A Carbon-13 Nuclear Magnetic Resonance Spectroscopic Study of Inter-Proton Pair Order Parameters: A New Approach to Study Order and Dynamics in Phospholipid Membrane Systems

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ABSTRACT We report a simple new nuclear magnetic resonance (NMR) spectroscopic method to investigate order and dynamics in phospholipids in which inter-proton pair order parameters are derived by using high resolution ^{13}C cross-polarization/magic angle spinning (CP/MAS) NMR combined with ^{1}H dipolar echo preparation. The resulting two-dimensional NMR spectra permit determination of the motionally averaged interpair second moment for protons attached to each resolved ^{13}C site, from which the corresponding interpair order parameters can be deduced. A spin-lock mixing pulse before cross-polarization enables the detection of spin diffusion amongst the different regions of the lipid molecules. The method was applied to a variety of model membrane systems, including 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC)/sterol and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)/sterol model membranes. The results agree well with previous studies using specifically deuterium labeled or perdeuterated phospholipid molecules. It was also found that efficient spin diffusion takes place within the phospholipid acyl chains, and between the glycerol backbone and choline headgroup of these molecules. The experiment was also applied to biosynthetically ^{13}C-labeled ergosterol incorporated into phosphatidylcholine bilayers. These results indicate highly restricted motions of both the sterol nucleus and the aliphatic side chain, and efficient spin exchange between these structurally dissimilar regions of the sterol molecule. Finally, studies were carried out in the lamellar liquid crystalline (L_α) and inverted hexagonal (H_{II}) phases of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE). These results indicated that phosphatidylethanolamine lamellar phases are more ordered than the equivalent phases of phosphatidylcholines. In the H_{II} (inverted hexagonal) phase, despite the increased translational freedom, there is highly constrained packing of the lipid molecules, particularly in the acyl chain region.

INTRODUCTION

Solid-state NMR methods are among the most useful approaches available for the study of molecular order and dynamics of lipids and proteins in biological membranes and model systems (Urbina and Oldfield, 1997; Cross and Opella, 1994; Opella, 1994; Auger, 1995; Ramamoorthy et al., 1995). Among them ^{2}H-NMR spectroscopy (Oldfield et al., 1971a; Smith and Oldfield, 1984; Seelig, 1977; Davis, 1983; Stohrer et al., 1991; Weisz et al., 1992) and the use of cross-polarization combined with magic angle spinning (CP/MAS) techniques (Pines et al., 1972; Pines et al., 1973; Urbina and Waugh, 1974; Schaefer and Stejskal, 1976), which allow high resolution ^{13}C- and ^{1}H-NMR spectra of unperturbed natural and artificial membranes to be obtained (Oldfield et al., 1987; Forbes et al., 1988a, b; Montez et al., 1993; Urbina et al., 1995), have produced detailed structural and dynamical information with atomic resolution. In addition, ^{31}P-NMR has proven particularly useful in the study of the orientational and dynamic properties of phospholipid headgroups (Seelig, 1978) and lipid polymorphism (Cullis et al., 1986; Cullis and De Kruijff, 1979).

The "order profile" of a membrane, or more specifically, the order profile of the hydrocarbon chain segments in a membrane, shows how order is a function of distance from the polar headgroup, and has been investigated extensively by using ^{2}H-NMR of selectively labeled molecules (Smith and Oldfield, 1984; Seelig, 1977; Davis, 1983; Urbina et al., 1995; Seelig and Seelig, 1977; Seelig and Browning, 1978; Seelig and Waespe-Sarcevic, 1978; Rance et al., 1980; Baeziger et al., 1991; Gally et al., 1979). The segmental order parameters, S_{cd}, can be directly obtained from the frequency separation ($\Delta\nu_q$) of the singularities of the experimental spectra of unoriented samples by using the relationship:

$$\Delta\nu_q = \frac{3}{4}(e^2qQ/h)\cdot S_{cd} \qquad (1)$$

where $\Delta\nu_q$ is the observed quadrupole splitting (the separation between the major singularities in the ^{2}H-NMR powder pattern), and e^2qQ/h is the nuclear quadrupole coupling constant for ^{2}H, typically about 168 kHz for C-D bonds.

