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# Effect of sterol structure on the bending rigidity of lipid membranes: A $^2$H NMR transverse relaxation study

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ABSTRACT

The effect of incorporation of 3–43 mol% sterol on the lipid order and bilayer rigidity has been investigated for model membranes of dimyristoylphosphatidylcholine or dipalmitoylphosphatidylcholine. $^2$H NMR spectra and spin-lattice relaxation rates were measured for macroscopically aligned bilayers. The characteristics of spectra obtained at temperatures between 0–60 °C are interpreted in terms of a two-phase coexistence of the liquid disordered and the liquid ordered phases and the data is found to be in agreement with the phase diagram published by Vist and Davis (Biochemistry 29 (1990), pp. 451–464). The bending modulus of the bilayers was calculated from plots of relaxation rate vs. the square of the order parameter at 44 °C. Clear differences were obtained in the efficiency of the sterols to increase the stiffness of the bilayers. These differences are correlated to the ability of the sterols to induce the liquid ordered phase in binary as well as in ternary systems; the only exception being ergosterol, which was found to be unable to induce $l_o$ phases and also had a relatively weak effect on the bilayer stiffness in contrast to earlier reports.



## 1. Introduction

Since the observation of the liquid ordered ($l_o$) phase [1,2] and its possible connection with rafts in biological membranes [3,4] the effect of cholesterol (CHOL) on domain formation in liquid crystalline bilayers has been of great interest for membrane biophysicists. The $l_o$ phase, characterized by a tight packing of the lipid chains, has been found in coexistence with the disordered analogue ($l_d$) in binary and ternary systems in which the incorporation of CHOL has a critical role. A multitude of experimental methods [5–13], as well as theoretical modelling [1,14–18] have been used to characterize domain formation in binary and ternary systems. The special issue on lipid interactions, domain formation and lateral structure of membranes (BBA 1788(1), 2009) provides a good overview of current research on this subject. Cholesterol has also been found to be a critical constituent for biological activity and its depletion is accompanied by loss of many biological functions.

Recent research has shown that other sterols also have the ability to induce lateral phase separation in lipid bilayers [19–21] and even to replace CHOL for structural function in living organisms [22–25], although with some possible modifications in raft functions [26]. Microdomains have also been found in vivo in plants [27], yeast [28] and *Drosophila melanogaster* [29], where other sterols are major constituents. Nevertheless, small variations in the sterol structure can drastically alter the ability of the sterol to induce domains in vitro as well as in vivo and it is an interesting problem to find out what properties distinguish "raft- from nonraft-forming" sterols. Some criteria have been suggested for this separation and different terms have been used, e.g. Bloch speaks of "functionally competent" sterols [30], while others speak about "membrane active" sterols [31] or "domain promoters" [32–34] but no consensus on the necessary criteria have so far been reached. It is also interesting to look into the possible reasons for the difference in structures of the major sterols found in animals (CHOL), plants (sitosterol (SIT) and stigmasterol (STI)) and fungi (ergosterol (ERG)). Finally, in order to understand the role of rafts in human diseases [35], and effects in the cell membrane due to defects in the CHOL biosynthesis [36], knowledge of the properties of sterol-containing lipid membranes will be of importance.

Several studies have indicated that small changes, such as introducing methyl groups and/or changing the position and number of double bonds in the sterol can have a large impact on the domain-forming properties [37 and references cited therein]. It is believed that the condensing effect of the sterol is crucial to the domain-forming propensity in that a tightly packed lipid bilayer would increase the van der Waals interactions. Therefore, the introduction of bulky methyl groups at the smooth surface of cholesterol or in the chain region would lower the domain-forming ability of the sterol. More unexpected was the finding that small variations in the stiff sterol ring skeleton can have a large effect. The introduction of additional double bonds or even a change in position of a double bond can drastically change the biophysical properties of the membrane in which the sterol is incorporated [37,38]. The reson for this is not clear but could involve a change in the relative orientation of the sterol rings, thereby making the sterol less flat.

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Our group has in a series of publications used the pulsed field gradient NMR method to study the influence of sterols on the lateral mobility of the lipids [13]. It has been shown that lateral diffusion is about 2–10 times smaller in the $l_o$ phase than in the $l_d$ phase and that this difference makes it possible to detect domain formation in model membrane systems. In a recent publication we studied the effect of several sterols of biological relevance and found large differences in their ability to induce lateral phase separation in a model membrane system [37]. This study extends the previous one by using deuterium relaxation NMR to study how these sterols affect the lipid chain order and bending rigidity of the membrane aiming to correlate raft-forming ability to physico-chemical properties of the bilayer.

## 2. Materials and methods

### 2.1. Materials

Six different sterols were used in the study (Fig. 1). Cholesterol (CHOL, 99.1%), lanosterol (LAN, 50–60% with the major impurity being dihydrolanosterol), stigmasterol (STI, 95%), ergosterol (ERG, 95%), and lathosterol (LATH, 98%) were all from Sigma Aldrich (St. Louis, MO); β-sitosterol (SIT, 78%) was from Steraloids (Newport, Rhode Island). The lipids dipalmitoylphosphatidylcholine (DPPC-$d_{62}$) and dimyristoylphosphatidylcholine (DMPC-$d_{54}$), deuterated in their hydrocarbon chains, were purchased from Avanti Polar lipids (Alabaster, AL). Deuterium depleted water, 99.9%, was obtained from Larodan Fine Chemicals AB (Malmö, Sweden).

### 2.2. Preparation of macroscopically oriented bilayers

Fully hydrated samples consisting of deuterated phospholipids with sterol contents of 0, 3, 13, 23, 33 and 43 mol% were prepared and oriented between glass plates according to previously published methods [39]. The water content could not be checked for the samples but is estimated to be between 30–40 wt.%, based on hydration kinetics of other samples of similar composition.

### 2.3. NMR measurements

NMR measurements were performed at the deuterium frequency of 61.48 MHz on a Chemagnetics Infinity NMR spectrometer equipped with a goniometer probe allowing the macroscopically aligned bilayers to be oriented with the bilayer normal at the desired angle with respect to the main magnetic field. The 90° and 0° orientations were set by maximizing the observed splittings at each of these positions (cf. Eq. (3) below). Most measurements were made with the bilayer normal parallel to the main magnetic field in order to obtain the largest frequency dispersion but some relaxation measurements were also made at the 90° orientation.

Composite pulses [40] were utilized for all pulse sequences to ensure complete spectral coverage. The hard 90° pulse length was measured to 11 μs, which gives a uniform excitation profile up to ±57 kHz and uniform inversion up to ±50 kHz for the composite pulses.

