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## Molecular order and dynamics of phosphatidylcholine bilayer membranes in the presence of cholesterol, ergosterol and lanosterol: a comparative study using $^2\text{H}$ -, $^{13}\text{C}$ - and $^{31}\text{P}$ -NMR spectroscopy

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### Abstract

We report the results of a comparative study of the molecular order and dynamics of phosphatidylcholine (PC) bilayer membranes in the absence and presence of cholesterol, ergosterol and lanosterol, using deuterium ( $^2\text{H}$ ) nuclear magnetic resonance (NMR) of deuterated phospholipid molecules, in addition to solid state  $^{13}\text{C}$  and  $^{31}\text{P}$ -NMR. Using dimyristoylphosphatidylcholines (DMPCs) specifically labeled at positions 2', 3', 4', 6', 8', 10' and 12' of the *sn*-2 chain together with the perdeuterated 2- $[\text{}^2\text{H}_{27}]$ DMPC derivative, the order profile for 9 of the 13 methylene groups of the *sn*-2 chain was established at 25°C for DMPC, DMPC/cholesterol, DMPC/ergosterol and DMPC/lanosterol membranes, at a fixed sterol/phospholipid mol ratio of 30%, and in the presence of excess water. The overall ordering effects were found to be ergosterol > cholesterol  $\gg$  lanosterol. Transverse relaxation ( $T_{2c}$ ) studies of these systems indicated that while for DMPC, DMPC/cholesterol and DMPC/ergosterol the relative relaxation rates were in qualitative agreement with models which assume cooperative motions of the bilayer molecules as the main relaxation mechanism, those in DMPC/lanosterol were anomalously high, suggesting alterations of lipid packing. Using dipalmitoylphosphatidylcholine (DPPC) deuterated at the trimethylammonium group of the choline moiety, we found that the differential ordering and motional effects induced by the sterols in the acyl chains were also reflected in the headgroup, both in the gel ( $L_\beta$ ) and liquid-crystalline phases.  $^{13}\text{C}$  and  $^1\text{H}$  spin dynamics studies of these systems, including cross-polarization, rotating frame longitudinal relaxation and dipolar echo relaxation rates showed that the mobility of the different regions of the phospholipid molecules in the binary lipid systems were inversely correlated with the ordering effects induced by the sterols. A novel combination of C-D bond order parameters (obtained by  $^2\text{H}$ -NMR) and  $^{13}\text{C}$ - $^1\text{H}$  cross polarization rates confirmed these results. The effects of the same sterols at the same molar proportion on the unsaturated lipid 1- $[\text{}^2\text{H}_{31}]$ palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine ( $^2\text{H}_{31}$ -POPC) at 25 and 35°C were different from those observed on DMPC and showed ordering effects which are largest for cholesterol, while ergosterol and lanosterol produced significantly smaller effects. Transverse relaxation studies indicate that while cholesterol does not perturb cooperative motions in POPC, both ergosterol and lanosterol do. Again, high-resolution solid state  $^{13}\text{C}$ -NMR studies support the conclusions of the  $^2\text{H}$ -NMR experiments. Titration experiments using both  $^2\text{H}$  and  $^{13}\text{C}$ -NMR show that ergosterol affects POPC bilayer structure up to 50 mol% but it increases the order of the phospholipid acyl chains only up to about 25 mol%. Beyond that level, it has a smaller ordering effect, possibly indicating aggregation or other more complex phase behavior. At > 30 mol% ergosterol,  $^{13}\text{C}$  spectra reveal the presence of a second form of the sterol. However,  $^{31}\text{P}$ -NMR spectra show that all POPC/sterol systems retain a bilayer configuration up to 30 mol% sterol. The concentration of ergosterol which induces maximum order in the POPC membranes coincides with that present in the plasma membranes of the protozoan parasite *Trypanosoma cruzi*. Taken together, our results indicate that the effects of sterols on PC bilayers are very complex, and depend on both sterol structure and on the fatty acids esterified to the phospholipid.

**Keywords:** Sterol function; NMR order parameter; Transverse deuteron relaxation; Cross polarization spin dynamics; Relaxation process,  $^{13}\text{C}$ ; Cooperative lipid motion

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## 1. Introduction

Sterols are complex molecules resulting from a long biochemical evolution [1] that are present in the plasma membranes of eucaryotic cells, where they seem to fulfill fundamental functions associated with the integrity and function of these structures [1–3].

The paradigmatic example of this group of compounds is cholesterol, which is present in vertebrate membranes but is absent in many other phylogenetic groups [1]. Many studies have been carried out in order to understand the molecular basis of the biological properties of this sterol, through e.g. the use of nuclear magnetic resonance spectroscopy [4–12]; electron paramagnetic resonance spectroscopy (EPR) [13–16]; differential scanning calorimetry (DSC) [17–23]; X-ray diffraction [17,24,25]; fluorescence methods [26–30]; and infrared and Raman spectroscopies [31–33]. From these studies, a consensus picture has emerged in which the cholesterol molecules intercalate into the phospholipid matrix, freeing the rotation and translation of the phospholipid molecules in the gel ( $L_\beta$ ) phase, while restricting these motions in the liquid-crystalline ( $L_\alpha$ ) phase. In some cases, phase diagrams have been constructed for phosphatidylcholine (PC)/cholesterol mixtures [7,8,10–12,15,16,30,34]. They showed that above about 25% mol% cholesterol in both saturated and unsaturated PCs, a liquid ordered phase ( $l_o$ ) or ‘liquid-gel’ phase is formed –the ‘intermediate fluid’ phase discovered by Chapman some years ago. This phase combines several properties characteristic of gel and liquid crystalline bilayers: high segmental order parameters [5–9], high lateral diffusion and rotational rates comparable to those of the fluid  $L_\alpha$  phase [35,36], but an area compressibility modulus [37,38] and bending rigidity [39] typical of the  $L_\beta$  phase.

Three main structural features of sterols such as cholesterol have been linked to its characteristic effects on lipid bilayer membranes: a planar cyclopentane-phenanthrene ring, a  $3\beta$ -OH group, and a long hydrophobic side-chain linked to C17 [1,40]. On this basis it has generally been assumed that sterols which share such properties with cholesterol (e.g., ergosterol, found in fungi, yeasts and protozoans, or sitosterol and its analogs, found in plants) induce similar properties and perform essentially equivalent functions in membranes, and thus are largely interchangeable. There are, however, several lines of evidence which do not fit the assumption particularly well. For example:

1. The sterols characteristically found in each phylogenetic group are essential growth factors which cannot be replaced by analog molecules which exhibit the above mentioned properties [41–49]. However, this ‘metabolic’ role requires only minute amounts of the characteristic sterol, and is different from the ‘bulk’ sterol function, which has less stringent structural requirements [1,44,49].

2. The functional reconstitution of several membrane transport proteins appears dependent not only on the type of phospholipid present in the reconstitution mixture, but also on its sterol composition. For vertebrate cation transporters, for example, optimal activity is achieved in the presence of cholesterol, while ergosterol supports much smaller activities [50].
3. The activity of polyene antibiotics, such as amphotericin B, on natural and artificial membranes is remarkably dependent on the type of sterol present in the membrane, being much more active in ergosterol-containing membranes than in those containing cholesterol [51,52]. Although a classical explanation for these effects has been the differential affinity of the antibiotic to the two types of sterols, recent studies have suggested that the physical state induced by the sterol on the phospholipid membrane also appears to be a critical factor which determines this differential antibiotic activity [51,53,54].

There have been very few detailed studies as to the effects of sterols other than cholesterol –and in particular of ergosterol –on the physical properties of phospholipid bilayer membranes. Early monolayers studies [55] noted that ergosterol was much less effective than cholesterol in the condensation of egg-PC monolayers, while Demel and de Kruffy [40] found that ergosterol and stigmasterol were less effective than cholesterol and its analogs in reducing the permeability of egg-PC liposomes to glycerol, glucose and rubidium ions. Semer and Gelerinter [56], using EPR of doxyl-fatty acid and cholestane spin label probes in egg-PC membranes, reported the observation that ergosterol, in contrast with cholesterol, only ordered acyl chains up to 15–20 mol%; above this ergosterol induced *disorder* of the acyl chains instead. However, no other related studies have been reported.

In this article we present the results of a study of the comparative effects of cholesterol, ergosterol and their common precursor in the biosynthetic pathway, lanosterol (Fig. 1), on the molecular order and dynamics of PC membranes, using  $^2\text{H}$ ,  $^{13}\text{C}$  and  $^{31}\text{P}$  solid state NMR methods.

