The Effect of Cholesterol, Lanosterol, and Ergosterol on Lecithin Bilayer Mechanical Properties at Molecular and Microscopic Dimensions: A Solid-State NMR and Micropipet Study

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Lecithin (DPPC) membranes doped with high concentrations (40 mol %) of three biologically relevant sterols (cholesterol, lanosterol, and ergosterol) were studied by spectroscopic and force measurements. Micropipet aspiration measurements of giant unilamellar vesicles provided the membrane area expansion modulus \( K \) on the microscopic scale while deuterium NMR experiments gave parameters such as the line shape, transverse relaxation, and molecular order, which are all based on a molecular scale at a given time resolution. Micropipet experiments at 10 °C gave \( K \) values for the three samples with ergosterol > cholesterol > lanosterol. From the NMR we obtained the highest CH\(_2\)-segmental molecular order and longest transverse relaxation time for cholesterol and the opposite for ergosterol. The lanosterol NMR parameters were found to be between those of the other two sterols but were closer to those of cholesterol. The extent of deformation of the sterol-doped vesicles in the magnetic field of the NMR magnet was lanosterol > cholesterol > ergosterol. The results suggest that membrane micromechanical properties are manifest in solid-state NMR parameters and suggest that high \( K \) values at the microscopic scale correspond to high molecular order and long transverse relaxation times at the molecular scale. Furthermore, the extent of magnetic field orientation of vesicles measured by NMR correlated well with \( K \) determined by micropipet aspiration. The results may help to appreciate why cholesterol has gained a dominant role over the other sterols in the course of cellular evolution.

Introduction

Cholesterol, lanosterol, and ergosterol are three structurally similar biologically relevant sterols which dominate the sterol fraction in the membranes of eukaryotes (cholesterol), of procaryotes (lanosterol), and of certain fungi or protozoans (ergosterol). From an evolutionary perspective, lanosterol is the precursor of cholesterol and the latter did not occur in nature before the Earth's atmosphere turned aerobic.\(^1\)\(^2\) It was long speculated that the known variations in the molecular structure (Figure 1) of the three sterols may be in part responsible for their distribution in the different species. But the possible relation between sterol structure and function in the cell membranes is still unclear. Several nuclear magnetic resonance (NMR) studies were devoted to the comparison between the sterols in model\(^3\)\(^5\) and natural\(^6\) membranes. They demonstrated that these sterols show significant differences in their effect on membrane order and NMR relaxation times and suggest that different viscoelastic and morphologic membrane properties may arise from each of these sterols. The influence of cholesterol on the mechanical properties of membranes is well documented.

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Figure 1. Molecular structures of cholesterol, lanosterol, and ergosterol.
Cholesterol substantially increases the area expansion modulus and the yield tension of membranes.\(^7\)\(^8\)\(^9\) Furthermore, it increases the bending rigidity. However, it appears that the effects of lanosterol and ergosterol on the mechanical properties of membranes have not been studied before.

In this paper, we combined physical methods on the molecular (deuterium NMR) and the microscopic (micropipet) level for the comparative study of lecithin bilayers doped with high concentrations of the three sterols. The sterol concentration in the bilayer (40 mol %) is in a range where for cholesterol the unique liquid-ordered (lo) phase was observed. Although it is not established yet whether this phase exists also for the other two sterols (ergosterol and lanosterol), a comparison at molecular and microscopic level can provide valuable insight into the mechanisms by which the sterols modulate membrane properties and how molecular properties translate into macroscopic function. Moreover, the combination of the two methods may help to assess how membrane micromechanic properties measured at the microscopic level manifest themselves at the molecular level.

