to magnetic field orientation. Moreover, the data indicates a significant change of the extent of this partial orientation for DPPC-d_{62} multilamellar vesicles containing 25 mol% cholesterol between 36–42°C. The semiaxes ratio of the (due to magnetic field orientation) ellipsoidal multilamellar vesicles changes over this temperature range by ≈25%. ^{2}H-NMR and FT-IR measurements indicate changes of the average orientational order at the bilayer center in the same temperature range and a significant increase of the number of end-gauche conformers while the majority of the methylene groups remain in an all-*trans* conformation. Additionally, specular reflection of neutrons shows a concomitant reduction of the bilayer thickness by 4 Å. Based on a model of the arrangement of DPPC and cholesterol in the lo phase, a molecular mechanism is proposed in which increasing the temperature between 30 and 45°C has the effect of driving cholesterol from the bilayer center towards the head group region.

INTRODUCTION

The effect of cholesterol, one of the most important lipid components of eucariotic plasma membranes, on phosphatidylcholine bilayers has been studied in the recent years by a variety of methods (for an overview, see, e.g., the work of Vist and Davis (37) and the references cited therein). In particular, the application of powerful spectroscopic methods such as ^{2}H-NMR, spin-label studies, FT-IR, neutron scattering, and DSC, as well as the compilation of the experimental results using theoretical models has drastically improved our knowledge about cholesterol/phosphatidylcholine interactions.

One of the most intriguing features of binary mixtures of cholesterol with dipalmitoylphosphatidylcholine (DPPC) is the formation of a presumably homogeneous phase at cholesterol concentrations above 20 mol%, designated as β-phase (11, 37) or liquid-ordered (lo) phase (15). This phase exhibits a combination of liquid-crystalline and gel phase features such as: an axially symmetric motion of DPPC that is fast on the ^{2}H-NMR timescale (27, 37) even at temperatures well below the $L_{\beta'} - L_\alpha$ transition of pure DPPC; a lateral diffusion coefficient of DPPC that is comparable to that of the fluid phase (1, 19, 20, 25), but a high orientational order (17, 37); and an area compressibility modulus (21) close to values obtained for the gel phase of the pure lipid. Although theoretical models of the lo phase structure exist that can reproduce the experimentally determined phase boundaries between the lo phase and the low cholesterol concentration region (15, 16, 28), experimental data about the structure of the lo phase are scarce.

Recently, Sankaram and Thompson (27) proposed a model of the lo phase structure based on a bilayer thickness determination using the ^{2}H-NMR order parameter profile. They suggested a partial interdigitation of the bilayer due to the presence of cholesterol and an increasing thickness of the bilayer with the cholesterol content between 25–50 mol%.

In an effort to improve the knowledge about the lo phase structure, we performed spectroscopic experiments on this phase using a variety of methods, such as ^{2}H-NMR, FT-IR, DSC, and neutron scattering. We were particularly interested in the question, whether the lo phase structure changes with temperature.

A further motivation for this work came from the observation of a significant temperature dependence of the degree of macroscopic magnetic field orientation of the DPPC/cholesterol multilamellar vesicles in the ^{2}H-NMR experiments. Since such an effect can obscure the interpretation of NMR lineshape changes with temperature as well as the results of numerical de-Pake-ing procedures, we performed comparative ^{2}H-NMR experiments using oriented multilayers and spherical sup-

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ported vesicles that were not undergoing partial orientation.

MATERIALS AND METHODS

1,2-perdeuterio-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC-d_{62}) was obtained from Avanti Polar Lipids Inc. (Alabaster, Alabama), DPPC-d_6 (both terminal methyl groups deuterated) was from Serdary Research Inc. (London, Ontario, Canada), and cholesterol was from Fluka (Buchs, Switzerland).

For all multilamellar vesicle preparations, DPPC-d_{62} and cholesterol were weighed at the corresponding proportion into glass test tubes, dissolved in chloroform (2 mg/ml), dried under a stream of nitrogen, followed by overnight vacuum desiccation. The lipid film was then redispersed in millipore water at a concentration of 20 mg/ml and incubated at 50°C for 30 min under gentle vortexing, For ^{2}H-NMR experiments, deuterium depleted water (Sigma Chemical Co., St. Louis, MO) was used.

For the preparation of oriented multilayers the multilamellar vesicles were sonicated using a Branson Tip sonifier (15 min at 30 W, pulsed mode with 50% duty cycle). The resulting small unilamellar vesicle solution was pipetted onto silicon wafer pieces. These pieces were cut from a 150 μm thick doubleside polished undoped silicon wafer (Virginia Semiconductor Inc., Virginia) so that a sandwich of 10 pieces would readily fit into a 10-mm plastic sample tube. The vesicle solution on the wafer pieces was dried under 80% humidity for 2–3 d and after that stacked to a sandwhich and transferred to the NMR sample tube (high pressure plastic caps from Wilmad Glass Co. Inc., Buena, NJ). The open tube was incubated in an atmosphere of 80% humidity (deuterium depleted water) for 24 h at a temperature of 80°C. The total DPPC-d_{62} concentration in the sample tube was 15 mg. A detailed description of the preparation procedure is given elsewhere (24). Spherical supported vesicles were prepared by condensation of small, unilamellar vesicles on silica beads of (640 ± 45) nm diameter according to previously published procedures (4). The silica beads were kindly provided as a gift by Dr. Müller from the Degussa Research Laboratories (Hanau-Wolfgang, FRG). These beads are prefectly spherical as judged by electron microscopy. The surface roughness of these beads is below 4 Å over an area of 10 nm^2 as measured by atomic force microscopy.

Deuterium NMR experiments were performed at 61 MHz using a Varian VXR-400 spectrometer (Palo Alto, CA) equipped with a high power probe. All spectra were obtained using the quadrupolar echo technique with a pulse separation of 20 μs and two 90° pulses of 6 μs duration. The repetition time for successive pulse sequences was 200 ms and 8K data points were collected with a dwell time of 1 μs. If not stated otherwise, 1,000 scans were accumulated for the multilamellar vesicles and oriented multilayers. For spherical supported vesicles 5,000 scans were acquired. All experiments were done on resonance with an 8 cyclops pulse cycling sequence (26) and no phase corrections were performed. The temperature of the sample was controlled by a Varian temperature control unit and was constant within ±1°C.