Despite the theoretical simplicity of this approach, the difficulty and cost of selective deuteration of large molecules, as well as the limited resolution of ^{2}H-NMR, has restricted its application somewhat. To circumvent this problem, other methods have been sought to obtain order profiles of lipid molecules, in a variety of systems. One of these approaches proposes a procedure (termed "smooth-

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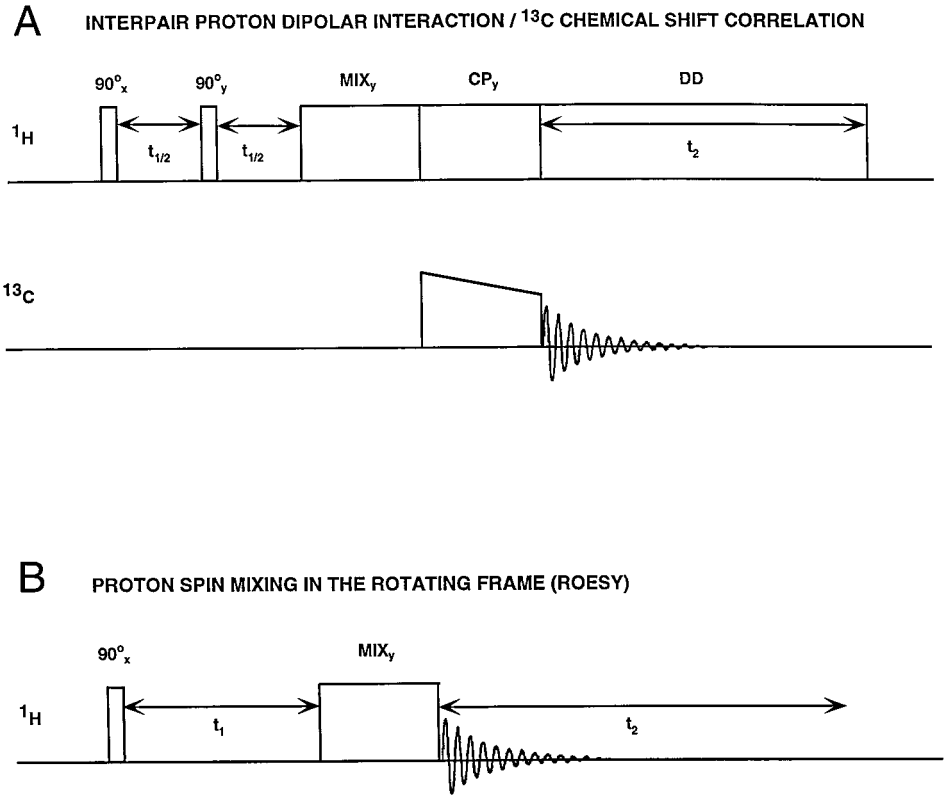

FIGURE 1 (*A*) Pulse sequence for a 2D-NMR experiment which correlates ^{1}H interpair dipolar interactions with the ^{13}C chemical shifts of the directly-attached carbon atoms. (*B*) Pulse sequence used to investigate ^{1}H spin mixing in the rotating frame (ROESY, see Vega, 1988).

ing") to extract order profiles from ^{2}H-NMR spectra of perdeuterated molecules in both bilayer (Lafleur et al., 1989; Salmon et al., 1987) and hexagonal phases (Thurmond et al., 1993), and assumes a monotonic decrease of S_{cd} as a function of segment position. However, it has recently been shown (Urbina et al., 1995) that smoothed order profiles may differ significantly from actual profiles obtained with specifically ^{2}H labeled lipids, indicating that structural conclusions drawn from smoothed profiles must be viewed carefully.