The observables of deuterium solid-state NMR are all dependent on microscopic motion in the sample under study with respect to a certain time scale. The time scale of NMR spans the millisecond–nanosecond range, and different observables are sensitive to one or several correlation times of the molecular reorientation. In our paper we have studied the observables line shape, order parameter, and transverse relaxation for samples having different membrane micromechanic properties. These membrane properties were determined by a method (micropipet aspiration) which is essentially time scale independent and is rather based on microscopic observation of membrane shape changes in response to the applied external force, i.e., the suction pressure of the pipet. It is now interesting to explore how the membrane microelastic properties determined by a static microscopic technique will show in a dynamical experiment on a molecular scale.

Materials and Methods

Materials. \(\alpha\)-Dipalmitoyl-phosphatidylcholine (DPPC), its perdeuterated analogue (DPPC-d\(_{62}\)), and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). Lanosterol and ergosterol were both purchased from Sigma-Aldrich Chemie (Germany). Deuterium depleted water was from Cambro Scientific (Germany).

Sample Preparation. For deuterium NMR, DPPC-d\(_{62}\) and the sterol were separately dissolved in 65:30:5 chloroform/methanol/H\(_2\)O and then volumetrically added to obtain a DPPC-d\(_{62}\)/sterol mixture of 6:4 at a DPPC-d\(_{62}\) concentration of 15 mg/mL. The mixtures were lyophilized using liquid nitrogen for 40 h and then rehydrated using deuterium-depleted water containing 20 mM Hepes buffer at pH 7.0 at 50 °C for 12 h under gentle vortixing. After this, the samples were cycled in temperature of 1 °C. The spectra were obtained by calculating a one-dimensional Fourier transform of the time domain data points starting at the top of the echo.

Freeze Fracture Electron Microscopy. Freeze fracture electron microscopy of the MLV solutions used for NMR were standard frozen in liquid propane, and the samples were fractured and carbon–platinum coated using a Balzers BAF 400 freeze fracture device. The samples were cleaned by floating on a detergent solution and afterward inspected using a Zeiss EM 902 electron microscope.

Micropipet Aspiration Measurements. Vesicles were observed in a chamber consisting of two parallel glass coverslips separated by a spacer of about 1 mm thickness. To allow easy access of the micropipet, the chamber was left open on two sides. Cylindrical glass micropipets with an inner diameter of about 5 μm were prepared as described elsewhere and connected to a micrometer-positioned water manometer which allowed application of specified suction pressures. A water hydraulic micromanipulator (Narishige, Tokyo, Japan) was used for positioning of the pipet. The chamber was mounted on the stage of an electron microscope of the Zeiss Axiolab 135TV series (Carl Zeiss, Jena, Germany), equipped with differential interference contrast optics (DIC). Vesicle aspiration was observed with a 40× long distance objective (LD Achroplan 40×/0.6 Carl Zeiss). Images were projected on the chip of a CCD camera (C2400-77, Hamamatsu, Hamamatsu City, Japan) and recorded to videotape. Digitized pictures with a resolution of 164 96× were taken at different pressures to determine the geometrical deformations of the vesicles. Image analysis was done using the program Scion Image from Scion Corporation. Data treatment was performed as described below.

Results

1. Deuterium NMR. \(^2\)H NMR spectra were obtained both for multimembranous vesicle dispersions (MLV) and for oriented samples of pure DPPC-d\(_{62}\) and of mixtures of DPPC-d\(_{62}\) with each of the three sterols (40 mol % sterol). We studied the samples at three temperatures: below and above the liquid–gel phase transition of pure DPPC-d\(_{62}\) at 20 and 50 °C, respectively, and at the physiological temperature of 36 °C. The MLV spectra were studied mainly for two reasons. First, we wished to compare the macroscopic magnetic field orientation of the MLV for the three DPPC-d\(_{62}/sterol\) mixtures with that of pure DPPC-d\(_{62}\).
Effect of Sterols on Lecithin Bilayers

Figure 2. Deuterium NMR spectra of multilamellar dispersions of pure DPPC-d62 and in binary mixture with 40 mol % of sterols, obtained at (a) 20 °C, (b) 36 °C, and (c) 50 °C. Here D, E, L, and C denote the spectra of pure DPPC-d62, DPPC-d62/ergosterol, DPPC-d62/lanosterol, and DPPC-d62/cholesterol, respectively.

d62. Beside that, the MLVs were used for a comparison of the transverse relaxation time T2 between the samples. Figure 2 shows representative spectra obtained for MLV samples at the three temperatures 20, 36, and 50 °C.