Spectra were first recorded with the quadrupole spin-echo method at three degree intervals from 60 to 0 °C with a 10 min delay after each change in temperature. This was followed by measurements at 15, 30 and 44 °C in order to check for possible hysteresis effects. No such effects were observed for any of the samples. Then the relaxation experiments were performed at a temperature of 44 °C. The longitudinal relaxation time ($T_{1Z}$) was measured by the phase cycled inversion recovery pulse sequence $180_x$–$\tau$–$90_x$–$\tau_1$–$90_y$–$\tau_1$-acquire in which $\tau$ was varied between 1–2000 ms in 11–18 steps with a refocussing delay $\tau_1$ of 30 μs. The relaxation delay was 2.5 s, which is five times the longest measured $T_{1Z}$. Experiments in which the quadrupole splittings had changed during the relaxation measurement were discarded, since this indicated that water had escaped from the sample during the measurement. Data was fit to a three-parameter equation using the fitting routines implemented in the software Spinsight (Varian Inc.). The fits indicated an efficiency of inversion of more than 80% in all cases and were usually more than 90%. In order to see whether the results were affected by poor inversion some experiments were performed under conditions where the inversion was insufficient. The same results were obtained even in cases where the inversion flip angle was close to 90° instead of 180°, showing that the results were not sensitive to the degree of inversion of the signals.

### 2.4. Determination of the relative change in elastic constant

The deuterium order parameters and the longitudinal relaxation rates can be used to investigate the elastic properties of the bilayers utilizing a model developed by Brown et al. [41]. The method has been used for pure phospholipid membranes as well as for binary systems incorporating detergents, sterols and peptides [41–45]. If the relaxation is affected by relatively slow motions, such as collective director

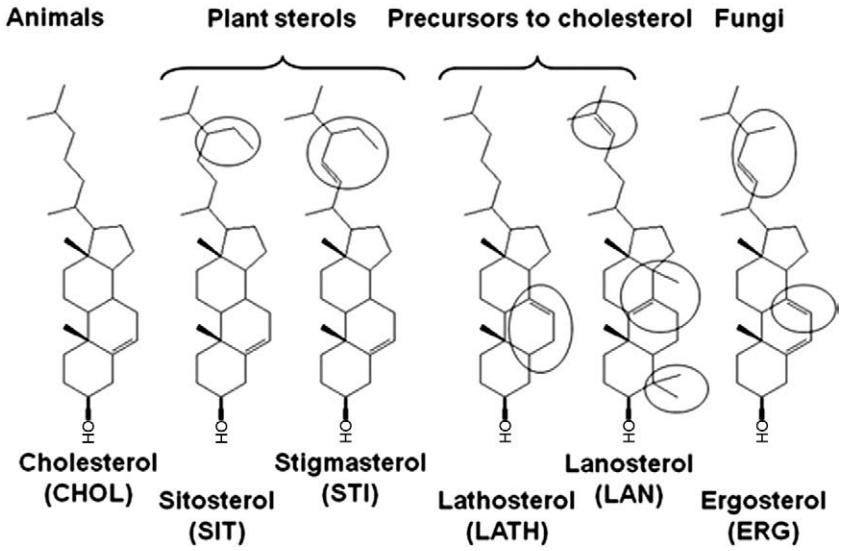


**Fig. 1.** Structures of the six sterols used in the study. The rings mark the difference in structure compared to CHOL.

fluctuations, the model predicts a linear dependence of the relaxation rate, $R_{1Z} = 1/T_{1Z}$, vs. the square of the order parameter [41].

$$R_{1Z} \propto K^{-3/2} S_{CD}^2. \quad (1)$$

Here, $K$ is a macroscopic elastic constant, equal to $\kappa/d$, where $\kappa$ is the bending rigidity and $d$ is the thickness of the bilayer. Thus, the relative effect of sterols on $K$ can be measured as

$$K_n = \frac{K}{K_0} = \frac{(\kappa/d)}{k_0/d_0} = \left(\frac{m_0}{m}\right)^{2/3} \quad (2)$$

in which $m$ is the slope of the line in a plot of $R_{1Z}$ vs. $S_{CD}^2$ and the subscript 0 stands for a lipid bilayer without sterol.

## 3. Results and discussion

### 3.1. NMR spectra

Fig. 2 shows representative stacked plots of inversion recovery measurements. Each C–$^2$H$_2$ segment of the lipid chains gives rise to Pake doublets, i.e. two lines at frequencies $\pm\nu$. The frequency separation of the peaks, so called residual quadrupole splitting ($\Delta\nu_q$), is given by

$$\Delta\nu_q = \nu_q S_{CD}\left(3\cos^2\theta_{LD} - 1\right) \quad (3)$$

in which the quadrupole coupling constant $\nu_q$ takes the value 127.5 kHz for a C–$^2$H-bond [46]. $S_{CD}$ is the order parameter of the C–$^2$H bond and $\theta_{LD}$ is the angle between the bilayer normal and the main magnetic field [47]. Due to differences in the order parameters among the C–$^2$H$_2$ segments, the spectra of well oriented samples consist of several Pake doublets corresponding to different residual quadrupole splittings. The innermost doublet with larger amplitude corresponds to the terminal methyl group of the chain. $\Delta\nu_q$ was considerably smaller for this group due to the additional averaging caused by the free rotation around the C–C bond. The small doublet marked with stars corresponds to the 90° peak for the methyl group in the powder pattern arising from the unoriented part of the sample.

For the samples without CHOL about 11 separate doublets were resolved (Fig. 2, left panel). The samples containing sterols generally showed less resolution and exhibited a considerable increase in the splittings due to the ordering effect of the sterol (Fig. 2, right panel). Note the extra splitting corresponding to a division of the methyl group signal into two doublets seen for the sterol-containing bilayer. This feature has been observed previously and has been taken as a characteristic of the liquid ordered phase [2,48]. Thus, the sterol-containing system shows characteristics of the $l_o$ phase. In the following section we will discuss the transition from the $l_d$ to the $l_o$ phase in more detail in order to address the question of whether or not there exists an equilibrium between these two phases in the studied binary systems.