T_2^{qe} measurements were performed by increasing the separation time t between the $\pi/2$ pulses in 10 steps up to 500 μs. At each separation t, 1,000 scans were accumulated. Semilogarithmic plots of the peak echo intensity versus $2t$ yielded linear dependences of slope $(T_2^{qe})^{-1}$ for both multilamellar vesicles and oriented multilayers.

The ^{2}H-NMR lineshapes for ellipsoidal vesicle shapes were obtained by Fourier transform of the simulated free induction decay calculated according to standard procedures discussed in Spiess (35) and in Saunders and Johnson (34) using a Fortran program running on a MicroVax II workstation (Digital, Maynard, MA).

The free induction decay was calculated according to

$$\mathrm{FID}(t) = \sum_{\theta=0}^{90} P(\theta) \exp(i\omega_\theta t) \exp\left(-\frac{t}{T_2(\theta)}\right). \quad (1)$$

A probability function $P(\theta)$ derived assuming an ellipsoidal distribution of director axes orientations (39).

$$P(\theta) = a \tan\theta \sec^2\theta\,(\tan^2\theta + a^4)^{1/2}/(a^2 + \tan^2\theta)^2, \quad (2)$$

was used, where a is the semimajor axis of the ellipse (the simiminor axis b is defined to be unity) and θ is the angle between the molecular director axis and the magnetic field applied for the spectra of ellipsoidal MLVs. Lorentzian line broadening and the experimentally determined values of $T_2^* = T_2(\theta)$ at the corresponding temperature, assumed to be orientationally independent, were used ($T_2^* = 350$ μs for all methylene deuterons and 1.2 ms for the methyl deuterons). The required values of $\Delta\nu_Q$ along the perdeuterated chain of DPPC-d_{62} were obtained directly from the oriented multibilayer sample spectrum at the corresponding temperature. To fit the experimental spectra of DPPC-d_{62}/cholesterol, the length of the semimajor axis a was varied as a parameter and the best fit was determined.

High sensitivity DSC measurements were performed with a MC-2 microcalorimeter (Microcal, Amherst, MA) at a scan rate of 40°C/h and a sample concentration of 2 mg/ml as reported elsewhere (2).

Fourier transform infrared spectroscopy (FT-IR) experiments were done in transmission mode using a Nicolet 60 SXR FT-IR spectrometer (Madison, WI) equipped with a liquid nitrogen cooled MCT detector. The DPPC-d_{62} was replaced by DPPC-d_6 for these measurements. The MLV sample of DPPC-d_6/cholesterol was kept between two CaF_2 windows separated by a 12 μm Zn-spacer in a cylindrical water bath thermostated jacket. The temperature was measured at the sample cell using a Pt 100 thermocouple that controlled the external water bath and was constant within ±0.2°C. The measurements were performed from lower to higher temperatures for each sample, at each temperature 1,000 scans were accumulated at a resolution of 2 cm^{-1} between 4,000–400 cm^{-1}. The temperature setting was varied and controlled by a homemade Macro program from the acquisition computer, the time for equilibration between each temperature step was 20 m. The background and reference (water) spectra were recorded separately at the same temperatures and the latter was interactively subtracted from the sample spectra. The frequency of the methylene and methyl stretching vibrations was determined as the maximum of the corresponding bands.

Neutron specular reflection measurements were performed using the TOREMA neutron reflectometer (32) at the Forschungszentrum Jülich (FRG). The sample was deposited as a single bilayer onto the large face of a 100 × 50 × 10 mm silica block (Westdeutsche Quarzschmelze, Garching-Hochbrück, FRG) in a thermostated sample cell. All preparation procedures, data acquisition and analysis, as well as the geometry of the sample cell are described in detail elsewhere (18). The specular reflection experiments were done at a neutron wavelength of 4.3 Å, and by variation of the grazing angle we reached a momentum transfer normal to the surface up to 0.7 Å^{-1}. Two cases of scattering contrast were considered. First the cell was filled with D_2O as bulk water, and in a second series of experiments water contrast matched to the silica block was applied. The reflectivity profiles from both series of experiments were fitted to a single box model using the optical matrix method (18, 23). The temperature was

controlled by an external water bath thermostat and was constant within ±1°C.

RESULTS

The ^{2}H-NMR spectra of a binary mixture of DPPC-d_{62} with mol% cholesterol for two different sample geometries (multilamellar vesicles and oriented multilayers) and at three temperatures (MLV only) are shown in Fig. 1. Compared with spectra obtained under similar conditions for pure DPPC-d_{62} MLVs (not shown), those in Fig. 1 exhibit the following interesting features. (*a*) The sharp Pake doublet arising from the terminal methyl groups (the one with the smallest quadrupolar splitting) is split into two singals that differ in their quadrupolar splittings $\Delta(\Delta\nu_Q)$ by 1.2 kHz in the temperature range between 20–42°C (Fig. 1 *B*). (*b*) The MLV spectra A and B show a significant degree of macroscopic orientation in the magnetic field as can be seen by their low intensity and shape of the shoulder region (corresponding to a parallel orientation of the molecular symmetry axis to the external field H_0). This macroscopic magnetic field orientation is most pronounced in spectrum A (i.e., at elevated temperature). For pure DPPC-d_{62} MLVs no such change of the lineshape can be observed for spectra obtained at 40 and 55°C, respectively. (*c*) Although the main phase transition temperature T_m of pure DPPC-d_{62} is 37.5°C, all spectra in Fig. 1 exhibit the lineshape characteristic for a liquid crystalline phase state of the lipids. (*d*) Finally, there is the well established increase of the average quadrupolar splittings (i.e., the first moment M_1) due to the presence of cholesterol (37).