For pure DPPC-d62 we can clearly distinguish between the fluid phase at 50 °C, exhibiting the well-known Pake doublet shape for axially symmetric reorientation of the molecular director axis and with a quadrupolar splitting of 62 kHz. In contrast, the gel phase (20 °C) shows nonaxial symmetric contributions and a much broader line shape as reported previously by Davis.12

Finally, the DPPC-d62 spectrum at 36 °C is a superposition of gel and fluid line shape contributions since the actual phase transition temperature of DPPC-d62 is very close at 37 °C.

The presence of the sterols in the DPPC-d62 increased for all three samples significantly the quadrupolar splitting and thus the molecular order of the palmitoyl chains. However, it is interesting to note that each of the three sterols modulated the MLV spectral line shape in a different way. This becomes most obvious at 20 °C where cholesterol exhibits a typical fluid state line shape characteristic for the so-called lo phase13 while ergosterol appears in a broader feature at the spectral shoulders, the origin of which is not clear yet (Figure 2c). The narrower component of this spectrum shows splittings comparable to those of pure DPPC vesicles. Similar to the line shapes, the measurement of the transverse relaxation time T2 for the three sterol samples at 36 °C showed a dependence on the individual sterol (Table 1). Here cholesterol gave the highest T2 value while ergosterol showed the shortest T2.

The MLV spectral line shapes from Figure 2 were analyzed for magnetic field orientation of the vesicles in the field of the NMR magnet, which occurred due to the anisotropy of the diamagnetic susceptibility of the fatty acyl chains of the phospholipids. This caused the fluid vesicles to adopt an ellipsoidal shape with the long axis parallel to the magnetic field direction B0,14 resulting in characteristic line shape distortions. Bending energy of the bilayers is one of the major forces to compensate the deformation toward ellipsoidal shapes. As a result, vesicles in the gel phase where the bilayer is virtually frozen and largely inelastic do not show magnetic orientation effects. However, in the fluid phase the extent of deformation may serve as a crude measure for the microelastic properties of the bilayer, in particular its bending energy.

The line shape analysis was performed by fitting the convolution of oriented spectra with ellipsoidal line shapes to the MLV spectra with the aspect ratio ε (short axis/long axis), i.e., the lower the value of ε, the higher the magnetic field orientation) as a parameter according to the line shape function

\[ p(\theta) \frac{\partial p}{\partial \omega} = -\frac{1}{2} \varepsilon^3 (2x - 2\omega + \varepsilon^2 x + 2\varepsilon^2 \omega) \frac{3(3x + 2\omega)}{(3x + 2\omega)^{5/2}} \]

derived by Pott et al.15 Here p(\theta) is the probability function of finding the normal of a surface element oriented at an angle \( \theta \) with respect to the magnetic field direction, ε is the aspect ratio (for ε = 1 results the well-known spherical line shape function (3x(3x + 2\omega)^{-2})\), \( \omega \) is the frequency of the MLV data, and x is the frequency of the oriented data. As can be inferred from Table 2, the MLV sample containing ergosterol (ε = 0.74) showed the lowest magnetic field orientation at 36 °C, while it was highest for lanosterol (ε = 0.60). The cholesterol MLV exhibited a slightly higher resistance toward magnetic field orientation (ε = 0.64) as compared to lanosterol. At 50 °C the magnetic field orientation for lanosterol is still slightly higher than that for cholesterol. The corresponding value for ergosterol is not given in Table 2 due to the complex (apparently superimposed) line shape of the spectrum.