#### 3.1.1. Phase behaviour in the CHOL systems

In order to put the spectral features into the context of general phase behaviour of binary lipid/sterol systems we decided to record the NMR spectra of samples containing 0–43 mol% sterol in the temperature interval of 0–60 °C. This is expected to span the interesting parts of the phase diagram. Next we will compare our data to known features of the proposed phase diagram for DPPC/CHOL [2,49] and similar results predicted theoretically[1,17]. According to these phase diagrams there is a two-phase coexistence area of $l_d$ and $l_o$ phases at temperatures above $T_m$, with an extension somewhere between 10–35 mol% sterol, depending on the temperature. At lower temperatures there is a two-phase coexistence area of $s_o$ and $l_o$ phases. If there is a difference in appearance of the NMR lineshape between the phases, e.g. a shift in the resonance frequency caused by a change in the quadrupole splitting for a specific line, one would expect changes in the observed lineshapes as one moves through the phase diagram by varying either the temperature or the sterol content. The lineshapes in regions where more than one phase coexist will depend on the degree of exchange the lipids undergo relative to the frequency difference between the lines in the different phases. If the exchange is slow two separate lines will be observed with intensities that reflect the relative populations of the phases, while a fast exchange will give rise to one single line, with a frequency that is the weighted mean value of the frequencies for the lines of the two phases. Finally, if the exchange falls in the intermediate time

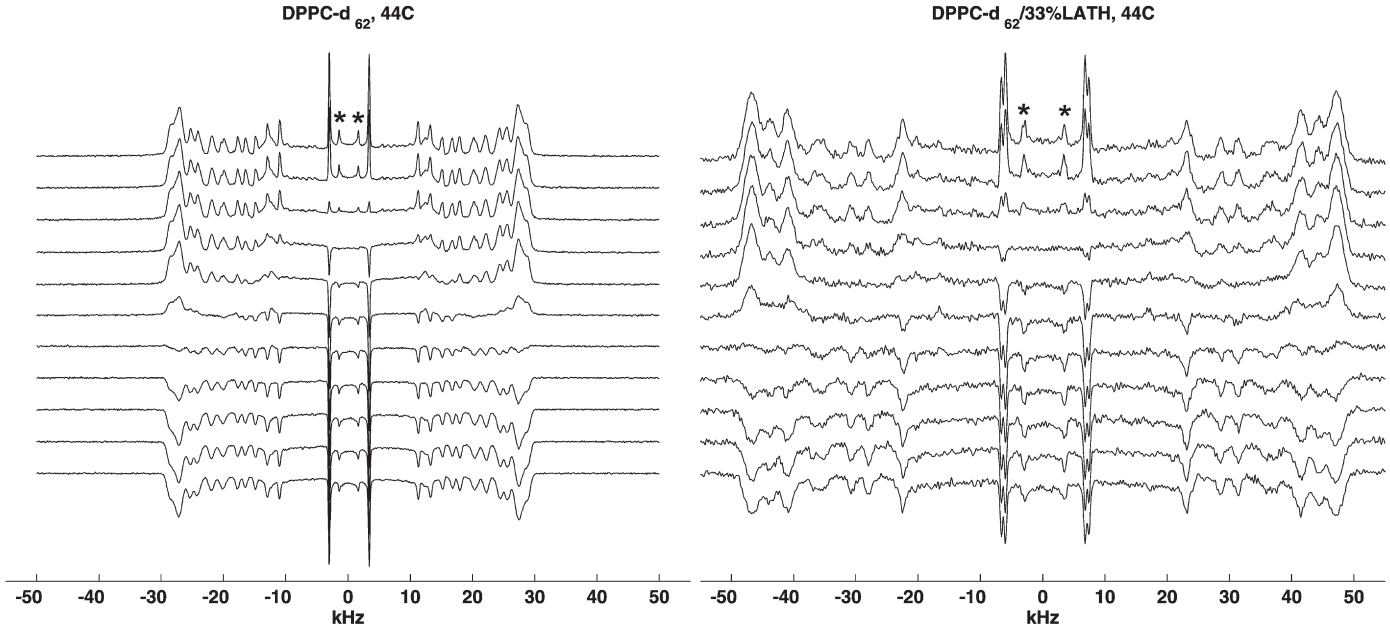


**Fig. 2.** Stackplots obtained in inversion recovery experiments at 44 °C with bilayers oriented at 0° with respect to the main magnetic field. Left: DPPC-$d_{62}$, right: DPPC-$d_{62}$ + 33 mol% LATH. The spectra corresponds, from bottom to top, to $\tau$-values of 1, 2, 4, 10, 20, 40, 100, 200, 400, 1000 and 2000 ms. The signal marked with stars belongs to the powder pattern arising from the unoriented part of the sample.

regime the two lines will coalesce into a broad, featureless shape in which all resolution is lost [50].

Fig. 3 shows six stackplots obtained from the DPPC/CHOL system, each one for a different sterol content and for $T=0$–60 °C, from bottom to top of the stackplot. (Since the spectra are symmetric, only the positive frequency part of the spectra is displayed from now on.) For the pure phospholipid the spectrum at high temperatures is typical for the $l_d$ phase. Between 36 and 39 °C the lineshape changes abruptly to one with very broad lines characteristic of the $s_o$ phase. The transition temperature is in good agreement with earlier published data for DPPC-$d_{62}$ [2]. The spectra for 3% CHOL is similar to that for 0%, also in good agreement with the phase diagram. At 13% CHOL, we see a broadening of the lines, starting at 39 °C. At the same time the $CD_3$ line splits into two. This corresponds to the entry into the two-phase $l_d/l_o$ region. At lower temperatures the methyl line broadens out but a relatively sharp line at $\pm 60$ kHz appears. As we will argue later, this line with a sharp cutoff close to the maximal possible value of 63.75 kHz (corresponding to a chain frozen in the all-trans configuration), corresponds to the $l_o$ phase at low temperatures. Thus, this sample starts off in the $l_d$ phase at high temperature and then passes through the $l_d/l_o$ coexistence region and finally ends up in the $l_o/s_o$ coexistence region, in agreement with the phase diagram.

For 23% CHOL the lineshape is broad and featureless at high temperatures but suddenly sharpens in the temperature interval 39–33 °C, as was also observed by Vist and Davis [2]. The $CD_3$ peak split appears at 45–42 °C and then follows the same fate as the 13% sample. These features can also be rationalized from the phase diagram if we assume that the linebroadening is caused by an intermediate exchange between the $l_d$ and $l_o$ phases. This approach has been used earlier both in binary and ternary systems [19,48]. The sharpening of the lines beginning at 30 °C corresponds to the entry into the one-phase $l_o$ region and the sample then remains in this phase as temperature drops further.

The sample with 33% CHOL has a split $CD_3$ line for all temperatures, indicating that the $l_o$ phase is present at all temperatures. However, there is a weak indication of exchange broadening at the highest temperatures which suggests that the two-phase region stretches up to ca 60 °C at 33 mol% CHOL.

At 43% the spectra are sharp all the way up to 60 °C. Therefore, most probably a single $l_o$ phase is present for all temperatures and this is the reason that we assigned the appearance of the sharp peaks at $\pm 60$ kHz to the $l_o$ phase as mentioned earlier.