## 2. Materials and methods

### 2.1. Labeled lipids

Specifically deuterated DMPCs, labeled at positions 2', 3', 4', 6', 8', 10' and 12' were synthesized and purified as described in detail previously [6]. DMPC with a perdeuterated acyl chain in the 2 position (2- $[\text{}^2\text{H}_{27}]$ DMPC), 1- $[\text{}^2\text{H}_{31}]$ palmitoyl-2-oleoyl-PC (1- $[\text{}^2\text{H}_{31}]$ POPC), and dipalmitoylphosphatidylcholine deuterated at the choline trimethylammonium group ( $[\text{}^2\text{H}_9]$ DPPE), as well as unlabeled 1-palmitoyl-2-oleoyl-PC (POPC) were obtained from Avanti Polar Lipids (Alabaster, AL) and were used without



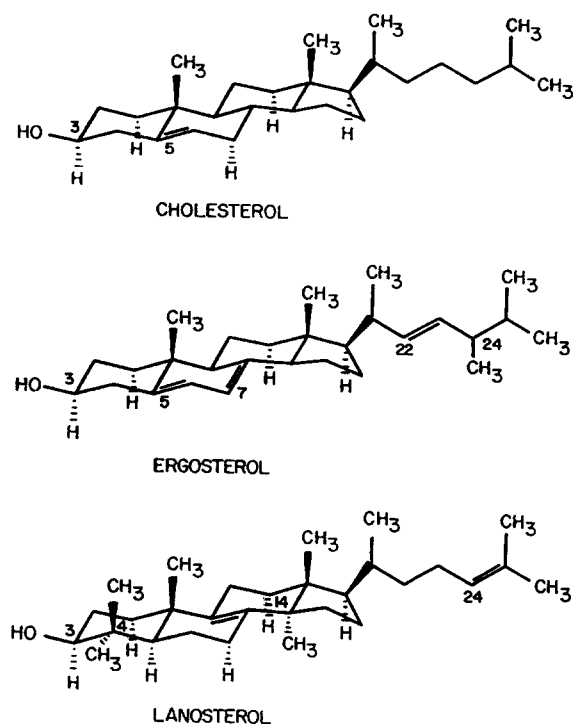


Fig. 1. Structures of cholesterol, ergosterol and lanosterol.

further purification. Cholesterol, ergosterol, 7-dehydro-cholesterol, stigmasterol and deuterium-depleted water were purchased from Sigma (St. Louis, MO). The sterols were recrystallized twice from ethanol immediately prior to use. Lanosterol was a product of Steraloids (Wilton, NH) and was used without further purification; its main impurity was 22-dihydrolanosterol.

## 2.2. Sample preparation

Samples for  $^2\text{H}$ -NMR spectroscopy were prepared by drying chloroform solutions containing the labeled phospholipids and sterols in the desired proportions under nitrogen, then eliminating residual solvent under high vacuum for at least 24 h. The lipid residues were resuspended using deuterium depleted water at 33 weight% solids, sealed in a glass tube, then subjected to 4–6 mixing and freeze ( $-20^\circ\text{C}$ ) thaw ( $50^\circ\text{C}$ ) cycles, prior to NMR spectroscopy. This procedure was found to be necessary in order to achieve good sample homogeneity and optimum spectral resolution. For  $^{13}\text{C}$  and  $^{31}\text{P}$ -NMR spectroscopy, the samples were prepared as above, except that the (unlabeled) dry lipids were resuspended at 50 weight% solids in  $^2\text{H}_2\text{O}$ .

## 2.3. $^2\text{H}$ -NMR spectroscopy

Deuterium NMR measurements were carried out at 55.25 MHz using a 'home-built' spectrometer equipped with an 8.45 Tesla 89 mm bore Oxford Instruments (Osney Mead, Oxford, UK) superconducting solenoid and a Nico-

let (Madison, WI) Model 1280/Explorer III-C computer for data analysis and instrument control. A composite-pulse version [57] of the quadrupolar echo sequence,  $(\pi/2)_y - \tau - (\pi/2)_x$ -acquire [58], provided relatively uniform radiofrequency excitation and was used in all cases. 4K complex data points were acquired using a dwell time of 2  $\mu\text{s}$ , and the resulting spin echoes were exponentially multiplied (100 Hz) prior to Fourier transformation. Typical  $90^\circ$  pulse widths were 1.8  $\mu\text{s}$ , the echo delay was 50  $\mu\text{s}$ , and the recycle delays were 300 ms. Transverse relaxation times ( $T_{2e}$ ) were obtained by varying the interpulse separation in an appropriate interval (50–4000  $\mu\text{s}$ ) and fitting the intensity of the acquired spectra to an exponential or gaussian function. For the analysis of the pulse frequency dispersion of the transverse relaxation a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence adapted to spin-1 nuclei was used  $(\pi/2)_y - \tau - ((\pi/2)_x - 2\tau)_n$ -acquire [59,60]. Temperature was controlled to  $\pm 1^\circ\text{C}$  by using a home-made variable temperature accessory, and was measured by inserting a thermocouple directly into the sample chamber of the NMR probe.

Order parameters for the CD bond vectors along the chain ( $S_{cd}$ ) were obtained from the quadrupolar splittings ( $\Delta\nu_q$ ), corresponding to the separation of the singularities of the experimental spectra, using the relationship:

$$\Delta\nu_q = \frac{3}{4} \frac{e^2 q Q}{h} S_{cd} \quad (1)$$

where  $e^2 q Q/h = 167$  kHz is the quadrupolar coupling constant for the deuteron in a CD bond [61].

First spectral moments,  $M_1$ , defined as:

$$M_1 = \frac{\int_0^\infty f(\nu) \nu d\nu}{\int_0^\infty f(\nu) d\nu} \quad (2)$$

where  $f(\nu)$  represents the spectrum as a function of frequency,  $\nu$ , measured from the center of the spectrum, were obtained using Nicolet software.

## 2.4. $^{13}\text{C}$ and $^{31}\text{P}$ -NMR spectroscopy

Cross-polarization magic-angle sample spinning  $^{13}\text{C}$ -NMR spectra were obtained using a Bruker AM-300 spectrometer operating at 75.472 MHz for  $^{13}\text{C}$  (6.7  $\mu\text{s}$   $90^\circ$  pulse, 3.9 kHz MAS spinning rate), or on a second 'home-built' spectrometer [62], using an Oxford Instruments 11.7 T 52 mm bore superconducting solenoid, equipped with a Nicolet 1280 computer, and a Doty Scientific (Columbia, SC) MAS NMR probe, operating at 125.720 MHz for  $^{13}\text{C}$  (9  $\mu\text{s}$   $90^\circ$  pulse, 3.2 kHz MAS spinning rate). All spectra were dipolar decoupled (40–100 watts  $^1\text{H}$  power). 16K complex data points were acquired and exponentially multiplied (7 Hz) prior to Fourier transformation. The following variations to the original CP double resonance method [63] were used to 'edit' [64] the



$^{13}\text{C}$ -NMR spectra, and extract different spin dynamics parameters in the membrane systems:

1. By allowing the  $^1\text{H}$  nuclei to relax in the rotating frame *before* the CP contact; this edits the subsequent CP  $^{13}\text{C}$  magnetization according to the  $T_{1\rho}$  behavior of the  $^1\text{H}$  nuclei directly interacting with each  $^{13}\text{C}$  nucleus. The  $^{13}\text{C}$  CP signals decay exponentially with the relaxation time of the  $^1\text{H}$  nuclei.
2. By allowing  $^{13}\text{C}$  spins to relax in their rotating frame *after* the CP contact. This allows the  $^{13}\text{C}$  magnetization to vary according to the  $T_{1\rho}$  behavior of the different  $^{13}\text{C}$  spins, while retaining the increased sensitivity of the CP experiment.
3. By inducing a dipolar echo [65–67] of the  $^1\text{H}$  spins and establishing the CP contact at the top of the echo. Varying the period between the phase-shifted  $90^\circ$   $^1\text{H}$  pulses leads to an exponential or gaussian decay (with a rate constant of  $1/T_{2e}(^1\text{H})$ ) of the maximum echo intensity and thus of the  $^{13}\text{C}$  signal directly generated by it. The decay of the dipolar echo amplitude for the acyl chains of phospholipids was found by Janes et al. [67] to follow a gaussian law, as predicted theoretically for loosely-coupled spin-1/2 pairs (i.e., methylene groups; [68]).
4. By using polarization inversion.

$^{31}\text{P}$ -NMR spectra (202.388 MHz) were obtained on the 500 MHz spectrometer using static samples and a Hahn echo sequence with proton decoupling and phase cycling. Typical length of the  $^{31}\text{P}$   $90^\circ$  pulse was 16  $\mu\text{s}$ . 4K complex data points were acquired and exponentially multiplied (50 Hz) prior to Fourier transformation.

### 3. Results

In this study we have compared several molecular properties of PC/sterol lipid bilayers using  $^2\text{H}$ ,  $^{13}\text{C}$  and  $^{31}\text{P}$ -NMR spectroscopy. *Ordering* of the phospholipid molecules has been inferred from the residual quadrupole splittings of specifically  $^2\text{H}$  labeled or perdeuterated phospholipid analogs, while long-range organization was obtained from  $^{31}\text{P}$ -NMR spectra. *Dynamical* properties were studied using transverse ( $T_{2e}$ )  $^2\text{H}$ -NMR relaxation rates, sensitive to slow ( $\sqrt{M_2} \ll \tau_2^{-1} \ll \omega_o$ , detectable by conventional spin echo decay) or ultraslow ( $\sqrt{M_2} \gg \tau_2^{-1}$ , detectable by the CPMG pulse sequence) motions. Independent confirmation was sought via analysis of  $^1\text{H}$  and  $^{13}\text{C}$  longitudinal relaxation rates in the rotating frame (sensitive to the spectral density of the molecular motions at the