| Table 1. ²H-NMR T2 Relaxation Times (in μs), Measured at 36 °C |
|---------------------------------|-----------------|-----------------|
| sample                          | temp. °C | T2 (μs) |
| DPPC/cholesterol                | 36       | 1.00              |
| 6:4 DPPC/cholesterol            | 36       | 0.64              |
| 6:4 DPPC/lanosterol             | 36       | 0.63              |
| 6:4 DPPC/ergosterol             | 36       | 0.60              |
| 6:4 DPPC/lanosterol             | 50       | 0.58              |
| 6:4 DPPC/ergosterol             | 50       | 0.74              |

* Here ε is defined as the ratio of the semiaxis perpendicular to the semiaxis parallel to the static magnetic field B0.

| Table 2. The Semiaxis Ratio ε Obtained at 36 and 50 °C for the Three Mixtures and for Pure DPPC-d62 Coated to a Silica Bead of 640 nm Diameter as a Control |
|---------------------------------|-----------------|
| temp. °C | semiaxis ratio ε |
| 36       | 1.00             |
| 50       | 0.74             |

there are significant differences regarding their temperature dependence. The ergosterol profile clearly showed the highest temperature dependence while the lanosterol profile was significantly less temperature dependent. At 36 °C the values of $S_{CD}$ were very similar for all three samples while at 50 °C the DPPC chains showed lowest order in the presence of ergosterol and highest $S_{CD}$ for cholesterol.

2. Micropipet Measurements. Micropipet aspiration experiments were performed in order to correlate micromechanic properties of the three sterol-doped DPPC bilayer (same sterol content as for the NMR experiment, i.e., 40 mol %) samples with the NMR results. In these experiments, we determined the area expansion modulus by the method developed by Evans and co-workers.17 Figure 6 represents a typical experimental setup where a giant unilamellar vesicle (GUV) of radius $r_0$ was aspirated to the micropipet (radius $r_p$). It is noteworthy that also in this preparation ergosterol appeared to increase vesicle size. Due to the aspiration pressure, $p$, applied to the vesicle, the excess membrane area (usually less than 5% of the total area)

$$\Delta A = 2\pi r_p \left(1 - \frac{r_p}{r_0}\right) \Delta L$$

was sucked into the pipet up to the projection length $\Delta L$ with respect to the projection length at vanishing aspiration pressure. Due to the suction, the bilayer surface experiences an isotropic surface tension

$$\tau = \frac{pr_p}{2 - 2r_p/r_0}$$

The change of area with tension is a measure for the deformation elasticity and is given by the compressibility modulus

$$K = \frac{d\tau}{d\alpha}$$

with $\alpha$ being the relative change in area, $\Delta A/\Delta \alpha$. Plots of $\tau$ vs $\alpha$ gave straight lines, the slope of which gave the area compressibility modulus $K$ (Figure 7). The data were independent of the history of the vesicle, i.e., increasing and decreasing the aspiration pressure yielded identical results. This observation excludes artifacts due to area loss or leaky membranes. The values of $K$ are represented


along with the critical lysis tension \( \tau_c \), in Table 3. At a certain critical tension \( \tau_c \), the vesicles collapse. We obtained similar values for all three sterol/DPPC GUV samples. However, K varied by nearly a factor of 2 with lanosterol giving the lowest K and ergosterol the highest one. Results for cholesterol-containing samples agree well with literature values.\(^8\) The variation in K correlated with the GUV size, where the ergosterol sample showed a larger diameter than that of lanosterol GUV. Similar to the case of the \( \epsilon \) values determined by NMR (Table 2), cholesterol gave K values between those obtained for the other two sterols but closer to lanosterol (Table 3).