In summary, all the features of the spectra can be rationalized in terms of phase coexistence in agreement with the earlier presented phase diagram, and our data indicates that the two-phase $l_d/l_o$ region extends up to 60 °C for a sterol content of 33%. In particular, the sharpening of the lineshapes observed in distinct temperature intervals would be difficult to explain if the bilayer consists of a single, homogeneous phase.

Now we concentrate the discussion on the signal from the terminal $CD_3$ of the chains, since this is the only signal that can be unambiguously assigned unless specific labelling is used. Fig. 4 shows the expanded spectral region from this group upon a change in either the CHOL concentration (left) or the temperature (right).

Both of these plots indicate a gradual transition between two lineshapes; one consisting of a single line and the other consisting of two lines at higher frequency. This is consistent with the phase diagram if we assign the lineshapes to the $l_d$ and the $l_o$ phases, respectively, assuming that the exchange rate between the two phases is fast. Similar assignments have been made earlier for binary systems [2] and for ternary systems, in which also conditions for slow exchange, giving a superposition of three lines from the methyl group, were observed [48]. Lineshape deconvolutions show that the integrated intensities of the two lines are equal, even though the linewidths may differ, supporting this interpretation of the spectra. Given the typical frequency differences of 2–5 kHz this means that the lifetimes in the

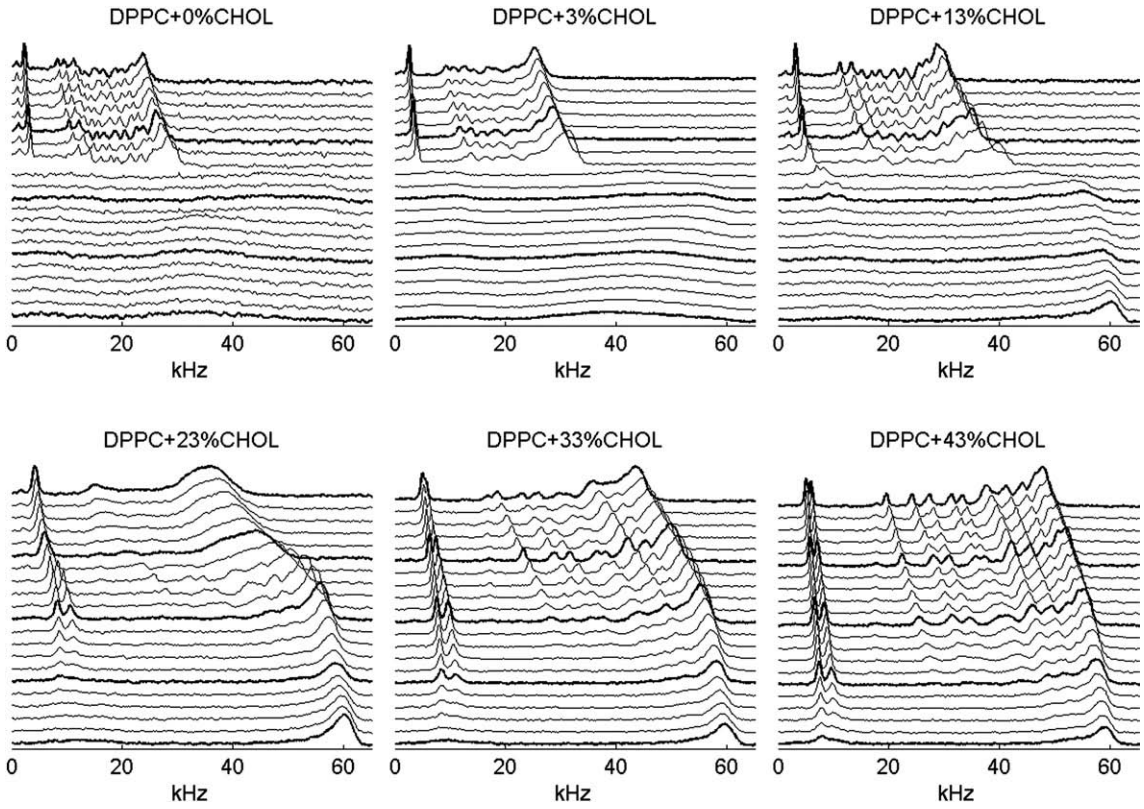


**Fig. 3.** Stacked half-spectra of oriented samples ($\theta_{LD}=0°$) of DPPC-$d_{62}$/CHOL. The temperature is varied from 0 (bottom) to 60 °C (top) in steps of 3°. The thicker lines indicate temperatures of 0, 15, 30, 45 and 60 °C.

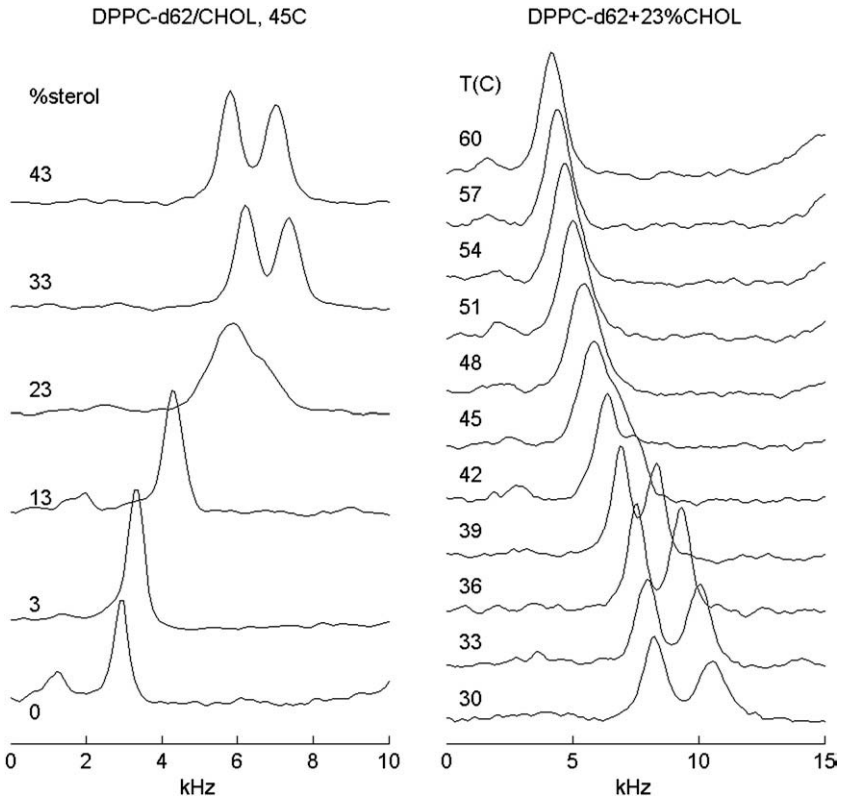


**Fig. 4.** Expanded spectra of oriented samples ($\theta_{LD}=0°$). Left: stackplots with increasing CHOL content at 45 °C. Right: stackplots with increasing temperature at a CHOL content of 23%. $\theta_{LD}=0°$.