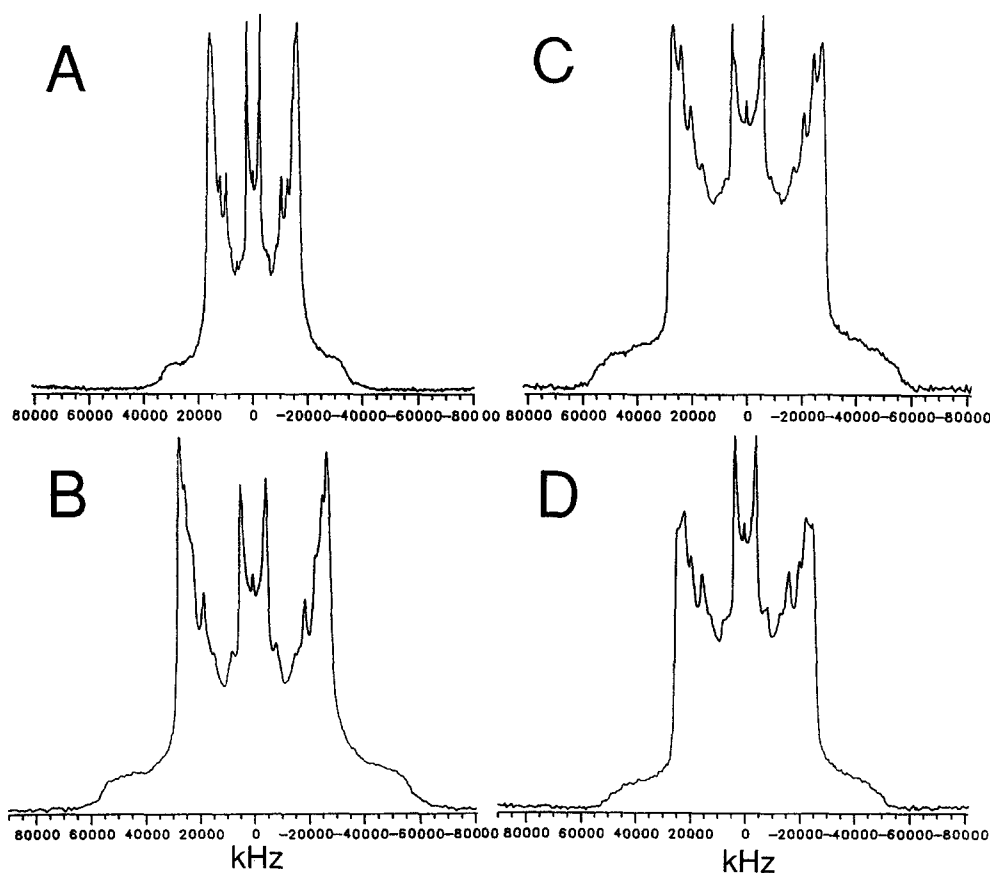


Fig. 2. 55.25 MHz  $^2\text{H}$ -NMR spectra of 2- $[\text{}^2\text{H}_{27}]$ DMPC in the absence (A) or presence of 30 mol% cholesterol (B), ergosterol (C) and lanosterol (D), in excess  $^2\text{H}$ -depleted water at  $25^\circ\text{C}$ . 3200 scans were accumulated for each spectrum.



rotating-frame resonance frequencies, which were 28–37 kHz in our experiments) as well as by the study of the  $^1\text{H}$ - $^{13}\text{C}$  cross-polarization and  $^1\text{H}$  dipolar echo decay rates, which provided information on the spectral density near zero frequency (for the well-matched Hartman-Hahn conditions of our experiments). Both saturated (DMPC) and unsaturated (POPC) PC-sterol membranes were studied, and the sterol/phospholipid mol ratio was 7:3 in all the experiments. At this mole ratio and at 25°C it is known that both DMPC/cholesterol and POPC/cholesterol membranes are in the liquid-ordered ( $l_o$ ) state [7,8,10–12,15,16,30,34]. Incorporation of other sterols was assessed by visual inspection of  $^{13}\text{C}$  MAS spectra in order to observe phase separated or ‘free’ sterol [62].

### 3.1. DMPC and DMPC / sterol membranes

#### $^2\text{H}$ -NMR studies

Fig. 2 presents the 55.25 MHz  $^2\text{H}$ -NMR spectra of 2- $[\text{}^2\text{H}_{27}]$ DMPC, 2- $[\text{}^2\text{H}_{27}]$ DMPC/cholesterol, 2- $[\text{}^2\text{H}_{27}]$ DMPC/ergosterol and 2- $[\text{}^2\text{H}_{27}]$ DMPC/lanosterol model membranes (30 mol% sterols, 33 wt% total lipids in  $\text{}^2\text{H}$ -depleted water, 25°C). Fig. 3A shows the order profiles (segmental order parameters,  $S_{\text{cd}}$ , of the methylene groups of the *sn*-2 chain as a function of chain position) of the different membrane systems. These were obtained from the individual spectra of specifically deuterated DMPCs labeled at positions 2',3',4',6',8',10',12' of the *sn*-2 chain, and by comparison of these spectra with those of DMPC containing a perdeuterated *sn*-2 chain (2- $[\text{}^2\text{H}_{27}]$ DMPC, Fig. 2), in the absence and presence of the three different sterols. It can be seen from Fig. 3A that the ordering effects of the sterols is ergosterol > cholesterol >> lanosterol. In Fig. 3B we plot the same order parameters, but ‘smoothed’ according to a procedure suggested by Laffleur et al. [69], that is, the data are sorted assuming a monotonic decrease of  $S_{\text{cd}}$  as a function of segment position. The sorted profiles are very similar in shape to those obtained by several groups using this procedure for the analysis of the spectra of perdeuterated saturated chains in both artificial and natural membranes [69–73] but are clearly quite different to the real experimental profiles (Fig. 3A). This is particularly evident in the region from C2/3 to C6/8 where in the actual profiles we find a pronounced increase in order with segment position, while the smoothed or sorted profiles show a constant or slowly decreasing order with segment position.

Transverse  $^2\text{H}$  relaxation processes in phospholipid membranes appear to be dominated by slow motions such as order director fluctuations [9,60,74–77] or surface undulations [78,79], although lateral diffusion of individual phospholipid molecules has also been proposed as a possible relaxation mechanism [59,80]. These models of order director fluctuations or surface undulations predict that the transverse  $^2\text{H}$  relaxation rates ( $1/T_{2e}$ ) should be proportional to the second power of the bond order parameter

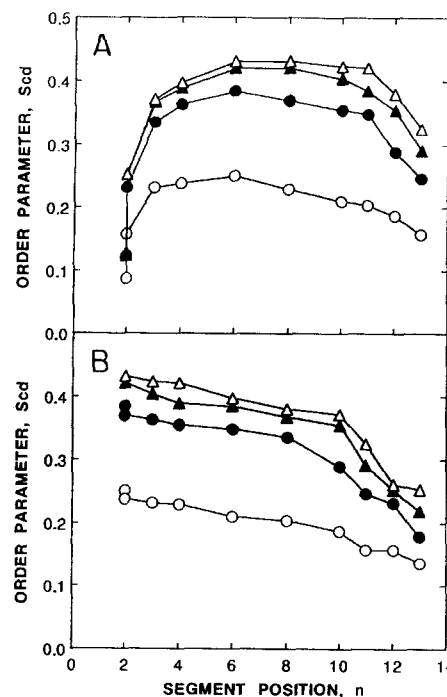


Fig. 3.  $^2\text{H}$ -NMR order profiles for lipid, lipid-sterol bilayers. (A) Order profile for DMPC and DMPC/sterol membranes at 30 mol% of sterols, in excess water at 25°C. The segmental order parameters were obtained from the quadrupolar splittings of DMPCs specifically deuterated at the 2',3',4',6',8',10' and 12' methylene segments of the *sn*-2 chain. The quadrupolar splittings for positions 11' and 13' were deduced by comparison of spectra of specifically-labeled lipids with those of DMPC containing a perdeuterated *sn*-2 chain (2- $[\text{}^2\text{H}_{27}]$ DMPC). No corrections were attempted for the small change in  $T_c$  of the  $d_{27}$ -lipid.  $\circ$ , pure DMPC;  $\blacktriangle$ , DMPC/cholesterol;  $\triangle$ , DMPC/ergosterol;  $\bullet$ , DMPC/lanosterol. (B) ‘Smoothed’ order profile for DMPC and DMPC/sterol membranes obtained from the data presented in the top panel. Symbols as in A.

( $S_{\text{cd}}$ ) and to  $\cos^2\theta_N \cdot \sin^2\theta_N$  (where  $\theta_N$  gives the orientation of the average director in the laboratory frame), while they are inversely related to the effective elastic constant,  $K$ , of the membrane, and to the coherence length,  $d$ , related to the membrane thickness [60,78]. Table 1 shows a common dependence of  $(1/T_{2e})$  with  $S_{\text{cd}}^2$  (measured from the intensities at the singularities of the spectra, i.e.,  $\theta_N = 90^\circ, 45^\circ$ ) for the relaxation of deuterons at positions 6' and 12' for DMPC, DMPC/cholesterol and DMPC/ergosterol, indicating that in these cases the contributions of order director fluctuations to the relaxation rates are similar [81]. In contrast, the DMPC/lanosterol membranes have larger transverse relaxation rates, despite the fact that their  $S_{\text{cd}}$  values are smaller.