As an alternative to ^{2}H labeling schemes, several methods have been proposed to extract order profiles from natural abundance, high resolution ^{13}C spectra, using two-dimensional variations of the CP/MAS experiment. Most of these methods were inspired by the early work of Waugh and co-workers (Hester et al., 1976) in which dipole-dipole spectra of spins with different chemical shifts were separated in a 2D experiment: the so-called separated local field experiment. The original procedure was restricted to single crystals and uniaxially oriented fibers, in which the limited number of molecular orientations allowed some resolution of the resonances in the chemical shift dimension, despite the presence of large chemical shift anisotropies (CSA). For polycrystalline or amorphous materials, MAS is required to average out the CSA's, but the generally weak heteronuclear dipolar information is then lost, since it is also scaled by a factor of $P_2 = 1/2(3\cos^2\theta - 1)$, where θ is the angle between the spinner axis and the external magnetic field. Recently, several methods have been developed to selectively recover heteronuclear dipolar interactions (from which the corresponding C-H bond order parameters can be directly calculated) from natural abundance high resolution ^{13}C-NMR CP/MAS spectra of lipids. One of the most

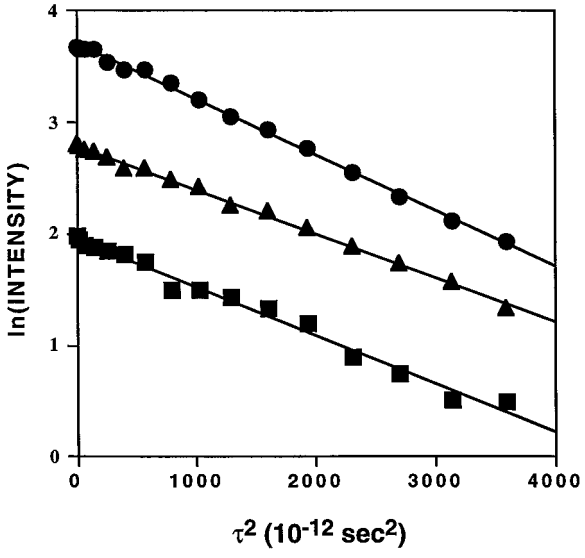

FIGURE 2 Intensity of selected ^{13}C resonances from the lipid acyl chains in the dipolar-echo edited cross-polarization MAS NMR spectra of DMPC, as a function of the square of τ, the interval between the two phase-shifted $\pi/2$ ^{1}H pulses ($t_{1/2}$ in Fig. 1). The intensities correspond to (—●—), C5-C11 methylene; (—■—), C3 methylene; and (—▲—), C13 methylene groups. Lines correspond to calculated linear regressions; the regression coefficients (R^2) vary between 0.985 and 0.995. The cross-polarization contact time was 10 ms.