The existence of two sharp Pake doublets of the terminal methyl resonance in DPPC-d_{62}/cholesterol mixtures has been reported previously by other authors (27, 37) but its temperature dependence was not considered. According to Sankaram and Thompson, the doublet results from a nonequivalence of the two deuterated terminal methyl groups because it cannot be observed when using DPPC-d_{31} which is chain deuterated in the sn-2 chain only. Using these lipids, the doublet signal with the larger quadrupolar splitting can be assigned to the sn-2 chain (Sankaram and Thompson, personal communication). The fact that the doublet can be observed only in the temperature region between 15–42°C indicates that membrane order in the bilayer centre undergoes a change with temperature in the liquid ordered (lo) phase.

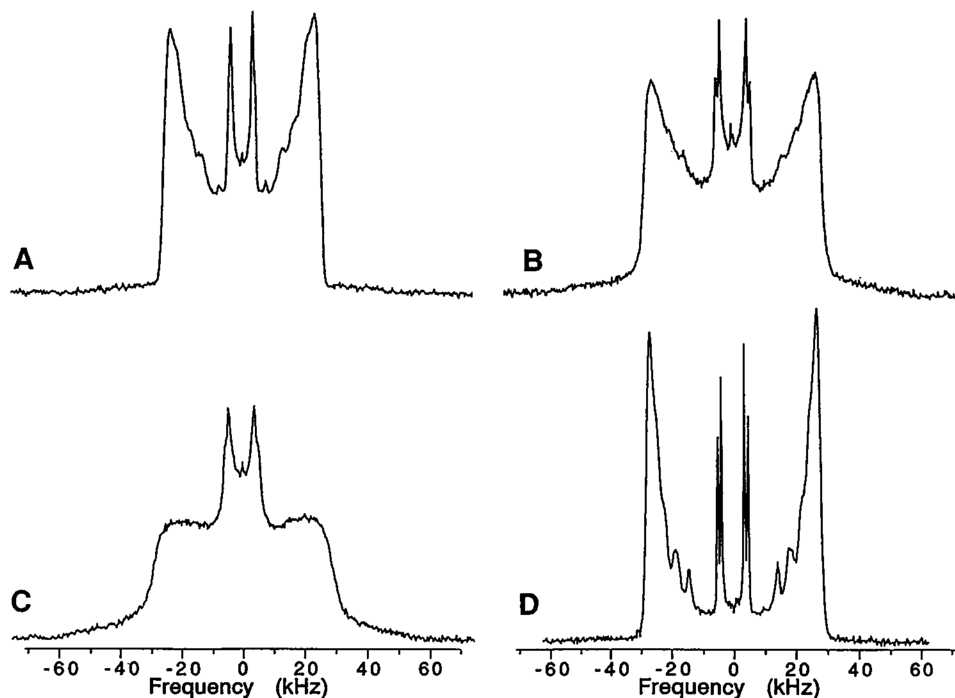

FIGURE 1 ^{2}H-NMR spectra of a binary mixture of DPPC-d_{62} with 25 mol% cholesterol at three temperatures. (*A*) T = 45°C; (*B*) T = 30°C; (*C*) T = 18°C. The spectra *A–C* were measured from multilamellar vesicles. For comparison, spectrum D was obtained from an oriented multilayer sample at the same temperature as spectrum B (T = 30°C).

Temperature dependence of the ^{2}H-NMR spectra and of their spectral moments

The temperature dependence of the quadrupolar splitting difference $\Delta(\Delta\nu_Q)$ between the two terminal methyl Pake doublets as well as of their absolute frequencies is shown in Fig. 2 *A*. At low temperatures (below 20°C) only one broad Pake doublet due to the terminal methyl groups can be observed and a splitting cannot be resolved. The lineshape of the spectrum is clearly axial symmetric but broadened. Obviously, the time scale of the molecular reorientations is near the intermediate regime (of order 1/(spectral width)) where the lineshape becomes sensitive to the details of the motion. However, at a temperature between 20°C (denoted T^a) and 24°C (denoted T^b) the reorientations become fast enough to exhibit a highly resolved axial symmetric spectrum where the Pake doublet due to the methyl groups shows a splitting of $\Delta(\Delta\nu_Q)$ = 1.2 kHz. Further increase of temperature does not change the value of $\Delta(\Delta\nu_Q)$ nor the lineshape up to a temperature of 36°C (denoted T^c), then the value of $\Delta(\Delta\nu_Q)$ continuously decreases and at 42°C (denoted T^d) the two signals coalesce into one Pake doublet ($\Delta(\Delta\nu_Q) = 0$). This process is completely reversible and no hysteresis can be observed.