two phases must be considerably less than 1 ms. However, if we consider the exchange broadening observed for the larger quadrupole couplings we can estimate a lower limit for the lifetimes. Assuming that the broadening implies a situation in which the exchange rates are roughly equal to the frequency difference between the lines of the different phases, we cannot have rates larger than 10–20 kHz. Thus, the lifetimes of the lipids in the phases are fixed in the interval of 10–100 μs. If the exchange is due to lateral diffusion of lipids this would correspond to domain dimensions of 10–50 nm if a value of 5 $\mu m^2/s$ is used for the lipid lateral diffusion [13]. However, any type of process in which the order of the chains is altered on this timescale would produce this effect, e.g. a system with large fluctuations in lipid order would produce the same kind of broadening [51].

In principle one could reduce the exchange broadening by orienting the samples at a different angle, making the quadrupolar splitting smaller. This will also reduce the frequency difference of lines in different phases and we will move into a region of fast exchange. This effect has been demonstrated in the ternary system of POPC-$d_{31}$/PSM/CHOL [13].

The DMPC system shows a remarkable similarity to the DPPC system, the only difference being a shift down in temperature by ca 15 °C and a maximum splitting that is slightly smaller than for DPPC (Fig. 5). It is also notable that the $CD_3$ splitting seems to be slightly larger in magnitude than for DPPC. Thus, we have the same appearance of the phase diagram for both lipids. Note that the exchange broadening for DPPC/23% CHOL is seen for DMPC both for 23 and 33% CHOL, indicating that the two-phase area extends to higher sterol content in the DMPC/CHOL system.

### 3.1.2. Phase behaviour in the other sterol systems

Fig. 6 summarizes the obtained results by concentrating on the $CD_3$ group signal of DPPC, showing stackplots for the most interesting sterol contents, i.e. 13–43%. For 3% sterol all systems exhibited similar spectra as for the sterol-free bilayers. The full spectra can be found in the supplementary information. Again, the spectra for the DMPC system were very similar to those for the DPPC system, when accounting for the difference in $T_m$. These spectra can also be found in the supplementary information.

The absence of a $CD_3$ splitting for the ERG system suggests that this sterol is unable to induce the $l_o$ phase. Instead, the spectra with decreasing temperature are indicative of a transition from $l_d$ to $s_o$, similar to that observed for all systems at low sterol content. This is in contrast to earlier published data in which split $CD_3$ lines were observed [52]. Also the splitting for the $CD_2$-groups in the plateau region (i.e. those with the largest splittings) was smaller than those reported by Beck et. al and Hsueh et. al [19,52] for temperatures above 30 °C, while the agreement was good at 30 °C.

All other sterols seem to induce an $l_o$ phase, although to different extents. LAN seems to be the weakest $l_o$ former, as the temperature range of the $CD_3$ splitting is less than for SIT, LATH and STI. Among the latter three sterols the order of increasing propensity of $l_o$ formation is LATH ≥ SIT ≥ STI, based on the magnitude of the splitting and the temperatures at which the splitting first occurs.

To summarize, the propensity of the sterols to induce an $l_o$ phase (as indicated by the inequivalence for the two $CD_3$ groups) follows the trend LATH ≥ CHOL ≥ SIT ≥ STI > LAN > ERG. This is similar to the propensity to form large domains in the ternary system of DOPC/eSM/sterol [37], except for ERG which formed large domains in the ternary system but gave no indications of $l_o$ formation in the binary system. Several other studies have also placed CHOL, SIT, LATH and STI into the group of domain-formers [32–34,53] but in the pfg-NMR study of Shahedi et al. [37] STI did not show any evidence of domain formation.

### 3.2. Bending rigidities

$T_{1Z}$ was obtained for each Pake doublet as the mean value obtained for the two peaks and the order parameter was calculated from the

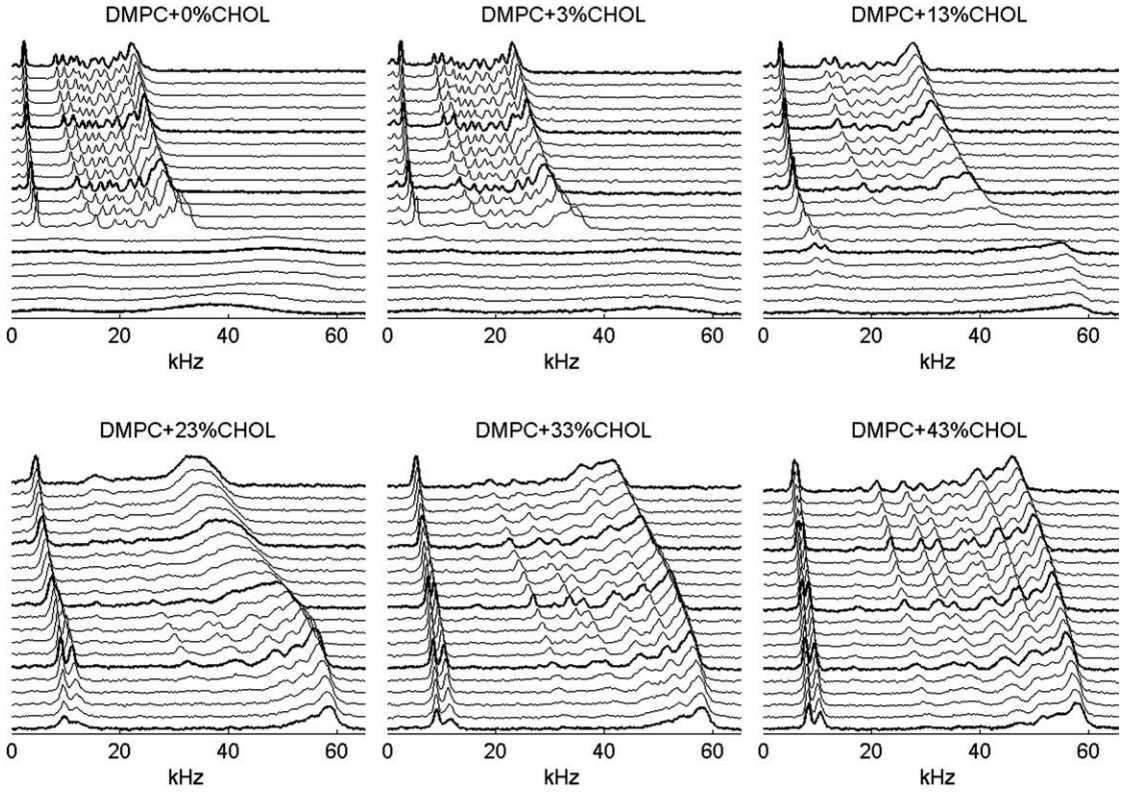


**Fig. 5.** Stacked half-spectra of oriented samples ($\theta_{LD} = 0°$) of DMPC-$d_{54}$/CHOL. Temperature is varied from 0 (bottom) to 60 °C (top) in steps of 3°.