We next investigated the effects of sterols on the quadrupole splittings and transverse relaxation rate of the choline headgroup, using  $[\text{}^2\text{H}_6]$ DPPE alone, and in mixed membranes containing different sterols, both below (25°C) and above (45°C) the main phase transition of the pure phospholipid. Table 2 shows the results of such studies. Here, it can be seen that the quadrupolar splitting of the headgroup of the pure phospholipid in the gel ( $L_\beta$ ) phase



Table 1  
Quadrupolar echo ( $T_{2e}$ ) relaxation in specifically  $^2\text{H}$ -labeled DMPC and DMPC/sterol membranes at  $25^\circ\text{C}$ <sup>a</sup>

System	$\theta_N$ ( $^\circ$ )	$\Delta\nu_Q$ (kHz)	$S_{cd}^2$	$1/T_{2e}$ ( $\text{s}^{-1}$ )
2-[6',6'- $^2\text{H}_2$ ]DMPC	90	32	0.063	2028
	45	16	0.016	2941
DMPC + cholesterol <sup>b</sup>	90	53.6	0.177	2101
	45	26.8	0.044	3817
DMPC + ergosterol <sup>b</sup>	90	55.2	0.187	2353
	45	27.6	0.047	3623
DMPC + lanosterol <sup>b</sup>	90	49.2	0.149	2882
	45	24.6	0.037	4878
2-[12',12'- $^2\text{H}_2$ ]DMPC	90	24	0.035	1631
	45	12	0.009	3030
DMPC + cholesterol <sup>b</sup>	90	45.2	0.126	1887
DMPC + ergosterol <sup>b</sup>	90	48.5	0.145	1961
DMPC + lanosterol <sup>b</sup>	90	37	0.084	3155
	45	18.5	0.021	3610

<sup>a</sup> Relaxation data were obtained using the quadrupolar echo pulse sequence, as described in the text. The interpulse delay varied between 100 and 1000  $\mu\text{s}$ , and 16000 spectra were accumulated for each time point.

<sup>b</sup> DMPC/sterol mol ratio = 7:3.

is significantly higher than that observed in sterol binary mixtures at the same temperature, suggesting a disruption of the packing of the phospholipid molecules by the sterols [82]. This idea receives additional support from the observation of an increase in the values of the transverse relaxation times. It must be noted, however, than the DPPC/ergosterol membranes have the largest quadrupolar splittings and the shortest relaxation times among all the sterol-containing membranes at low temperature, and that this effect is carried over to the high temperature ( $l_o$ ) phase, where the ergosterol containing membranes display the highest headgroup quadrupolar splittings among all systems studied. These results suggest that the higher order induced by ergosterol in the acyl chains of the phospholipid may also be reflected in the polar headgroup region, possibly in part due to decreased rigid body motions.

Table 2  
Quadrupolar echo ( $T_{2e}$ ) relaxation in [ $^2\text{H}_9$ ]DPPC and [ $^2\text{H}_9$ ]DPPC/sterol membranes<sup>a</sup>

System	Temperature ( $^\circ\text{C}$ )	$\Delta\nu_Q$ (kHz)	$1/T_{2e}$ ( $\text{s}^{-1}$ )
[ $^2\text{H}_9$ ]DPPC	24	1.7	629
	45	0.93	696
[ $^2\text{H}_9$ ]DPPC/CHOL <sup>b</sup>	25	1.0	482
	45	0.83	554
[ $^2\text{H}_9$ ]DPPC/ERG <sup>b</sup>	24	1.33	617
	45	1.07	484
[ $^2\text{H}_9$ ]DPPC/LANO <sup>b</sup>	24	1.13	495
	45	0.93	320

<sup>a</sup> Relaxation data were obtained using the quadrupolar echo pulse sequence, as described in the text. The interpulse delay was varied between 100 and 4000  $\mu\text{s}$ , and 256 spectra were accumulated for each time point.

<sup>b</sup> DPPC/sterol mol ratio = 7:3.

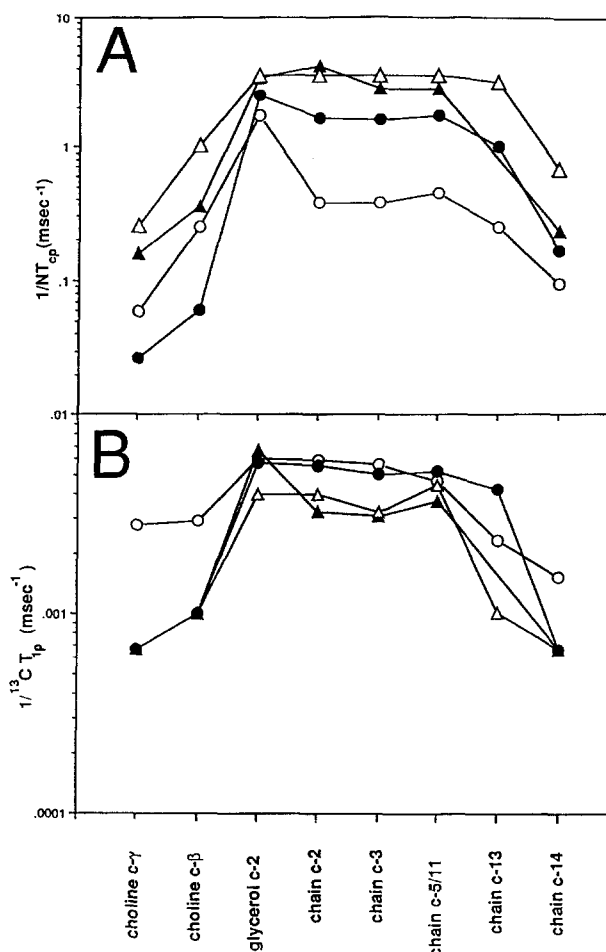


Fig. 4. Carbon-13 NMR CP results for DMPC, DMPC/sterol bilayers. (A) Cross-polarization and (B)  $^{13}\text{C}$  longitudinal relaxation rates in the rotating frame ( $T_{1\rho}$ ,  $\omega_{rf} = 28$  kHz) for the different carbon sites illustrated in DMPC and DMPC/sterol membranes. Symbols used are as in Fig. 3. Phospholipid/sterol mol ratio, 7:3; temperature  $25^\circ\text{C}$ .

In parallel with the above  $^2\text{H}$ -NMR experiments, we investigated the same phospholipid/sterol systems by  $^{13}\text{C}$  CP-MAS NMR methods. We measured, using the procedures described above, the values of several spin dynamics parameters for 8–9 individual resonances in DMPC and DMPC/sterol binary mixtures (7:3 mol ratio), at  $25^\circ\text{C}$  and in the presence of excess water, and the results are presented in Fig. 4 and Fig. 5. The cross-polarization rates, ( $1/T_{CP}$ ; Fig. 4A), were obtained from both CP signal intensity versus mixing time curves and from CP-polarization inversion experiments. Their values are quite accurate given the very large ( $>$  two orders of magnitude) difference between the cross-polarization and rotating frame spin-lattice relaxation rates for all lipid systems [83]. These rates, which give a measure of the strength of the local  $^{13}\text{C}$ - $^1\text{H}$  dipolar interactions, and the degree of motional freedom of the spins involved, map the mobilities of the different parts of the phospholipid molecules. The glycerol backbone is shown, as expected [62] to be the most rigid part, while the acyl chains and the choline headgroup



display the familiar gradients of mobility, increasing towards their 'free' ends. More interestingly, the presence of sterols in the membranes decreases the mobility of essentially *all* regions of the phospholipid molecules, the effects being most dramatic (approximately a 10-fold reduction in  $T_{CP}^*$ ) for the first 2/3 of the acyl chains, but also being clearly felt in the terminal parts of the chains, as well as for the glycerol backbone *and* the choline headgroup. The effectiveness of the various sterols in inducing these effects was ergosterol > cholesterol >> lanosterol, in accord with the  $^2\text{H}$ -NMR results on DMPC and DPPC presented above (Fig. 2, Table 1 and Table 2). No evidence for phase separation was seen in the  $^{13}\text{C}$  MAS NMR spectra.  $^1\text{H}$   $T_{1\rho}$  values (Fig. 5A), which are sensitive to motions in the range of the rotating-frame resonance frequency were found to be very similar in the different regions of the phospholipid molecules, indicating spin diffusion effects. However, the  $^1\text{H}$   $T_{1\rho}$  values do reflect an increase in the density of motions in the kilohertz range in the sterol-containing membranes when compared with the pure phospholipid;

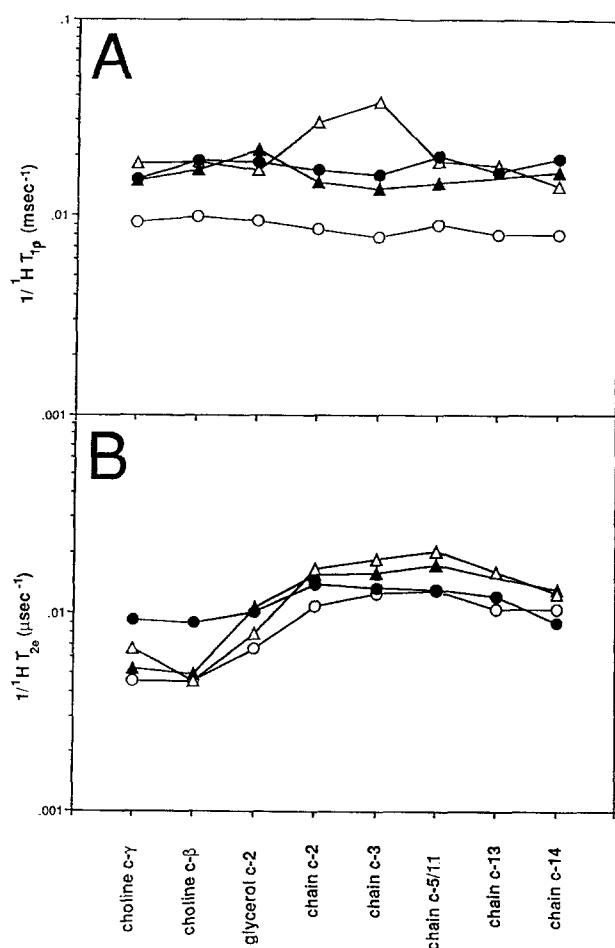


Fig. 5.  $^1\text{H}$  longitudinal relaxation rates in the rotating frame ( $T_{1\rho}$ ,  $\omega_{rf}^H = 28$  kHz (A) and  $^1\text{H}$  dipolar echo decay rates for protons directly attached to different carbon atom sites in DMPC and DMPC/sterol membranes (B). Symbols as in Fig. 3. Phospholipid/sterol mol ratio, 7:3; temperature 25°C.