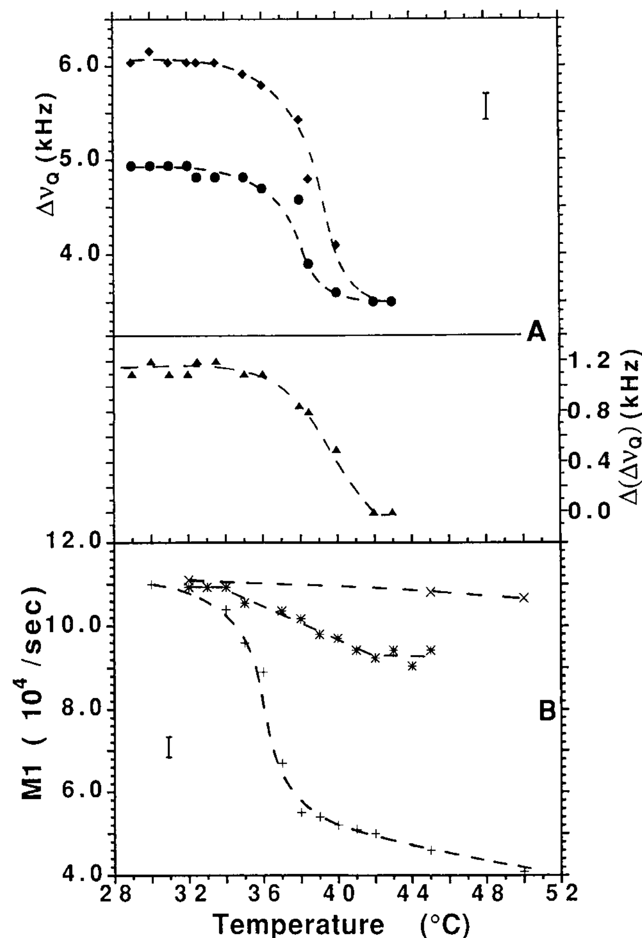

FIGURE 2 ^{2}H-NMR spectral data of a binary mixture of DPPC-d_{62}/cholesterol (25 mol%). (*A*) Temperature dependence of the frequency of the sn-1 (●) and sn-2 (◆) terminal methyl doublet signal and of the difference quadrupolar splittings $\Delta(\Delta\nu_Q)$ (△). (*B*) Temperature dependence of the first moment M_1 obtained for multilamellar vesicles (*) and for oriented multilayers with the bilayer normal perpendicular to the magnetic field (X) of a binary mixture of DPPC-d_{62} with 25 mol% cholesterol. For comparison the variation of M_1 with temperature, obtained for an MLV sample of pure DPPC-d_{62} is shown (+). The broken lines are drawn to guide the eye.

The variation of $\Delta(\Delta\nu_Q)$ with temperature (Fig. 2 *A*) provides evidence that subtle changes of the bilayer structure occur in the lo phase between $T^c - T^d$, leading to a nonequivalence between the sn-1 and sn-2 methyl group of DPPC-d_{62} below T^c. This nonequivalence can be attributed either to different degrees of motional averaging of the two chains or to different average orientations of the terminal C—C bonds of the sn-1 and sn-2 chain, respectively (37).

We studied this nonequivalence between T^a and T^d in the cholesterol concentration range 23–50 mol% and found a drastic dependence of $T^a - T^d$ on the concentration (Table 1). While T^a, T^b, and T^c decrease with increasing cholesterol concentration, T^d increases up to 30 mol%. At 50 mol% cholesterol, we obtained the same value for T^d (50 ± 2°C) as for 30 mol% cholesterol, suggesting that T^d is constant over the 30–50 mol% concentration range. This significant broadening of the transition region between T^c and T^d was also found using FT-IR (see below). However, the intensity ratio of the two split methyl Pake doublets is independent on the cholesterol concentration, in agreement with results of Vist and Davis (37). This indicates that the splitting does not result from two different lipid environments such as a lateral phase separation. In the following, we have

TABLE 1 Dependence of the temperatures T^a, T^b, T^c, and T^d on the cholesterol concentration

Cholesterol concentration (mol%)	T^a	T^b	T^c	T^d
23	24	27	37	40
25	20	24	36	42
30	10 ± 2	14 ± 2	29	50 ± 2
50	—	—	25	50 ± 2

All temperatures are in °C. If not indicated otherwise, the error of the temperature values is ±1°C. The error of the cholesterol concentration is ±1 mol%.

focussed our investigations mainly on the structural changes in the temperature range between T^c and T^d.

A well established tool of ^{2}H-NMR for the detection of changes of membrane order (e.g., phase transitions in membranes) is the method of moments (10). While the first moment M_1 corresponds to the average quadrupolar splitting, the second moment M_2 is very sensitive to intensity changes at the wings of the spectra. In Fig. 2 *B*, the first moments are plotted over the temperature region between T^c and T^d for MLVs and for oriented multilayers. For comparison, the change of M_1 obtained for a MLV sample of pure DPPC-d_{62} is shown. While the moments obtained for pure DPPC-d_{62} exhibit a sharp decrease with increasing temperature upon exceeding its phase transition temperature of 37.5°C (10), those obtained in the presence of 25 mol% cholesterol are found to be dependent on the geometry of the sample: the MLV sample shows a continuous reduction of M_1 with increasing temperature between T^c and T^d, but the oriented multilayer does not exhibit any particular changes of M_1. Similar behavior can be observed for the second moment M_2. This example shows the necessity to consider different sample geometries in order to obtain reliable results. As demonstrated below, the differing temperature dependences obtained for the two types of samples containing cholesterol (MLV and oriented multilayer) can be explained by a macroscopic magnetic field orientation of the MLV sample. Therefore, the only reliable measure of the changes of the moments with temperature at high magnetic fields is obtained from the oriented multilayers. These spectral moments of the lo phase are comparable to those of pure DPPC-d_{62} in the gel phase state, indicating that the chains are nearly in their all-*trans* conformation. However, a significant change of these moments between T^c and T^d was not observed.

Temperature dependence of ^{2}H-NMR relaxation times

In order to obtain information about changes of the membrane dynamics due to the structural changes between T^c and T^d, the temperature dependence of the transversal relaxation time T_2^{qe} was measured. The value of T_2^{qe} is sensitive to ultraslow motions such as lateral diffusion of phospholipids (6). We measured a significant increase of the value of T_2^{qe} between T^c and T^d by 160 μs (from (350 ± 20) μs at T^c to (510 ± 20) μs at T^d) for the MLV sample in comparison to an increase of (70 ± 20) μs obtained for the oriented multilayer sample (bilayer normal perpendicular to H_0). This result stresses again the importance of the sample geometry. We interpret the changes of T_2^{qe} obtained for MLVs as caused mainly by lateral diffusion (6). As demonstrated below, the MLVs undergo a significant increase of their macroscopic magnetic field orientation between T^c and T^d. The resulting highly eccentric ellipsoidal shape of the MLV with its long axis parallel to H_0 diminishes the number of molecules in areas where the phospholipids can rapidly change its orientation to H_0 by lateral diffusion on the surface of the ellipse. Thus, the more eccentric the ellipse the lower the contribution of lateral diffusion to T_2^{qe}, i.e., the average must increase.