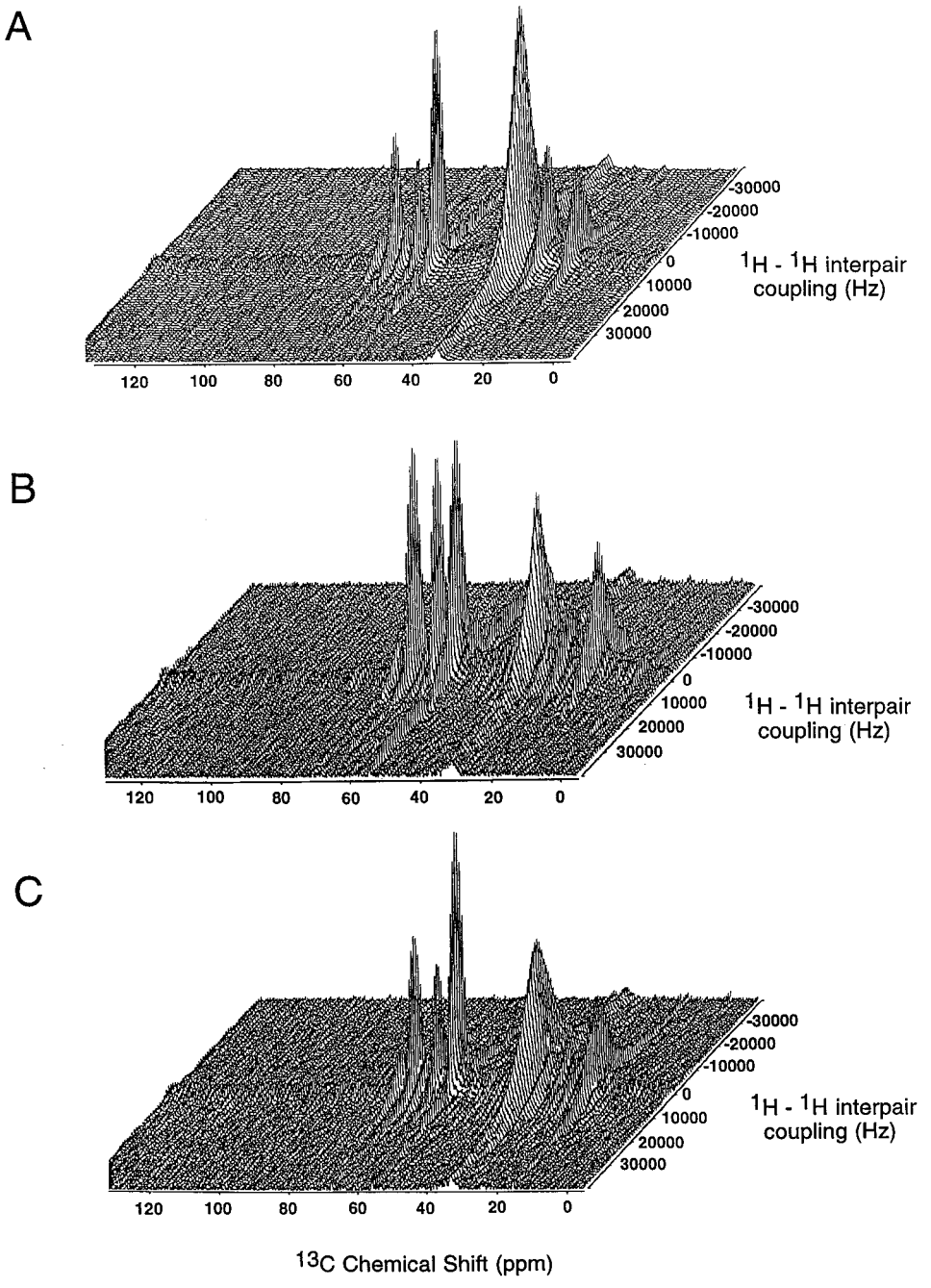

FIGURE 3 125.69 MHz 2D-NMR spectra obtained with the sequence presented in Fig. 1 *A* of (*A*) DMPC; (*B*) DMPC/cholesterol = 7:3 (mol/mol); and (*C*) DMPC/ergosterol = 7:3 (mol/mol). All lipids were 50% by weight in D_2O. Temperature, 25°C. The cross-polarization contact time was 10 ms.

successful approaches, proton-detected local field spectroscopy (PDLF, Nakai and Terao, 1992), has been performed in static samples consisting of small molecules magnetically oriented in nematic liquid crystals and in polycrystalline lipid samples. Frequency selection (Schmidt-Rohr et al., 1994), switched-angle sample spinning (Hong et al., 1995b; Nakai and Terao, 1992) or off magic-angle spinning during the whole experiment, (Hong et al., 1995a) have been used to recover the magnitude and sign of the dipolar interactions. Another experimental approach, dipolar recoupling on-axis with scaling and shape preservation (DROSS, Gross et al., 1997), uses MAS to eliminate I-I interactions (this is restricted to materials such as membranes and liquid crystals with fast molecular motions; Forbes et al., 1988b), together with rotor-synchronized π pulses to reintroduce I-S interactions during the evolution period, followed by high resolution detection under MAS and I-S decoupling.