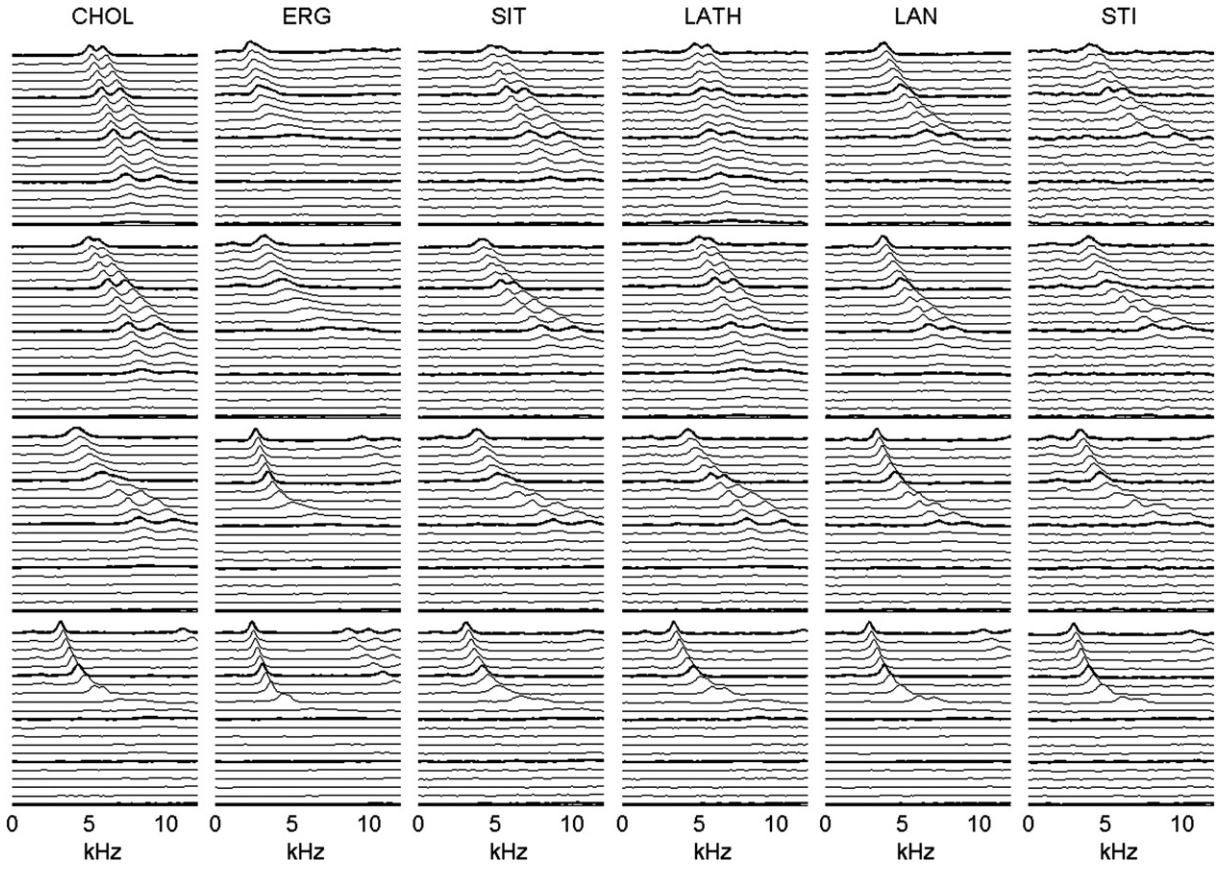


**Fig. 6.** Overview of the behaviour of the $CD_3$ signal for sterol contents of 13, 23, 33 and 43 mol% (from bottom to top). The stackplots shows spectra ($\theta_{LD} = 0°$) for temperatures from 0 to 60 °C in steps of 3°, with a thicker line for 0, 15, 30, 45 and 60 °C.

frequency difference of the peaks according to Eq. (3). In Fig. 7 $R_{1Z}$ is plotted against the square of $S_{CD}$ for the two samples shown in Fig. 2. In both cases a linear relationship according to Eq. (1) was assumed, in which the slope is proportional to $K^{-3/2}$. The supplementary information contains the linear fits for all sterols and all concentrations. In the worst case of linebroadened spectra only two points could be used (DPPC + 33% ERG) but for all other samples at least four and generally more than six points could be used in the fits.

It is clear from the slopes of the regression lines that $K$ increases with added sterol. The method does not give absolute values of the bending rigidity, but the relative effects of the sterols, monitored by $K_n$ according to Eq. (2), can be studied. In a preliminary study we measured $K_n$ for 33 mol% sterol by this method at two different bilayer orientations, 0 and 90°. The results (Table 1) indicated that *i*) the results were the same for both lipids within error margin, *ii*) $K_n$ obtained for the 90° orientation was consistently smaller than that for the 0° orientation and *iii*) when compared to results obtained for randomly oriented bilayers these values were found between the 0 and 90° results obtained by us.

The reason for this is most probably the orientational anisotropy of the relaxation that is not accounted for. The orientation dependence of $T_{1Z}$ is small for pure PC bilayers but when CHOL is added it becomes much larger and depends on the chain position, such that $T_{1Z}(90°) > T_{1Z}(0°)$ for carbon numbers 2–6, while the opposite is true for carbons 8–ω [55–58]. This leads to the conclusion that the calculated $K$ will be larger for 90° than for 0°. Thus, the normalized bending rigidity will be larger for the 90° orientation, in agreement with our results. It also makes sense that the results obtained for random orientations are located between those at the 90 and 0° orientations, since the dePaking routine is used to extract spectra corresponding to one orientation. Such a procedure will mix intensities from all orientations into the final lineshape and this will influence the obtained relaxation rates, if they depend on the orientation.