Discussion

The most interesting result of our study is the correlation between micromechanic membrane properties measured by a (static) method at the microscopic scale with parameters derived from a time-resolving method (NMR) which is sensitive at molecular scale. We observed a correlation between the NMR transverse relaxation time \( T_2 \) (Table 1), the NMR lineshape (expressed by the aspect ratio \( \epsilon \), Table 2), and the area compressibility modulus K (Table 3) for the three DPPC/sterol samples studied. This correlation seems quite understandable from the physical point of view. The shortest \( T_2 \) was measured for ergosterol, indicative for the most rigid (solidlike) bilayer, and its \( \epsilon \)-value indicated the lowest deformation in a magnetic field and it gave the highest K. On the other side, lanosterol showed medium \( T_2 \) (in agreement with a previous study of Urbina et al.\(^4\)) and it had the highest magnetic field deformation and the lowest K. Obviously the micromechanical properties of membranes must be most profoundly influenced by the molecular packing of its constituents. Therefore the observed correlation between the results of deuterium NMR line shape and relaxation, which is sensitive to molecular packing, and micropipet aspiration, testing the elastic properties of membranes, was no surprise. As a consequence, this finding implies that deuterium NMR may be used to study aspects of membrane micromechanic properties. An interesting question is why the order parameter profiles measured did not show such a clear correlation (Figure 5). After all, \( S_{CD} \) is highly

![Figure 6](image1.png)

**Figure 6.** Representative picture of a giant lipid bilayer vesicle (here 6:4 DPPC/ergosterol) with 32 \( \mu \)m diameter aspirated to a micropipet. The projection length of the excess membrane area can easily be detected by the change of gray level in the micropipet (arrow).

![Figure 7](image2.png)

**Figure 7.** Typical plot of the membrane tension \( \tau \) vs area dilatation \( \alpha \). The slope of \( \tau \) vs \( \alpha \) corresponds to the area compressibility modulus K.

<table>
<thead>
<tr>
<th>Lipid–Sterol Mixture</th>
<th>Area Compressibility Modulus, K (mN/m)</th>
<th>Lysis Tension</th>
</tr>
</thead>
<tbody>
<tr>
<td>6:4 DPPC/cholesterol</td>
<td>740 ± 100</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>6:4 DPPC/lanosterol</td>
<td>610 ± 90</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>6:4 DPPC/ergosterol</td>
<td>1100 ± 100</td>
<td>25 ± 9</td>
</tr>
</tbody>
</table>
sensitive to the molecular area accessible to the chain segments and the other results strongly suggest that a different degree of lipid area condensation due to the sterol presence is crucial for modulating the membrane micromechanical properties. It should also be noted that in a paper of Urbina et al.4 the overall order in DMPC/sterol mixtures (30 mol % sterol) of MLV was reported as ergosterol > lanosterol > cholesterol. To resolve this discrepancy, we have to consider the temperatures where the $S_{CD}$ measurements were performed. Owing to the possible contribution of gel phase to the ergosterol spectrum at 20 °C, we have chosen to analyze the order parameters for the spectra obtained at 36 and 50 °C only. However, Figure 5 shows clearly that the temperature dependence of the methylene $S_{CD}$ is quite different for the three samples with the ergosterol sample showing the highest sensitivity to temperature changes. Since the micropipet measurements were performed at low temperature, we would need to extrapolate $S_{CD}$ to this temperature to compare correctly. However, this approach may introduce severe errors owing to possible nonaxial symmetric contributions to the spectra. Therefore, we have studied the order parameter of the terminal methyl groups which are not affected in their axially symmetric reorientation down to temperatures far below 0 °C.12,19 Figure 8 shows $S_{CD}$ of the terminal CD$_3$ group for the three samples as a function of temperature. It demonstrates that at 20 °C, the ergosterol sample shows indeed the highest order while this changes with increasing temperature to lowest order of all three samples at 50 °C. A rationale for this significantly higher temperature sensitivity of the ergosterol sample is not given here; we can only surmise that some uniaxial rotation of this sterol might become activated in this temperature range, thereby increasing the separation between adjacent DPPC molecules.