Here, we present a particularly simple 2D-NMR experiment which permits the determination of *inter*-proton pair order parameters as well as spin diffusion from high resolution ^{13}C MAS NMR spectra of lipids (or other condensed systems) having loosely coupled proton pairs, and we apply this new approach to analyze lipid order and dynamics in a variety of lipid and lipid-sterol systems.

MATERIALS AND METHODS

Sample preparation

Dimyristoyl(1, 2–14:0)-phosphatidylcholine (DMPC), 1-palmitoyl(16:0)-2-oleoyl(18:1)-phosphatidylcholine (POPC), 1-[$^2H_{31}$]-palmitoyl-2-oleoyl-phosphatidylcholine (d_{31}-POPC) and 1,2,-dioleoyl-phosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Alabaster, AL) while cholesterol, ergosterol and lanosterol were obtained from Sigma Chemical Company (St. Louis, Missouri). ^{13}C-labeled ergosterol was isolated from *Saccharomyces cerevisiae* grown in YPB medium (Cushley and Filipenko, 1976) in the presence of [1-^{13}C]-acetate (Cambridge Isotopes, Andover, MA) and purified using silicic acid column chromatography and preparative thin-layer chromatography. The final product was recrystallized twice from ethanol, in the dark at −20°C. Lipids were dissolved in chloroform in the desired proportions and solvent eliminated under high vacuum for 12 h. The solid residues were resuspended in D_2O at 50 weight % solids, sealed in glass tubes, then subjected to 4−6 mixing and freeze/thaw cycles to ensure homogeneity.

NMR methods

125.69 MHz cross-polarization "magic-angle" spinning ^{13}C-NMR spectra were acquired on a "home-built" NMR spectrometer using an Oxford Instruments 11.7 T 52-mm bore superconducting solenoid, under the control of a Tecmag (Houston, TX) Aries pulse programmer, using the MacNMR 5.5 software package (Tecmag) run on an Apple PowerMac 7100/80 computer. Typical $\pi/2$ pulse lengths for both ^{1}H and ^{13}C were 6.5 μs using a Doty Scientific (Columbia, SC) 5 mm MAS NMR probe and the MAS rate was controlled at 3000 ± 2 Hz via a Doty Scientific spin-speed controller. Ramped-amplitude cross-polarization (where the carbon H_1 field strength was varied linearly during CP while the proton amplitude was kept constant (Le Guernevé and Auger, 1995; Metz et al., 1994) was used to facilitate efficient cross-polarization in the different segments of the lipid molecules. For the 2D-spectra, 32 × 2048 complex data points were acquired with the inter-$\pi/2$ pulse separation ($t_{1/2}$ in Fig. 1 *A*) incremented in steps of 4 μs with 256 scans if the F1 dimension. The spectra were zero filled once in the first dimension and twice in the second. Exponential multiplication (5 Hz) in the first dimension and Gaussian broadening (100 Hz) in the second were applied before 2D-Fourier transformation using the Tecmag MacNMR 5.5 sofware package. For the 499.80 MHz ^{1}H ROESY (Vega, 1998) NMR experiments 256x2048 complex data points were acquired with the evolution time (t_1, Fig. 1 *B*) incremented in steps of 100 μs, a dwell time of 100 μs during t_2 and 32 scans in the F1 dimension. The spectra were zero filled twice in the second dimension and exponential multiplication (5 Hz) in both the first and second dimension was applied before 2D-Fourier transformation using Tecmag MacNMR 5.5. Temperatures were controlled to within ±1°C using an Omega temperature controller.