Thermodynamical behavior

In order to determine whether thermodynamical changes in the membrane can be attributed to the observed spectral behavior between T^a and T^d, we performed DSC and FT-IR experiments. Both methods did not show any significant changes in the $T^a - T^b$ temperature region. However, between T^c and T^d an endothermic feature can be observed by DSC and the FT-IR indicates drastic changes at the terminal methyl groups. Some results of the experiments performed in the $T^c - T^d$ temperature range are shown in Fig. 3. The DSC thermograms exhibit at 23, 25 and 30 mol% cholesterol broad endothermic features (as the one shown in Fig. 3 *A*) whose onset temperature and completion temperature agree within ±2°C with the values of T^c and T^d as given in Table 1. No other signals outside this region can be observed between 5–65°C.

By FT-IR measurements, the temperature dependence of the frequencies of the methylene stretching mode and of the asymmetric methyl stretching mode of DPPC/cholesterol between T^c and T^d were obtained. For a MLV sample of pure DPPC, a plot of these wavenumbers versus temperature shows the well known sigmoid shape where the steepest slope can be attributed to the main phase transition temperature of the lipid (8). The drastic increase of the number of gauche conformers at the phase transition causes a positive frequency shift of the stretching modes by ≈ 3.5 cm^{-1}.

Since the ^{2}H-NMR measurements indicate a specific motional restriction of the terminal methyl group, we were particularly interested in distinguishing the IR bands of this group from that of the methylenes. This was achieved by using DPPC-d_6, where both terminal methyl groups are deuterated. The deuteration bypasses the problem of band overlap between the two vibrations, thus permitting the separate recording of the temperature dependence of the frequencies of both groups without interference. For pure DPPC-d_6, a sharp phase transition at 39.5°C was measured using DSC (data not shown). This is 2°C higher than that measured for pure DPPC-d_{62} (37.5°C) but still 1.8°C lower than the transi-

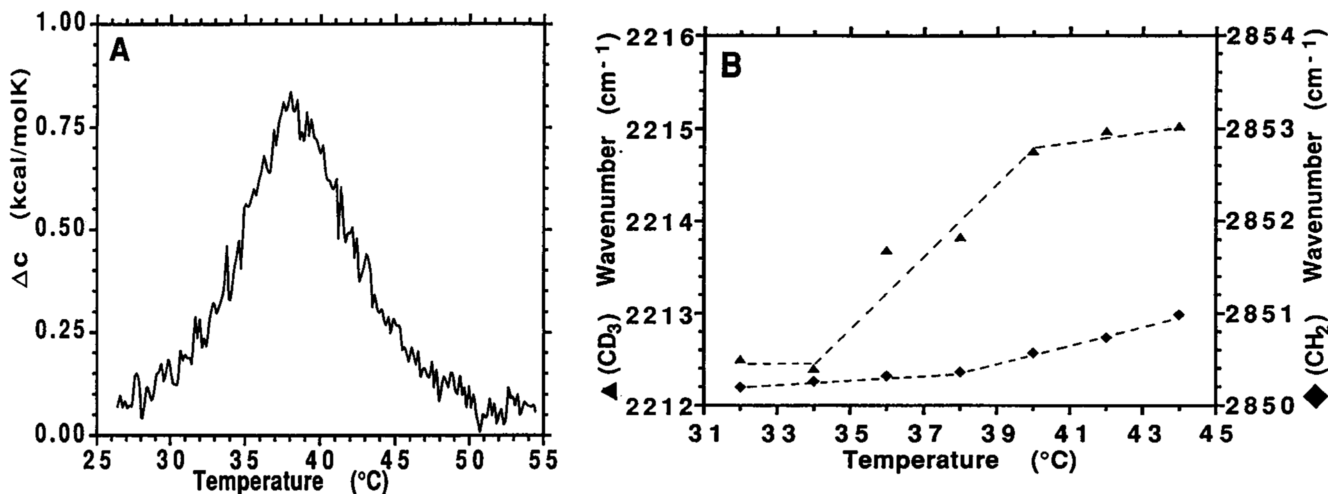

FIGURE 3 (*A*) DSC endotherm obtained for multilamellar vesicles of DPPC-d_{62}/cholesterol (30 mol%) (*B*) Temperature dependence of the frequency of the CD_3 asymmetric stretching vibration (Δ) and of the CH_2 symmetric stretching vibration (◆) of multilamellar vesicles of DPPC-d_6 with 25 mol% cholesterol. The broken lines are drawn to guide the eye.

tion temperature obtained for pure protonated DPPC (41.3°C).

The FT-IR data obtained for DPPC-d_6 with 25 mol% cholesterol (Fig. 3 *B*) reveal a strikingly different temperature dependence of the frequencies of the methyl and methylene groups, respectively. While the asymmetric methyl vibrations exhibit an increase of 3 cm^{-1} between T^c and T^d, the symmetric methylenes change over this temperature range by less than 0.5 cm^{-1}. The absolute frequency of the latter at T^d (2851.0 ± 0.2 cm^{-1}) indicates that the vast majority of the methylene groups of DPPC-d_6 is still in an all-*trans* conformation, in agreement with the ^{2}H-NMR results. In contrast, the methyl groups exhibit a higher vibrational frequency in the presence of cholesterol at T^d (corresponding to a high amount of end-gauche conformers) than in a pure DPPC-d_6 sample above its phase transition temperature. This high amount of end-gauche conformers at T^d does not agree with data presented previously by other authors (36), where an increase of the asymmetric methyl vibration frequency of less than 1 cm^{-1} was observed for protonated DPPC with cholesterol. A likely explanation for this discrepancy is an obstruction of their vibration frequency determination due to heavy band overlap between the asymmetric methyl and the symmetric methylene vibration, respectively.

Our FT-IR results indicate a local melting of the acyl chains (i.e., an increase of the number of end-gauche conformers) at the terminal methyl groups while the majority of the methylene groups remain in an all-*trans* conformation.