However, the terminal methyl group order parameter at low temperature suggests that indeed high molecular order seems to correspond to the high area compressibility modulus $K$ obtained for the ergosterol sample. At low temperature (20 °C), our results are also in agreement with those of Urbina et al.4

It is interesting to discuss our results in terms of the differences in the molecular sterol structure (Figure 1). It appears quite conceivable that cholesterol with its flat sterol body at one side can achieve a higher packing in the DPPC bilayer and consequently force the acyl chains to adopt a higher order compared to lanosterol. The latter exhibits due to its additional three methyl groups a (in the cross section) bulkier sterol body. These differences in packing along with the resulting different dispersion forces in the bilayer plane may account for the higher values of $K$ and $\epsilon$ observed for cholesterol over lanosterol. It is less obvious which molecular features of ergosterol enable this sterol to cause an even higher molecular order than cholesterol, along with the highest values for $K$, $\epsilon$, and vesicle size. Its sterol body exhibits a similar flatness as cholesterol, so we assume that the modifications of the short alkyl chain may account for an even stronger van der Waals interaction between the palmitoyl chains of the DPPC (possibly at the expense of membrane fluidity) and thus cause this high membrane bending stiffness. A conceivable sensitive mechanism might be a slightly different average position of the ergosterol center-off-mass in the membrane with respect to the bilayer normal direction due to the modifications in the acyl chain as compared to cholesterol.

An important question regarding our comparison of the modulation of DPPC membrane properties by the three sterols studied is whether the lo phase reported for cholesterol is also a feature of the other two sterols. Unfortunately, no published work is available on DPPC/lanosterol or DPPC/ergosterol phase diagrams. However, our NMR results obtained at 20 °C (Figure 2) indicate that lanosterol shows a similar axially symmetric spectrum as cholesterol and also an increase of the DPPC-$d_{40}$ quadrupolar splittings. This indicates that at least the fluid/gel phase transition of DPPC-$d_{40}$ at 36 °C is abolished by the presence of lanosterol in a similar way as was observed for cholesterol. Indeed, a microcalorimetry (differential scanning calorimetry, DSC) measurement of the DPPC/lanosterol sample did not show any remaining phase transition. Also for DPPC/ergosterol the DSC gave no detectable thermal event. However, the NMR spectrum of DPPC/ergosterol at 20 °C (Figure 2) may indicate contributions of gel phase, but at 36 °C it still appears largely axially symmetric and thus indicative for a fluid phase. All three different types of membranes behaved like fluid membranes in the micropipet experiments, i.e., membranes that were not tensed exhibited large thermal fluctuations. Remaining plastic deformations were not observed following aspiration and expulsion of vesicles. Thus the membranes are fluid on a micrometer length scale. From these observations we deduce that all three sterols render the DPPC to maintain its fluid state below the DPPC phase transition temperature but force the palmitoyl chains to adopt a significantly higher molecular order. Further studies will be required to verify or dismiss the existence of a lo phase for the ergosterol and lanosterol samples.

It is very tempting to view the results of our study from the perspective of cellular evolution. All three sterols are found in cells, most prominently cholesterol in eukaryotes, but lanosterol exists in prokaryotes while ergosterol is essential in some fungi or protozoan cells. From the results of our study we can speculate that the proven ability of these sterols to modulate the micromechanical properties of lecithin bilayers to a different degree may have functional reasons and originates from an evolutionary optimization process. However, further studies of mixtures with different lipids and even proteins at various amounts of sterols will be required to substantiate our surmise. Nevertheless, cholesterol seems to be the perfect molecule to keep a delicate balance between membrane rigidity to allow for large cell volumes as is the case for eukaryotes and membrane softness and fluidity to enable complex membrane function.

Conclusion
The comparison of the membrane effects of the three sterols suggests at least two molecular mechanisms by which these molecules may modulate membrane properties. One likely candidate is the average depth of immersion of the sterol molecule with respect to the hydrophobic region of the bilayer. The second may be the extent to which the sterols are able to perform fast uniaxial reorientation with respect to the bilayer normal and the thermal energy barrier at which this rotation is activated.

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