Due to the time consuming measurements we decided to perform the main experimental series only on the 0° orientation, where we have the best frequency resolution of the lines.

The results are displayed in Fig. 8. It is evident also from this figure that the effect of the sterols on $K_n$ is approximately linear. This feature has been observed in several other studies [42,59–62] and it therefore seems appropriate to report the rigidifying effect as the slope of a linear fit to the data. It is also clear from the figure that the effect is similar for both lipids, thus the lines in the plots are best linear fits to all data points. Note that for all sterols, there seem to be a maximum in $K_n$ at 33%, after which $K_n$ levels off. This would be expected since the systems enter the one-phase $l_o$ area at approximately this concentration and additional sterol will have a smaller effect on the overall phase behaviour. However, the relatively large scatter in the data points makes it difficult to state this unambiguously and the lines are fits to all data points.

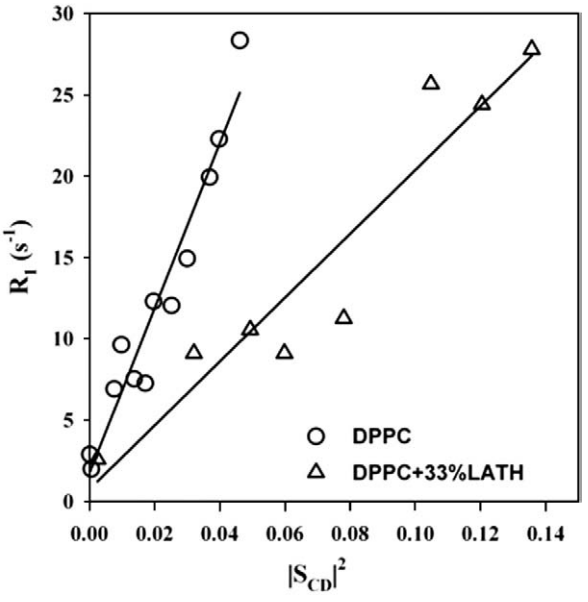


**Fig. 7.** Plot of the relaxation rate, $R_{1Z}$, vs. the square of the order parameter, $S^2_{CD}$, for DPPC-$d_{62}$ (circles) and DPPC-$d_{62}$ + 33% LATH (triangles) at 44 °C, and with the bilayer normal parallel to the main magnetic field.

**Table 1**
Summary of relative changes in elasticity constant caused by the addition of 33% sterol to lipid bilayers at 44 °C, as measured by the $^2$H-NMR method on oriented and non-oriented (powder) bilayers.

| | 90° | | 0° | | Powder |
|---|---|---|---|---|---|
| | DMPC | DPPC | DMPC | DPPC | DMPC |
| CHOL | 3.6 | 3.7 | 2.3 | 2.0 | 2.9[a] |
| | | | | | 3.6[b] |
| ERG | 2.1 | 2.8 | 1.6 | 1.6 | |
| SIT | 2.9 | 2.8 | 2.0 | 2.0 | |
| LATH | 4.6 | 3.6 | 2.0 | 1.9 | |
| LAN | 2.3 | 2.3 | 1.7 | 1.2 | 2.1[b] |
| STI | 2.7 | 2.7 | 1.9 | 1.6 | |

[a] DMPC, 44 °C, 33% [42].
[b] DMPC, 44 °C, interpolated to 33% [54].

The slopes of the lines are summarized in Table 2, together with previously published results using the same method as well as a method for measuring the bending rigidity of lipid bilayers based on the observation of thermal fluctuations in vesicles [60–62]. The results of the two methods are not directly comparable, since the measured quantity in the relaxation experiments is $K_n = \kappa_n \cdot d_0 / d$ (Eq. (2)), in which the relative change in bilayer thickness is included. This will render the measured $K_n$ to be slightly smaller than $\kappa_n$. However, this will only shift the values by approximately 10% and will be approximately the same for all sterols [63,64]. Thus, at least for comparisons between the sterols, the values of $K_n$ will be comparable with the values of $\kappa_n$.

We observe a significant variation among the sterols (Table 2), a variation that is similar for all methods. The value obtained for unoriented bilayers by the Brown group [42,54] is larger than the values found by us, due to the angular anisotropy of the relaxation as discussed above. An even larger value was obtained from transverse relaxation by Althoff et al. [59].

Similar results have been obtained using microscopy image analysis in which fluctuations in vesicle shape can be interpreted in terms of bending rigidity [65] for DMPC/CHOL at 40 °C [62], and a slightly larger value was found at 30 °C [60]. It is interesting to note that the systems of unsaturated palmitoyloleoylphosphatidylcholine with addition of CHOL, ERG and LAN at 30 °C give comparable results to those for the saturated lipid at 44 °C [61] (Table 2). In particular, the relative differences for different sterols are well mirrored. This means that the effect of the sterols is similar with regard to the bilayer stiffness for both saturated and unsaturated bilayers. Finally, the results can be compared with those obtained from shape analysis of vesicles, in which $l_o$ and $l_d$ phases coexist. In vesicles of DOPC/eSM/CHOL the $l_o$ phase, which is expected to be enriched in CHOL and saturated lipids, was found to have a bending modulus five times bigger than that of the $l_d$ phase [66]. Also this value is in reasonable agreement with our results, even though the composition of the two phases in the vesicles was unknown.

The sterols can be ordered in decreasing stiffening capability as CHOL ≥ LATH ≥ SIT = STI > LAN > ERG, which is similar to the propensity of forming an $l_o$ phase discussed in section 3.1.2.

Our results indicate that ERG, a major sterol present in detergent insoluble domains in yeast [28] and *Drosophila melanogaster* [29], is less potent than CHOL in rigidifying the lipid membranes. This is