The behavior in the $T^c - T^d$ temperature region was studied by both methods in the 23–50 mol% cholesterol concentration range. We found a similar variation of both temperatures with the cholesterol concentration as reported above by ^{2}H-NMR. However, DSC measurements were performed up to a concentration of 35 mol% only, because at higher concentrations the determination of T^c and T^d is severely restricted due to the broadness and the low intensity of the endotherm.

Magnetic field orientation

The ^{2}H-NMR spectra of DPPC-d_{62} obtained from MLV (Fig. 1) exhibit lineshapes characteristic for macroscopic magnetic field orientation (13, 31). The negative diamagnetic susceptibility anisotropy $\Delta\chi_{eff}$ of the fatty acyl chains causes a preferential orientation of the molecular symmetry axis perpendicular to the external magnetic field H_0. This is balanced by the elastic properties of the membrane, i.e.,. its bending stiffness and by the volume of the water trapped inside the vesicles. As a consequence, at high magnetic fields the MLV undergo a transformation from their originally spherical shape into a prolate ellipsoidal one with the long axis parallel to H_0 (cf. the paper of Brumm et al., companion paper).

Since ^{2}H-NMR spectra of pure DPPC-d_{62} obtained at temperatures above the main phase transition temperature (data not shown) exhibit a significantly lower extent of magnetic field orientation, the presence of cholesterol must either cause a drastic reduction of the membrane bending stiffness or provide a considerable contribution to the negative value of $\Delta\chi_{eff}$.

A salient feature of the ^{2}H-NMR spectra of MLV in

Fig. 1 is a significant increase of magnetic field orientation in the $T^c - T^d$ temperature range. In order to obtain a quantitative measure of these shape changes, we calculated ^{2}H-NMR spectra assuming ellipsoidal distributions of quadrupolar frequencies and fitted their axis ratio $r_{ex} = a/b$ (a denotes the long axis parallel to H_0) to the experimental spectra obtained at T^c and T^d, respectively. The result for a spectrum at a temperature T^c is shown in Fig. 4. For this spectrum, a value of $r_{ex} = 2.0$ gave the best fit between the theoretical and the experimental lineshape. The remaining discrepancy between theoretical and experimental spectra is mainly due to the neglect of the orientational dependence of T_2 and to the errors that arise from the use of the order parameter profile of the oriented multibilayer (which has a hydration of ≈20 wt%) in the simulation of the MLV spectra. We refer to the companion paper of Brumm et al., where ^{31}P-NMR lineshape calculations were performed for the same system and experimental conditions, but including the orientational dependence of T_2. Nevertheless, the good agreement between experimental and calculated spectra indicates that the MLV spectra of DPPC-d_{62} with cholesterol are indeed drastically affected by macroscopic magnetic field orientation and that the assumption of an ellipsoidal shape of the MLV is justified.

The obtained values for r_{ex} are 2.0 at T^c and 2.4 at T^d, i.e., with increasing temperature there is a significant shape change of the MLV towards a higher eccentricity of a prolate ellipsoidal shape. A comparison with the value of $r_{ex} = 1.4$ obtained for a sample of pure DPPC-d_{62} at T^d shows the drastic effect of cholesterol on the MLV shape in a magnetic field strength of 9.4 Tesla.

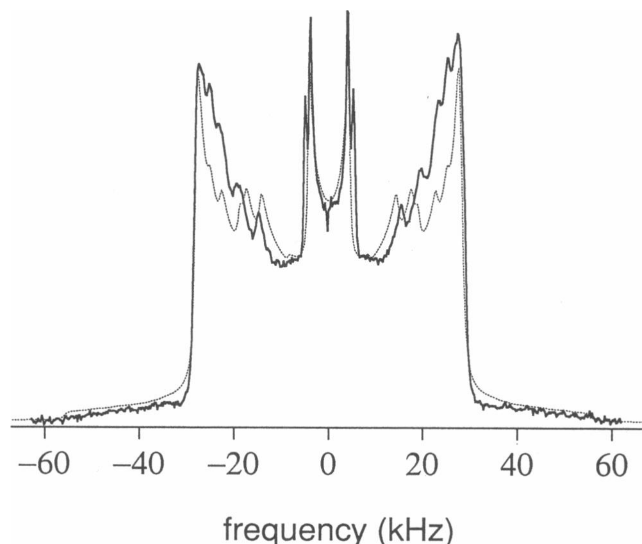

FIGURE 4 ^{2}H-NMR spectra of DPPC-d_{62} with 25 mol% cholesterol at 35°C. MLVs and the calculated spectrum (*dotted line*) assuming an ellipsoidal distribution of director axes.

A method to prevent the macroscopic field orientation effects that can obscure spectral moments and relaxation times has recently been devised by us (3, 4) with the introduction of so-called spherical supported vesicles (SSV). These SSVs consist of a single bilayer supported by a spherical glass bead of well defined size, where the bilayer is separated from the latter by an ultrathin water layer. Such SSVs give powder lineshapes corresponding to a spherical distribution of director axes, independently of temperature and magnetic field strength (cf. the work of Brumm et al., companion paper in this issue). An advantage of the SSVs over the oriented multibilayers used in our work (Fig. 1 *D*) is that the former can be used in excess water.

Bilayer thickness

The ^{2}H-NMR spectral moments of chain deuterated lipids are sensitive to changes in the bilayer thickness (29). Therefore, a satisfactory interpretation of the observed spectral changes between T^c and T^d will rely on the knowledge about changes of the average bilayer thickness over this temperature region.

The increase of the average bilayer thickness d_b due to the presence of cholesterol in a DPPC-d_{62}/cholesterol (25 mol%) mixture was estimated recently to be ≈4 Å from the segmental order parameter profile using a model for the arrangement of cholesterol and lipid molecules in the bilayer, assumed to be unique at this concentration (27).