Table 3

Quadrupolar echo ( $T_{2e}$ ) relaxation in  $[^2\text{H}_{31}]\text{POPC}$  and  $[^2\text{H}_{31}]\text{POPC/sterol membranes}^a$

System	Temperature (°C)	$\Delta\nu_Q$ (kHz)	$S_{cd}^2$	$1/T_{2e}$ ( $s^{-1}$ )
$[^2\text{H}_{31}]\text{POPC}$	25	25	0.038	1342
	34	24	0.035	1408
$[^2\text{H}_{31}]\text{POPC/CHOL}^b$	25	43.6	0.117	1724
	35	41.2	0.104	1585
$[^2\text{H}_{31}]\text{POPC/ERG}^b$	25	31.2	0.060	1776
	34	28	0.048	1795
$[^2\text{H}_{31}]\text{POPC/LANO}^b$	25	31.6	0.061	2101
	35	29.2	0.052	2033
$[^2\text{H}_{31}]\text{POPC/DHC}^{b,c}$	25	39.5	0.096	3195
$[^2\text{H}_{31}]\text{POPC/STG}^{b,d}$	25	39	0.094	3413

<sup>a</sup> Relaxation data were obtained using the quadrupolar echo pulse sequence, as described in the text. The interpulse delay was varied between 100 and 1000  $\mu\text{s}$  and 5600 spectra were accumulated for each time point.

<sup>b</sup> POPC/sterol mol ratio = 7:3.

<sup>c</sup> DHC, 7-dehydrocholesterol.

<sup>d</sup> STG, stigmasterol.

the effectiveness of the different sterols in inducing these effects parallels their effects on  $T_{CP}^*$ .

$^{13}\text{C}$   $T_{1\rho}$  values (Fig. 4B), sensitive to the same range of frequencies as the  $^1\text{H}$   $T_{1\rho}$  but without impediment from spin diffusion effects, show, as expected, distinctly different values in the different regions of the phospholipid molecule. However, only minor changes in these parameters are observed in the presence of sterols. Finally,  $^1\text{H}$   $T_{2e}$  values (Fig. 5B), obtained from dipolar echo-edited CP spectra of the different  $^{13}\text{C}$  resonances (gaussian decays in all cases) indicate a gradient of mobility within the phospholipid molecules and relative effects of the different sterols on them essentially identical to those derived from the analysis of  $T_{CP}^*$  values, despite the effects of spin diffusion.

### 3.2. POPC and POPC / sterol membranes

When we studied the effects of the three sterols on a phospholipid containing an unsaturated acyl chain in the *sn*-2 position ( $1-[^2\text{H}_{31}]\text{POPC}$ ), we obtained results rather different to those described above for the saturated PCs, at the same sterol molar proportion (30%) and temperature (25°C). Fig. 6 and Table 3 show that the quadrupolar splittings in this case decrease in the order POPC/cholesterol > POPC/ergosterol = POPC/lanosterol > POPC. Furthermore, it can be seen from Table 3 that the transverse relaxation data for POPC/lanosterol and POPC/ergosterol depart from the correlation between  $1/T_{2e}$  and  $S_{cd}^2$  observed for POPC and POPC/cholesterol, since they have faster transverse relaxation rates than POPC/cholesterol, despite the fact that their  $S_{cd}$  values are smaller.

We also explored the effects of different sterols on the ultraslow ( $\tau_2^{-1} \ll \sqrt{M_2}$ ) motions present in PC mem-



branes, detectable using the CPMG pulse sequence [59,60]. Fig. 7 shows for 1- $^{2}\text{H}_{31}$ POPC and 1- $^{2}\text{H}_{31}$ POPC/cholesterol that the echo decay for the CPMG sequence departs from a simple quadrupolar echo decay for all interpulse delays tested ( $2\tau = 100, 220$  and  $420 \mu\text{s}$ ), indicating that  $\tau_2 \gg 420 \mu\text{s}$  ( $\omega_2 \ll 2.4 \text{ kHz}$ ). However, for 1- $^{2}\text{H}_{31}$ POPC/ergosterol and 1- $^{2}\text{H}_{31}$ POPC/lanosterol, we obtain a significant departure from the quadrupolar echo decay (down to  $\sim 20\%$  of the original echo amplitude) only when the interpulse delay was  $100 \mu\text{s}$ , indicating that for these systems the characteristic correlation time *decreases* by at least a factor of four.

To investigate the possibility that sterols may be incorporated to different extents into bilayer membranes [13], thereby influencing the results observed, we carried out titration experiments with POPC and DMPC sterol mixture using high resolution  $^{13}\text{C}$ -NMR (simple Bloch decays with MAS), as well as  $^2\text{H}$ -NMR. Typical results are shown in Fig. 8 for ergosterol and suggest that both ergosterol and lanosterol (not shown) are completely incorporated into the bilayers up to about 30 mol%, above which significant broadening and the appearance of new peaks are observed, indicating the appearance of a second phase. Fig. 9 shows that ergosterol increases linearly the first moment of the

$^2\text{H}$ -NMR spectrum of the acyl chains of 1- $^{2}\text{H}_{31}$ POPC up to about 25 mol%, but beyond this level the ordering effects decrease and stay relatively constant up to 50%. Diphasic effects were also observed with the transverse relaxation rates ( $1/T_{2e}$ ) which, however, increased up to the highest concentrations, despite the fact that the first moment did not vary significantly above 25%.  $^{31}\text{P}$ -NMR spectra of all POPC/sterol systems (not shown) demonstrated that, at 25–30 mol%, they all display a bilayer configuration of POPC, as shown by the typical axially symmetric chemical shift anisotropy ( $\Delta\sigma \sim 48 \text{ ppm}$ ).

These same lipid membrane systems were also investigated using the CP-polarization inversion and dipolar echo-CP sequences under MAS conditions (see Materials and methods) and the results are presented in Fig. 10 and Fig. 11. It can be seen that the relative rigidity of the acyl chain and glycerol backbone atoms (indicated by the intensities of their nuclear resonances in the polarization inversion-edited spectra, Fig. 10, and the values of their  $^1\text{H}$  dipolar echo decay rates, Fig. 11) in the different membranes follow the sequence: POPC/cholesterol > POPC/ergosterol  $\sim$  POPC/lanosterol > POPC. For the dipolar echo decay rates this was most evident in the head-group region. These results are in sharp contrast with those obtained with the DMPC systems (Fig. 4 and Fig. 5)

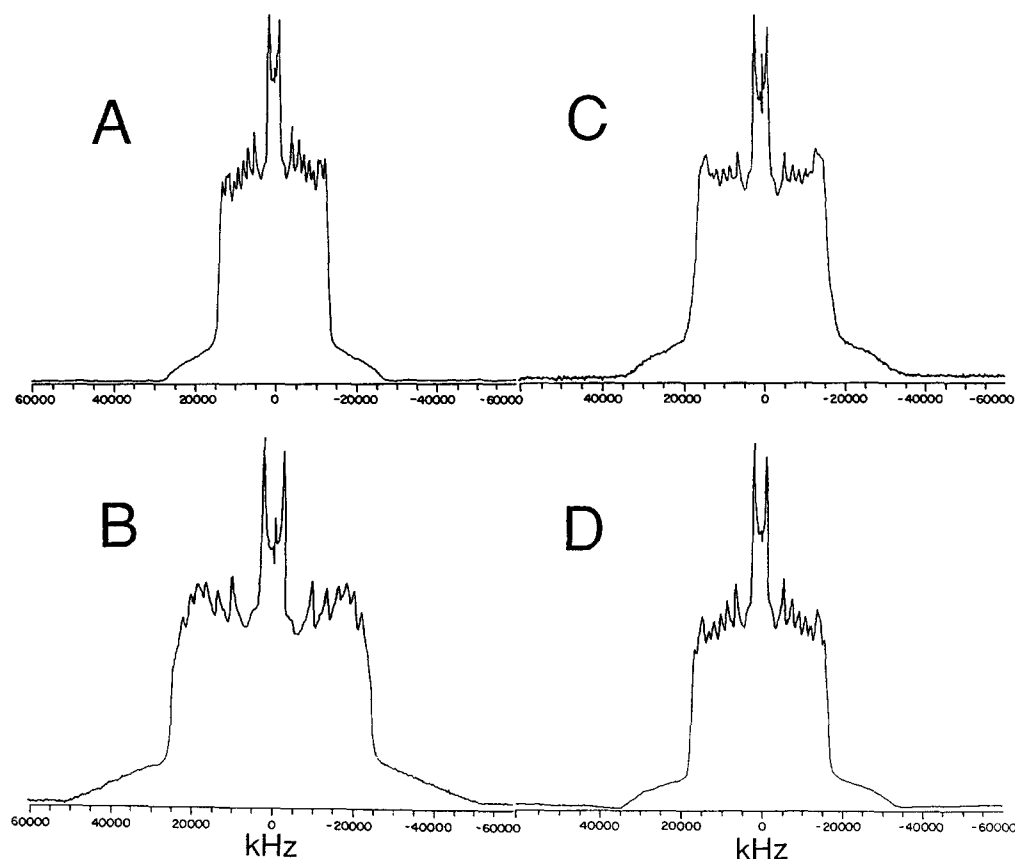


Fig. 6. 55.25 MHz  $^2\text{H}$ -NMR spectra of 1- $^{2}\text{H}_{31}$ POPC in the absence (A) or presence of 30 mol% of cholesterol (B), ergosterol (C) and lanosterol (D), in excess  $^2\text{H}$ -depleted water at  $25^\circ\text{C}$ . 32 000 scans were accumulated for each spectrum.