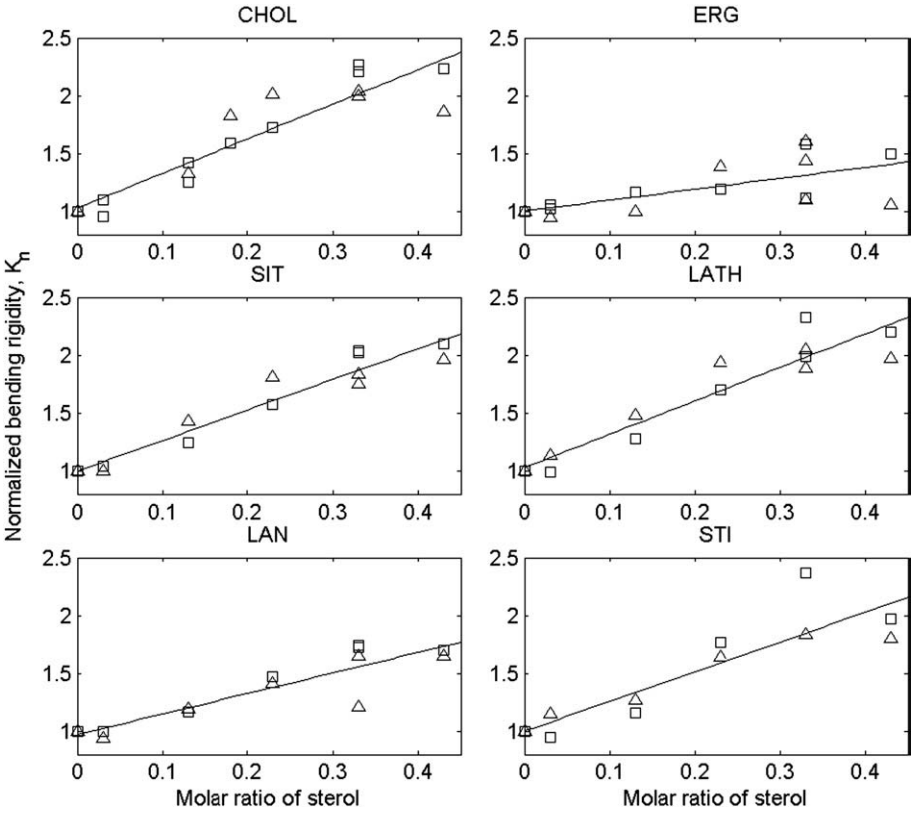


**Fig. 8.** Normalized bending rigidities obtained at 44 °C for the DMPC (squares) and DPPC (triangles) systems. Since the data for both lipids overlap within error margins all data in each plot is fit to a straight line. The slopes of the lines are given in Table 2.

somewhat surprising since ERG has been shown to induce phase separation in both binary [19,67] and ternary [33,34,37] systems. Studies on other membrane properties such as permeability [68], lateral tension [69], as well as bending rigidity [70] show that ERG has a larger effect than CHOL in unsaturated bilayers, while the effects are more dependent on temperature in saturated bilayers. This could be a consequence of the ability of CHOL to form $l_o$ phases at high temperatures in saturated systems, where ERG only forms the $l_d$ phase.

Micropipette aspiration experiments showed that ERG induced more rigid DPPC bilayers than CHOL at 10 °C [71], while the effect is about the same for both sterols at 25 °C [72]. The bending rigidities obtained by us at 44 °C are in general accord with this trend.

**Table 2**
Slopes of the lines in Fig. 8, together with corresponding literature values.

| | $^2$H NMR $T_{1Z}$ relaxation | | $^{31}$P/$^2$H $T_2$ relaxation | Image analysis of vesicle fluctuations |
|---|---|---|---|---|
| | 0° | Powder | | |
| CHOL | 3.0 | 7.7[a,b] | 13.3[f] | 3.9[c]<br>3.9[d]<br>7.7[e] |
| LAN | 1.8 | 3.2[b] | | 3.0[c] |
| LATH | 2.8 | | | |
| STI | 2.6 | | | |
| ERG | 1.0 | | | 1.9[c,*] |
| SIT | 2.6 | | | |

[a] DMPC, 44 °C [42].
[b] DMPC, 44 °C [54].
[c] palmitoyloleoylphosphatidylcholine, 25 °C [61].
[d] DMPC, 40 °C [62].
[e] DMPC, 30 °C [60].
[f] DMPC, 40 °C [59].
* The value at 30% is excluded from the extrapolation, since ERG is poorly soluble in palmitoyloleoylphosphatidylcholine.

Our study of the phytosterols indicates that these sterols are less efficient rigidifiers than CHOL. Domain formation has been observed in ternary systems, in which fluorescence quenching measurements show that both SIT and STI promote domain formation to a larger degree than CHOL [33], while large domains were observed by pfg-NMR only for SIT but not for STI [37]. In vivo studies have shown that SIT could replace CHOL as a growth factor, while STI lacked that ability [25].

In summary, SIT has the largest similarity to CHOL concerning its influence on the bilayer properties, but it is hard to draw any firm conclusions, since the results also depend on the degree of lipid unsaturation. It is not surprising that phytosterols, encountering lipids of a high degree of unsaturation, will have slightly different properties than sterols found in animals. The large amount of different sterols in plants might also reflect the need to keep the membrane in a defined state over larger temperature regions [52].

For LAN and LATH, two precursors of CHOL at an early and late step in the biosynthetic pathway, respectively, we found that LATH was more efficient than LAN in influencing the bilayer properties. It was early suggested that the biosynthetic pathway found in animal cells would reflect the gradual refinement of the sterol structure towards an evolutionary optimum in terms of a regulator of the state of the bilayer [73,74]. In this context one would assume that the effect of LATH on lipid bilayers would have a closer resemblance to CHOL than LAN, in accordance to our results. This is supported by studies showing that LAN is unable to induce domain formation in bilayers [32,34,37,75], while LATH is even more potent in driving the phase separation than CHOL [37,53,76]. Furthermore, LATH supports growth in CHOL deficient cells, while LAN is toxic to the cells [25]. Earlier studies also showed that CHOL is more effective than LAN with respect to stiffening of the bilayers [70,71]. However, LATH seems to have a lower capability than CHOL to increase ordering and rigidity, or to decrease permeability [22,38,68].

## 4. Conclusions

In this study we have examined the phase behaviour of binary systems composed of either DMPC or DPPC, mixed with sterols of various structures. The interpretation of $^2$H NMR spectra in terms of a fast to intermediate exchange between the $l_d$ and $l_o$ phases is compatible to the general phase behaviour previously predicted by both theory and experiment. The double quadrupole splittings observed for the methyl peak are a clear indication of the $l_o$ phase. The phase behaviour is remarkably similar for DMPC and DPPC, if one accounts for the differences in chain melting temperature. Evidence is obtained for all of the sterols, except ERG, for inducing an $l_o$ phase.

The effect of different sterols on the bilayer stiffness was investigated by NMR relaxation. A correlation was found between the propensity of the sterol to form an ordered phase and the increase in the bending rigidity.

All the studied sterols, except ERG and LAN, were found to affect the bilayers of saturated lipids similarly to CHOL. It should be noted that different sterols might have different effects on unsaturated lipids, demanding further studies. A "true" comparison between domain-forming features of different sterols and their impact on bilayer properties probably requires the use of bilayers with both lipids and sterols in compositions similar to those encountered in vivo.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2009.06.019.

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