The motionally averaged interpair second moments, $M_{2(inter,av)}$, were obtained from the decay of the intensities of individual resonances as a function of t_1 (Gaussian decays in all cases):

$$M_{2(inter,av)} = 8/(T_{2e})^2 \quad (2)$$

where T_{2e} is the Gaussian decay time, or from the widths of the slices in the 2D spectrum at each ^{13}C resonance frequency:

$$M_{2(inter,av)} = 2(\Delta\nu_{1/2})^2/\ln 2 \quad (3)$$

where $\Delta\nu_{1/2}$ is the half-width at half-height of the Gaussian line.

202.165 MHz ^{31}P NMR spectra were acquired on the same spectrometer described above using Hahn's echo sequence, without proton decoupling. Typical $\pi/2$ pulse lengths were 5.5 μs and the interpulse delay was 50 μs.

TABLE 1 Motionally averaged interpair second moments, $M_{2(inter,av)}$ ($\times 10^{-9}$ s^{-2}) for DMPC and DMPC sterol systems at 25°C, in excess D_2O

Carbon Site	DMPC	DMPC/ Cholesterol	DMPC/ Ergosterol	DMPC/ Lanosterol
C2-Chain	0.945	2.016	2.222	1.587
C3-Chain	1.245	2.150	2.645	1.422
C5/C11-Chain	1.349	2.462	3.332	1.385
C13	0.886	1.633	2.081	1.161
C14	0.800	1.385	1.349	0.638

The motionally averaged interpair second moments were obtained from the decays of the intensities of individual resonances as a function of t_1 (Gaussian decays in all cases, Eq. 2), or from the widths of the slices in the 2D spectra at each ^{13}C resonance frequency (Eq. 3).

RESULTS AND DISCUSSION

NMR background

The response of a rigid dipolar coupled spin system formed by identical spins I = 1/2 to the pulse sequence $(\pi/2)_0 - \tau - (\pi/2)_{90}$ is the well known "solid echo" described by the function (Powles and Strange, 1963; Oldfield et al., 1971b):

$$E(t') = 1 - M_2(t' - \tau)^2/2! + M_4(t' - \tau)^4/4! + M_{4\epsilon}t'^2\tau^2 \quad (4)$$

where M_2, M_4 are the second, fourth moments of the absorption spectrum and $M_{4\epsilon}$ is a fourth moment-like error term.

However, it has been shown both theoretically and experimentally (Boden and Mortimer, 1973; Boden et al., 1974; Janes et al., 1990; Boden et al., 1975) that for a system of *loosely coupled* spin 1/2 pairs, that is a system in which the intra-pair dipolar interaction is assumed to be considerably larger than its inter-pair counterpart, the re-

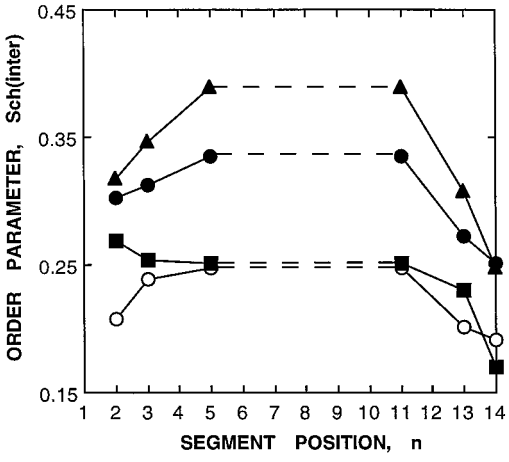

FIGURE 4 Inter-proton pair order parameters for DMPC (—○—), DMPC/CHOL (—●—), DMPC/ERG (—▲—), and DMPC/LAN (—■—) systems as a function of the segment position in the acyl chains. The order parameters were calculated from the values of $M_{2(inter,av)}$ using Eq. 6. Positions 5–11 were not resolved and a common value was assigned. However, the lineshape in the ^{1}H dimension for those segments could be fitted very well by using a single Gaussian function ($R^2 \geq 0.99$).