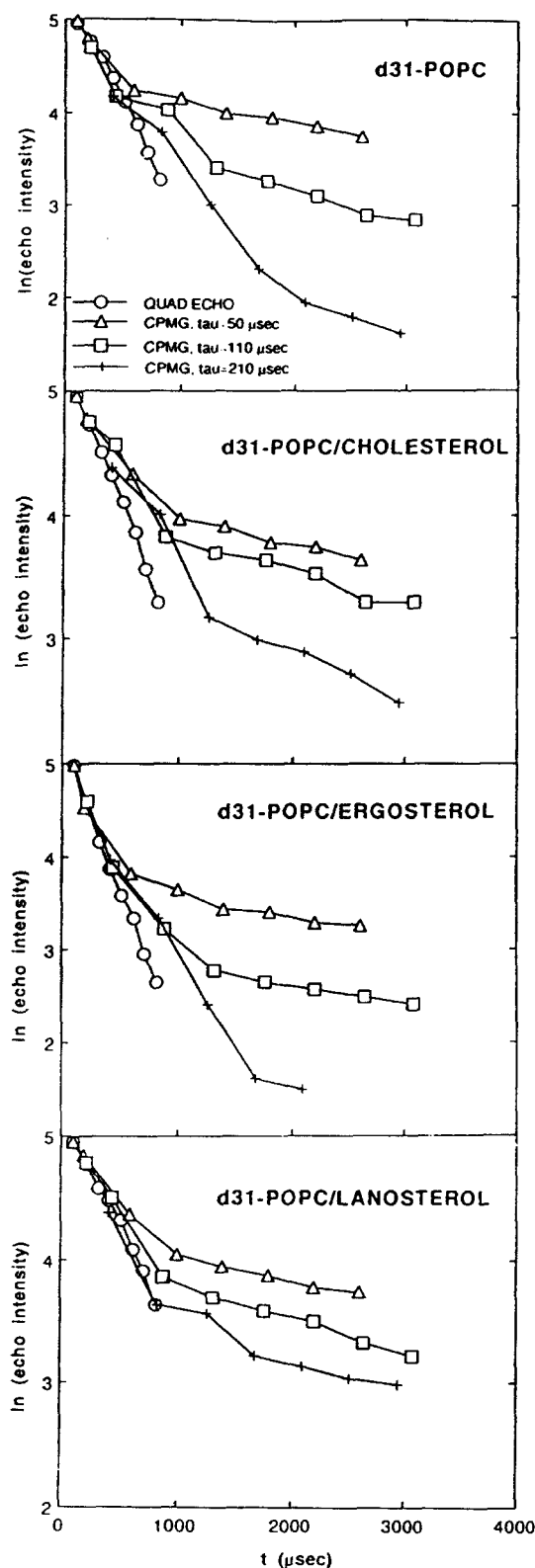


Fig. 7. 55.25 MHz  $^2\text{H}$ -NMR spin-echo decays using conventional quadrupolar echo or CPMG sequences for deuterons of  $1\text{-}[^2\text{H}_{31}]\text{POPC}$  in the absence or presence (from top to bottom) of 30 mol% of cholesterol, ergosterol and lanosterol in excess water at  $25^\circ\text{C}$ .  $\circ$ , Conventional quadrupolar echo decay;  $\Delta$ ,  $\square$  and  $+$ , CPMG echo decays for interpulse delays ( $2\tau$ ) of 100, 220 and 420  $\mu\text{s}$ , respectively. 1400 echoes were accumulated for each data point.

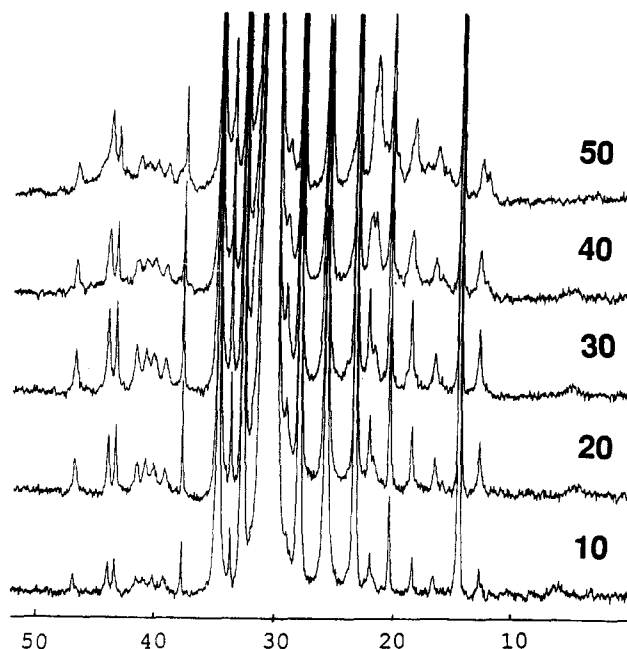


Fig. 8. 11.7 Tesla  $^{13}\text{C}$  MAS NMR spectra of POPC/ergosterol at the mol% ergosterol indicated. Spectra were recorded using  $^1\text{H}$  dipolar decoupled Bloch decays.

but in accord with results obtained on  $1\text{-}[^2\text{H}_{31}]\text{POPC}$  and  $1\text{-}[^2\text{H}_{31}]\text{POPC/sterol}$  membranes by  $^2\text{H}$ -NMR (Table 3 and Fig. 7).

#### 4. Discussion

The order profiles obtained from the  $^2\text{H}$ -NMR spectra of specifically labeled DMPCs with singly deuterated methylene groups in the *sn*-2 chain, and from the same molecule but with a perdeuterated *sn*-2 chain (Fig. 3A), for pure DMPC and DMPC/cholesterol, agree very well with previous reports from this and other laboratories on DMPC and other saturated PCs [6,8,82,84,85]. In particu-

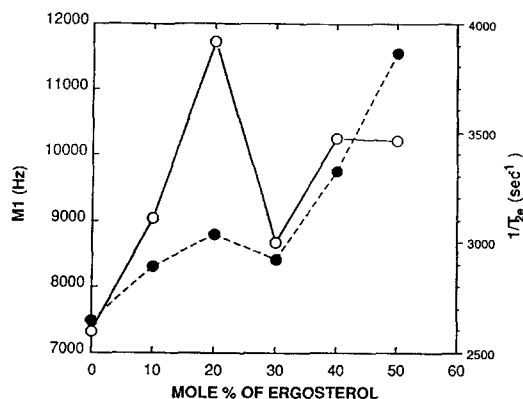


Fig. 9. Spectral first moments ( $M_1$ ,  $\circ$ ) and transverse relaxation rates ( $1/T_{2e}$ ,  $\bullet$ ) of  $1\text{-}[^2\text{H}_{31}]\text{POPC}$ , in the presence of increasing concentrations of ergosterol, in excess water, at  $25^\circ\text{C}$ . 8000 scans were accumulated for each spectrum.



lar, the low values of the segmental order parameters for the methylene segments at chain positions 2' and 3' (comparable to those of the terminal methylene segments) is a characteristic feature of the *sn*-2 chain, due to its particular packing restrictions in the bilayer. No previous reports on the effects of ergosterol on the order or dynamics of the acyl chains of saturated PCs exist, but our results using both  $^2\text{H}$  and  $^{13}\text{C}$  solid state NMR clearly indicate that this sterol at a mol ratio of 30% induces higher order of the acyl chains than does cholesterol, and that this ordering effect extends to the terminal methylene segments and the choline headgroup. Presumably, the bulkier and stiffer

(due to the  $\Delta^{22}$  double bond, see Fig. 1) side chain of ergosterol restricts the rapid segmental motions of the terminal methylene segments and the rotational motion of the whole molecule more effectively than does cholesterol. On the other hand, the effects of lanosterol on the acyl chain order of DMPC are consistent with its molecular structure and with previous studies using a variety of techniques. In particular, the presence of three bulky methyl groups at positions 4,4' and 14 as well as the side chain  $\Delta^{24}$  double bond, appear to introduce steric restraints onto the  $\alpha$  face of the sterol for its interaction with the acyl chains of the phospholipids [1], a conclusion which has