Recently, we introduced the method of specular reflection of neutrons in combination with the variation of the scattering contrast as a sensitive tool for determining the thickness of a single bilayer (18). Using this method, we measured the total thickness d_b and the scattering length density n_b of a single DPPC/cholesterol (25 mol%) bilayer on a planar support (silica plate) at two temperatures (below T^c and above T^d). The bilayer thicknesses we obtained from fitting the reflectivity profiles to a single layer model (cf. the experimental section for details) are $d_b = (50.5 \pm 1)$ Å at 30°C and $d_b = (46 \pm 1)$ Å at 45°C. The scattering length density n_b of the single bilayer increases by (8 ± 3)% with increasing temperature in the range 30–45°C. Since this experiment was performed under conditions where the reflectivity is solely determined by the single bilayer (the water and the planar support were matched in scattering contrast), the observed changes indicate a significant alteration of the internal structure of the DPPC/cholesterol bilayer between T^c and T^d. The most likely reason for the concomitant change of d_b and n_b is a loss

of segmental order in the bilayer and an increase of the volume per molecule, i.e., the creation of excess volume in the temperature range between T^c and T^d.

Recently, we determined the thickness of the fully hydrated phosphatidylcholine head group to be d_h = (8 ± 1.5) Å by the same method (18). Calculating the thickness of the tail region per monolayer d_t by $d_t = (d_b - 2d_h)/2$ yields d_t = (17.3 ± 1.5) Å at 30°C (below T^c) and d_t = (15 ± 1.5) Å at 45°C (above T^d). This provides evidence that the acyl chains are even below T^c not in a complete all-*trans* conformational state because the theoretical length of an all-*trans* palmitoyl chain is 19.15 Å (33). The 2 Å difference between the theoretical and experimental value can account for an average of 1.5 gauche conformers per acyl chain below T^c (at 30°C). The observed reduction of d_t with increasing temperature is in agreement with the predictions from a microscopic interaction model of a fully hydrated DPPC bilayer containing cholesterol which was recently proposed by Ipsen, Mouritsen, and Bloom (17).

It is interesting to compare our bilayer thickness data with those obtained by Sankaram and Thompson (27) from ^{2}H-NMR order parameter profile measurements. These authors obtained a value of 44.9 Å for the total bilayer thickness at 52°C and at the same cholesterol content, and a value of 15.3 Å for the effective chain length from the carbonyl to the terminal methyl carbon. These values are in excellent agreement with those of d_b and d_t, that we obtained at 45°C using the specular reflection technique. However, this requires that there is no further reduction of the bilayer thickness between 45–52°C.

DISCUSSION

Phase diagram

Vist and Davis (37) proposed a phase diagram based upon DSC and ^{2}H-NMR data that has been successfully reproduced by the theoretical calculations of Ipsen (16) and Scott (28) using a lattice model for the arrangement of cholesterol and DPPC in the membrane without invoking mechanisms such as lateral phase separation. According to this phase diagram, all cholesterol concentrations studied in this work give the so-called β- or liquid ordered (lo)-phase. The data obtained by various methods in this study provide evidence that subtle structural changes occur in this phase which so far has been considered as a homogeneous phase. The most significant changes occur in the $T^c - T^d$ temperature range and can be summed up as follows.

(*a*) The drastic increase of the end-gauche conformers of DPPC-d_{62} indicates a local melting of the chains near the bilayer center, while the majority of the methylene groups remain in an all-*trans* state.

(*b*) The nonequivalence of the two methyl resonances of DPPC-d_{62} observed below T^c gradually diminishes with increasing temperature and at T^d the two methyls are equivalent as regards their average orientation or degree of motional averaging.

(*c*) The MLVs become increasingly prone to macroscopic magnetic field orientation. This causes the ellipsoidal shape of the MLV at T^c (axis ratio r_{ex} = 2.0) to become even more eccentric (r_{ex} = 2.4) at T^d.

(*d*) A significant reduction of the bilayer thickness by 4 Å with increasing temperature.

In order to understand the above changes of some physical properties of the bilayer with temperature it will be useful to compare our data with the established phase boundaries of the cholesterol/DPPC-d_{62} phase diagram. The values we obtained for T^d at various cholesterol concentrations using ^{2}H-NMR, DSC, and FT-IR (cf. Table 1) meet exactly the phase boundary between the lo phase and the fluid coexistence region (ld-lo phase). This is not surprising for this boundary has been determined in the work of Vist and Davis (37) from a "sharpening of the resonances." Presumably, the high degree of macroscopic magnetic field orientation at T^d contributes to this sharpening. The change of field orientation between T^c and T^d was not observed in the previous ^{2}H-NMR work (27, 37) because all these experiments were performed at a 25% lower field strength and the orientation effect is proportional to the square of the applied field. The boundary which can be described with our T^d values extends further towards higher temperatures with increasing cholesterol concentrations, while in the phase diagram it has not been established above 25 mol% cholesterol. However, at 30 and 50 mol% cholesterol, respectively, we find a similar value of T^d = 50 ± 2°C. Provided that our T^d values are representative for the lo – (ld-lo) phase boundary, this would suggest a deflection of this boundary into a horizontal line above 30 mol% cholesterol. This is in contradiction to the predictions based upon theoretical calculations by Ipsen (15), where the existence of an upper critical point terminating the ld-lo coexistence is suggested. Recently, based upon spin label data, Sankaram and Thompson (27) suggested the existence of an upper critical point for this mixture at a concentration of 40 mol% cholesterol and a temperature of 70°C. We have no conclusive explanation for this discrepancy with our data, but suspect that the several orders of magnitude difference in the characteristic timescales of the two resonance methods make a comparison ambiguous.

Structural changes in the lo phase

The structural changes in the lo phase can be interpreted in terms of a time averaged model of the arrangement of DPPC and cholesterol which requires the following assumptions.