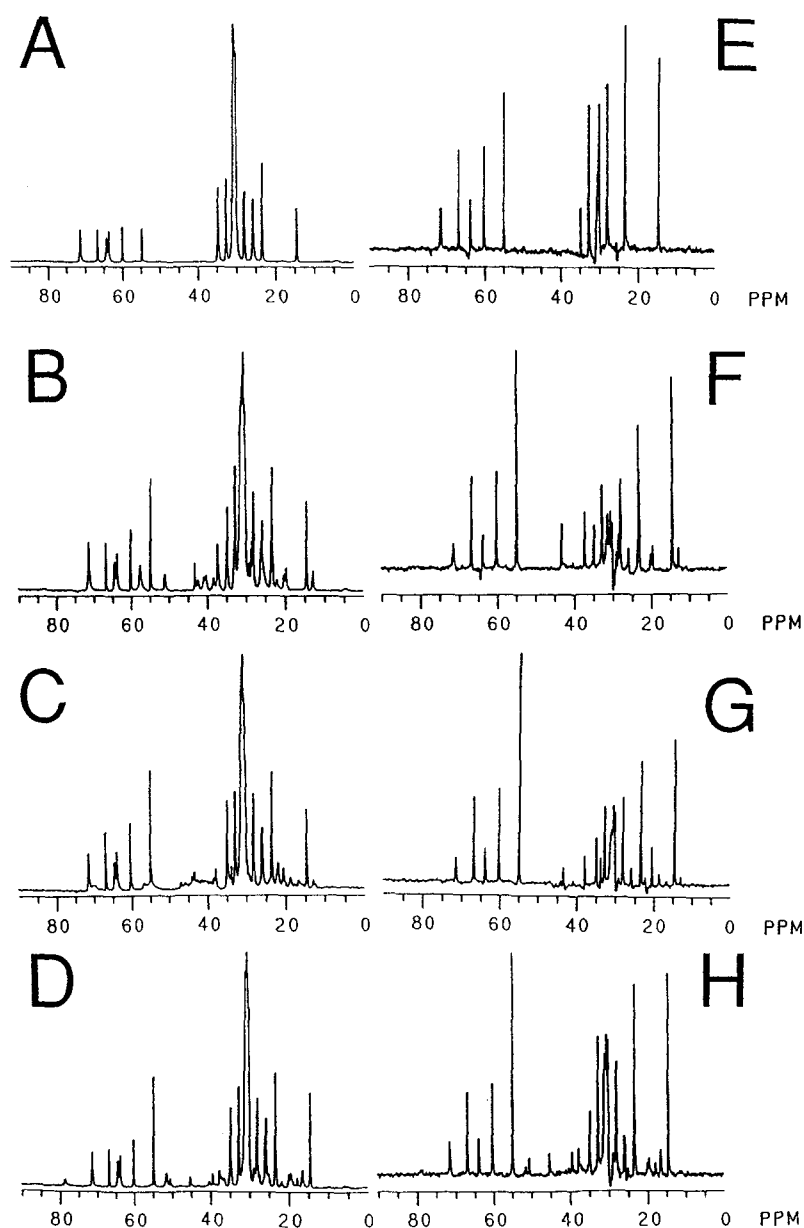


Fig. 10. Polarization inversion-edited 125.71 MHz  $^{13}\text{C}$  CP/MAS NMR spectra of POPC and POPC/sterol membranes, in excess  $^2\text{H}_2\text{O}$  at  $25^\circ\text{C}$ . Conventional CP/MAS spectra (20 ms mix time, A–D) and polarization-inversion-edited CP/MAS spectra (20 ms mixing time, 500  $\mu\text{s}$  total polarization inversion time, E–H) were acquired for POPC in the absence (A,E) or presence of 30 mol% of cholesterol (B,F), ergosterol (C,G) or lanosterol (D,H). Spectra E–H were amplified  $4\times$  (vertically) to facilitate comparison with (A)–(D). 2000 scans were accumulated for each spectrum. Chemical shifts are in ppm from external TMS.



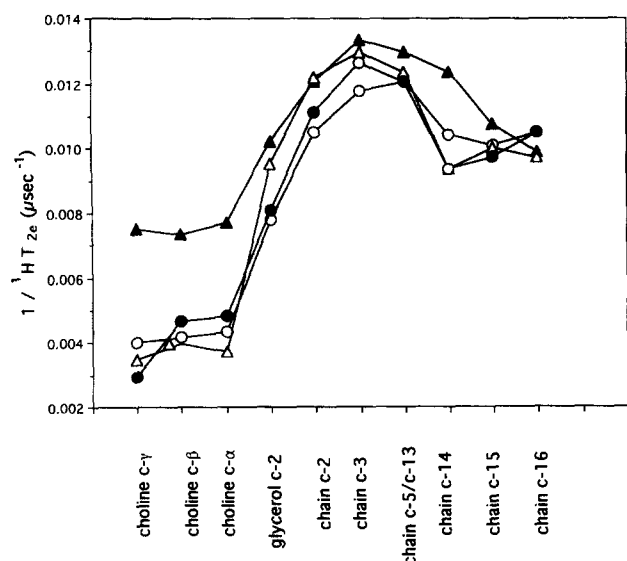


Fig. 11.  $^1\text{H}$  dipolar echo decay rates for protons directly attached to different carbon sites in POPC and POPC/sterol membranes. Phospholipid/sterol mol ratio, 7:3; temperature  $25^\circ\text{C}$ .  $\circ$ , Pure POPC;  $\blacktriangle$ , POPC/cholesterol;  $\triangle$ , POPC/ergosterol;  $\bullet$ , POPC/lanosterol.

been drawn from EPR of spin-label fatty acids in PC vesicles [86], fluorescence depolarization measurements in artificial and natural membranes [87–89], and  $^2\text{H}$ -NMR studies of artificial [2,90] and *Mycoplasma capricolum* [91] membranes. In all these studies it has been shown that the trimethylated sterol has a very limited capacity to order acyl chains in phospholipid membranes. This in turn is consistent with the limited capacity of lanosterol to restrict the passive permeability of polar molecules such as glucose through these structures [92]. Concerning our experimentally derived order profiles, their similarity with the original data of Seelig and Seelig [94] for the 1-chain in POPC should be noted, as should their differences from those obtained from the same spectral data obtained using the ‘smoothing’ procedure proposed by Lafleur et al. [69] and Sternin et al. [93]. Previously reported monotonically decreasing order profiles as a function of ‘carbon number’ or ‘segment position’ often only represent order profiles as a function of *line position*, and it seems that any structural results derived from such smoothed profiles must be viewed carefully. Properties inferred from *mean values* of the segmental order parameters (which are, of course, independent of the sorting procedure) are of course correct.

$^2\text{H}$  transverse relaxation in phospholipid membranes has received increased attention in recent years since it can give interesting information on slow and ultraslow movements of phospholipid molecules. These are expected to modulate and in turn be affected by the interaction of the lipids with other molecules found in natural membranes [9,59,60,74,79,81,95]. In the present study, we have found that for DMPC, DMPC/cholesterol and DMPC/ergosterol membranes, the transverse relaxation rates are approximately correlated with  $S_{\text{cd}}^2$ , and are generally consistent

with the  $\cos^2\theta_N \cdot \sin^2\theta_N$  dependence (Table 1) predicted by theoretical models which propose that order director fluctuations or surface undulations are dominant transverse relaxation mechanisms in fluid  $L_\alpha$  or  $l_o$  phases [9,60,74–79]. In contrast, the relaxation rates for DMPC/lanosterol membranes fall outside the correlation defined by the other three systems, since their values are much greater than those expected from the value of bond order parameters. Huang et al. [91] have also found in membranes of *Mycoplasma capricolum* containing lanosterol that transverse relaxation rates are greater than those observed for membranes from organisms grown in the presence of cholesterol, although the segmental order parameters were smaller for the lanosterol-containing membranes. These results indicate that the density of collective phospholipid motions in DMPC membranes is increased by lanosterol –most probably as a consequence of a decrease in the coherence length and/or a decrease in the elastic constants of the membrane, due to disruption of lipid–lipid interactions, as proposed by Watnick et al. for the interaction of chlorophyll *a* with DMPC [74,81].

The relative effects of the different sterols on the order and dynamics of the DMPC molecules, as detected by  $^2\text{H}$ -NMR, were independently investigated by using solid-state  $^{13}\text{C}$ -NMR experiments, via CP-MAS techniques (Fig. 4 and Fig. 5). It is of some interest that the strong similarity of the shapes and relative amplitudes of the curves shown in Fig. 3A ( $^2\text{H}$ ) and 4A ( $^{13}\text{C}$ ) is, in fact, expected on theoretical grounds [83], since:

$$(1/T_{\text{cp}}) \propto S_{\text{CH}}^2 J_x(\Delta\omega_c) \quad (3)$$

where  $T_{\text{cp}}$  is the cross-polarization time,  $S_{\text{CH}}$  the bond order parameter, and  $J_x(\Delta\omega_c)$  is the cross polarization spectral density at a frequency ( $\Delta\omega_c$ ) equal to the difference between the effective resonance frequencies of proton and carbon nuclei in the rotating frame (which is almost zero under our experimental conditions). When we plotted the  $^{13}\text{C}$ - $^1\text{H}$  cross polarization rates measured in the different lipid systems against the corresponding bond order parameters (obtained from  $^2\text{H}$ -NMR, Fig. 3A; the bond order parameters for the methyl groups in the acyl chains and choline headgroup were corrected using the corresponding geometrical factors assuming fast rotation) we found (Fig. 12A) the expected power ( $n=2$ ) function dependency. The scatter of the data most probably reflects the variation of the spectral densities, which are sensitive to the details of the dynamic processes occurring. Also of interest, we have found that the ratio of the cross polarization rates divided by the square of the (measured) bond order parameters which according to Eq. (3) should give us a quantity directly proportional to the cross polarization densities at zero or near zero frequency) yields a series of profiles which show that the sterols restrict the mobilities of all the acyl carbons atoms in DMPC proportionally to their ordering effects (Fig. 12B). For the headgroup choline methyl groups, both cholesterol and ergosterol restrict



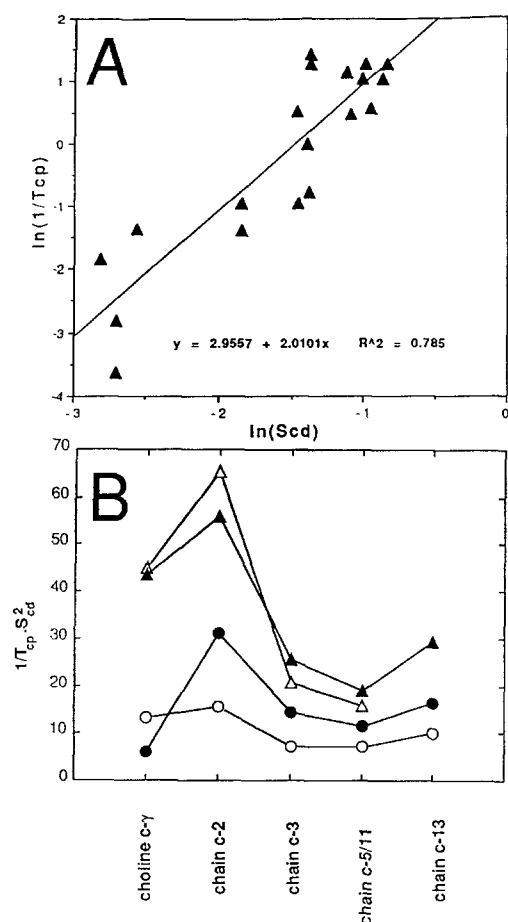


Fig. 12. (A) Correlation between  $^1\text{H}$ - $^{13}\text{C}$  cross polarization rates (obtained from CP/MAS  $^{13}\text{C}$ -NMR experiments), and bond order parameters ( $S_{cd}$ , obtained from  $^2\text{H}$ -NMR experiments) for carbon atoms in DMPC and DMPC/sterol bilayer membranes in excess water, at 25°C. The observed slope is 2.01 (the theoretical value is 2), and the Pearson correlation coefficient,  $R^2$ , is 0.785. (B) Graphical representation of  $1/(T_{cp} \cdot S_{cd}^2)$  a quantity proportional to the cross polarization spectral density at zero frequency (well-matched Hartmann-Hahn condition) for different carbon atom sites in DMPC and DMPC/sterol bilayers. Symbols as in Fig. 3.

motion, but lanosterol does not. Confirmatory evidence for these differential effects was obtained from the  $^1\text{H}$  dipolar echo decay rates (Fig. 5B), despite the presence of spin diffusion as manifest in the  $^1\text{H}$   $T_{1\rho}$  values (Fig. 5A).

Previous studies using  $^{13}\text{C}$  and  $^{19}\text{F}$  longitudinal relaxation rates in the laboratory and rotating frames [96–98] have indicated that cholesterol increases the ratio of  $T_{1\rho}$  to  $T_{1z}$  in pure phospholipids and phospholipid mixtures extracted from biological membranes, which suggests a marked increase in low frequency ( $10^5$  to  $10^7$  Hz) motions in the bilayer. These results are confirmed in this work (Fig. 5A), and are extended to ergosterol and lanosterol, the former being more effective but the latter less effective than cholesterol, in increasing the density of motions in the  $10^4$  to  $10^5$  Hz frequency range, in DMPC bilayers.

The effects of different sterols on saturated phospholipid (DPPC) headgroup order and dynamics (Table 2, Fig.

4 and Fig. 5) clearly reflect some of their interactions with the acyl chains of the lipids. Thus, the effects of ergosterol on both order and dynamics in these regions of the saturated phospholipid molecules indicate motions which are restricted when compared to the other DPPC/sterol membrane systems, consistent with the higher orientational order of the acyl chains of DMPC with ergosterol. In addition, these results may also reflect restricted rotational motion of the phospholipid molecule as a whole. Such effects are also seen in the high temperature fluid phases.

The relative effects of cholesterol and ergosterol on the unsaturated phospholipid, POPC, are different and more complex than those observed with the saturated DMPC, both in chain ordering and in dynamics, as shown independently from  $^2\text{H}$  and  $^{13}\text{C}$ -NMR experiments. Thus, although ergosterol was significantly more effective than cholesterol in inducing order and restricting mobility in the saturated PC, it appears less effective, at 30 mol%, when interacting with the unsaturated analog, where its behavior is more similar to that of lanosterol (compare Fig. 2 and Fig. 6). The limited capacity of ergosterol and lanosterol to induce order in the methylene segments of the acyl chains of the unsaturated phospholipid is matched by their effects on the transverse relaxation of the chain deuterons (dominated by quadrupolar effects) or protons (dominated by dipolar interactions). These results show that the inclusion of these sterols into POPC membranes produces, in addition to a decrease in the elastic constant and/or the coherence length of the membranes, an increase in the frequency of the ultra-slow collective motional modes, indicating altered packing of the phospholipid molecules. The titration experiments using both  $^{13}\text{C}$  and  $^2\text{H}$ -NMR (Fig. 8 and Fig. 9) strongly suggest (but do not prove) that both ergosterol and lanosterol are indeed incorporated into POPC bilayers, but above  $\sim 25$  mol%, there is a small decrease in ordering caused by ergosterol, and at higher sterol levels (above  $\sim 28\%$ ) a possible new phase is formed, as suggested by the anomalous transverse  $^2\text{H}$  relaxation rates and broad features in the  $^{13}\text{C}$  MAS NMR spectrum of POPC/ergosterol membranes, Fig. 8. However, the  $^{31}\text{P}$ -NMR data show that at these mole ratios the bilayer configuration is fully retained in all DMPC/sterol systems. Taken together, our results indicate that while the POPC/cholesterol (7:3 molar ratio) membranes exhibit molecular properties consistent with the previously characterized liquid-ordered ( $l_o$ ) or 'liquid gel' phase [11,12], the POPC/ergosterol and POPC/lanosterol systems at the same molar proportion are in a qualitatively different, more disordered bilayer state. One possible reason for these complex effects is that the rigid and bulky ergosterol molecule (or the methylated  $\alpha$  face of lanosterol, see Fig. 1) is unable to interact effectively with the phospholipid molecules when an unsaturated acyl chain is present. Both the presence of two double bonds in the B ring of the tetracyclic nucleus and the C-24 methylated side chain of ergosterol seem to be responsible for this limited capacity



to order the 2-chain unsaturated phospholipid molecules, as evidenced by the behavior of 7-dehydrocholesterol and stigmasterol in the same system (Table 3). These results are consistent with previous studies which have shown that ergosterol (at 30 mol%) is less effective than cholesterol in the condensation of unsaturated (egg) PC monolayers [55], and in the reduction of the passive permeability of polar molecules and ions through mixed egg-PC/ergosterol bilayers [99]—where phase separation was noted at 26 mol% ergosterol. Furthermore, our results are in agreement with those reported by Semer and Gelerinter [54] using EPR of doxyl-fatty acid and spin-labeled cholestane probes in egg-PC liposomes, which indicate that up to ~15–20 mol%, ergosterol and cholesterol have similar ordering effects on egg-yolk phosphatidylcholine bilayers, while above that level the apparent ordering effect of ergosterol decreases, while the ordering of cholesterol increases linearly, up to 50 mol%. Interestingly, recent results from our laboratories (Contreras, L.M. and Urbina, J.A., unpublished data) indicate that in *Trypanosoma cruzi*, a protozoan human parasite which requires ergosterol and other 24-alkylated sterols for growth [100–104], the sterol content of the plasma membranes, which are rich in unsaturated fatty acids, is 23 mol% (of which 3/4 are ergosterol or its 24-ethylated analogs). This molar proportion is significantly smaller than that observed in many mammalian plasma membranes, in which cholesterol is typically present at 30–50 mol%. However, this mole ratio is at the top of the chain  $^2\text{H}$ -NMR first moment versus molar concentration of ergosterol curve, Fig. 9. Based on these results and the previous ESR studies [56] it appears that the parasite may regulate its ergosterol content to achieve *maximum* order of its unsaturated phospholipid acyl chains.

In conclusion, the effects of cholesterol and ergosterol on the physical properties of PC membranes differ. At 30 mol% and 25°C ergosterol is more effective at increasing order and restricting the mobility of DMPC than is cholesterol, while the opposite is true with POPC bilayers. The trimethylated precursor, lanosterol, is less effective than the other two sterols, with both saturated and unsaturated lipids. These results show that the interactions between phosphatidylcholines and sterols are complex, and depend on the details of sterol structure and the types of acyl chains present in the phospholipid molecules. A more complete understanding of these complex interactions will require at a minimum construction of phase diagrams for binary mixtures of saturated and unsaturated PCs containing the different sterols, and potentially a consideration of the effects of proteins on these phase diagrams as well.

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