(*a*) No microscopic lateral phase separation occurs in the cholesterol concentration range of 25–50 mol%. The lipid-cholesterol interaction can be described by a lattice model with sites occupied either by cholesterol or DPPC, as recently proposed (15, 16). (*b*) Cholesterol causes a straightening of disordered acyl chains of DPPC-d_{62} in the liquid crystalline phase to an all-*trans* conformation. As a result, the sn-1 chain extends $\approx (2 \pm 1)$ Å further into the bilayer in the lo phase (30). (*c*) Cholesterol can bind from its hydroxyl group to the DPPC molecule via a hydrogen bond either to the sn-2 carbonyl (14, 38) or to the phosphate moiety (27).

In the temperature range $T^b - T^c$, both methyls and methylenes of the DPPC-d_{62} acyl chains are essentially in an all-*trans* conformation according to the FT-IR results, the ^{2}H-NMR spectral moments, and the neutron specular reflection data. Under these conditions, cholesterol can be expected to be in close contact with the acyl chains (15) while retaining a fast rotational diffusion about its long axis (7). Together with the axially symmetric motion of the DPPC-d_{62} in the lo phase and its fast lateral diffusion (20), this arrangement should give on average an equal occupation of the interaction sites with the sn-1 and sn-2 chain. Moreover, cholesterol has been suggested to bind via a hydrogen bond to the sn-2 carbonyl (14, 38). In this case, cholesterol would extend into the opposite monolayer by 2–4 Å, considering the value we obtained for the effective chain length from the sn-2 carbonyl to the methyl group of d_t = 17.2 Å and a length of the cholesterol molecule of 20–22 Å (9). Thus, a partial interdigitation between the two monolayers via cholesterol (27) is likely exhibiting a rather high membrane bending stiffness. The higher quadrupolar splitting of the sn-2 terminal methyl is probably caused due to the location of this group ≈ 2 Å off the bilayer center where the order parameter is slightly higher so that this group undergoes a different degree of orientational averaging.

This situation changes in the $T^c - T^d$ temperature region. The local melting of the acyl chains near the bilayer center, while retaining the all-*trans* conformation of the methylene groups off of the bilayer center, will have the following consequences. First, the length mismatch between the two acyl chains is obliterated with increasing temperature so that the two methyl groups become equivalent at T^d. Second, the increased number of end-gauche conformers of DPPC (cf. Fig. 3 *B*) causes packing problems with the rigid cholesterol and reduces its interaction energy with the former by increasing the average intermolecular distance. This energetically unfavorable situation can be relieved by lifting the cholesterol off of the bilayer center towards the headgroup region where the methylene groups remain in an all-*trans* conformation. Cholesterol could accomplish this by changing its hydrogen bond site to DPPC from the sn-2 carbonyl to the phosphate moiety. This bond can compensate for part of the increased hydrophobic free energy cholesterol acquires due to its partial exposure to the interfacial region. As the distance between the carbonyl and the phosphate group projected to the DPPC long axis is 6.25 Å (22), this change of the hydrogen bond site would sufficiently withdraw cholesterol from the bilayer center to enable both monolayers to come into closer contact with their disordered terminal chain parts, thereby reducing the total bilayer thickness by 4 Å as measured by neutron specular reflection. The value of d_t = (15 ± 1.5) Å obtained at 45°C indicates that the average number of gauche conformers per chain is about twice as high above T^d as compared with temperatures below T^c, where an average of 1.5 gauche conformers are present.

The assumption of a withdrawal of cholesterol from the bilayer center between T^c and T^d can also explain the observed changes of the macroscopic magnetic field orientation over this temperature range. There are two obvious features that prevent a complete orientation in the magnetic field: the membrane bending stiffness and the volume of the water trapped inside the multilamellar vesicles. The bending stiffness of the membrane is certainly reduced due to the lifting of cholesterol because this enables the two monolayers to slide readily along the bilayer midplane in order to accommodate higher curvatures. The withdrawal of cholesterol increases the contrast between the protonated heads and the deuterated tail of the bilayer, thus increasing the scattering length density of the latter. The resulting excess volume (because the volume expansion of the trapped water can be neglected) is well established to cause the formation of nonspherical shapes in unilamellar vesicles (5, 12). However, these shape changes with temperature cannot be observed for MLVs because in the absence of a strong magnetic field a collective orientation of all the single bilayers in an MLV is unlikely due to the interbilayer interaction. Presumably, both processes (change of bending stiffness and excess volume effects) account for the formation of highly eccentric ellipsoidal MLVs above T^d in high magnetic fields. The two structural states of the MLV bilayer according to the proposed model is depicted in Fig. 5.

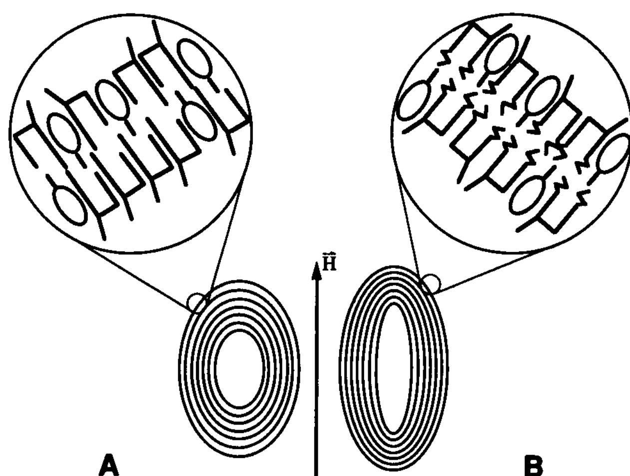

FIGURE 5 Schematic drawing of the effect of temperature on the bilayer structure and shape of multilamellar vesicles containing 23–50 mol% cholesterol. (*A*) $T = T^c$; (*B*) $T = T^d$.

CONCLUSIONS

Using various spectroscopic methods we have demonstrated that changes of shape and bilayer structure of multilamellar vesicles occur in the high cholesterol concentration lo phase over a rather narrow temperature range. These effects can be explained by a decrease of orientational order near the bilayer center and a change of the location of cholesterol inside the bilayer. Since these changes take place in a physiologically relevant temperature range they might be of some relevance for the understanding of the regulatory function of cholesterol in eucariotic cells.

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