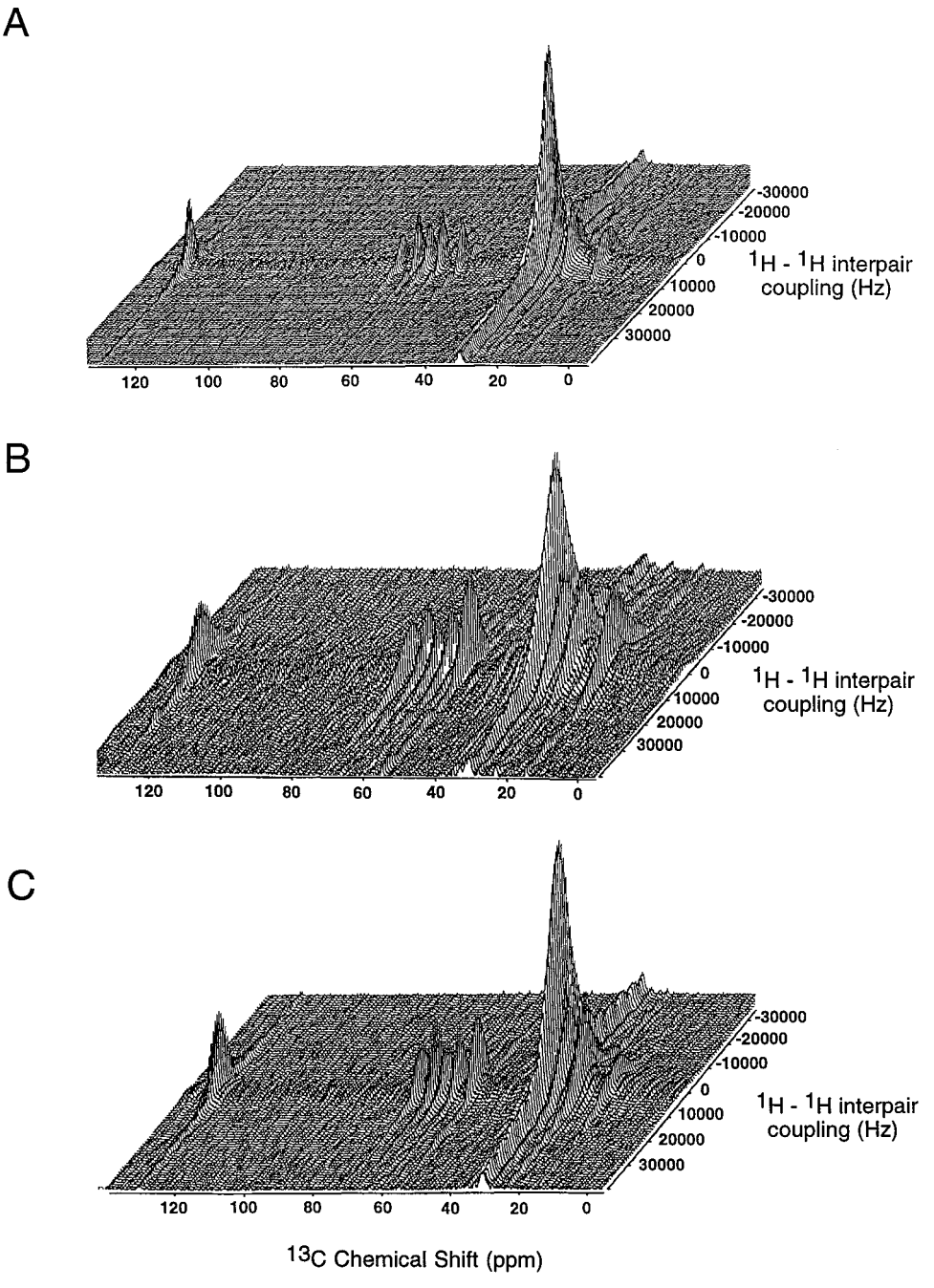

FIGURE 5 125.69 MHz 2D-NMR spectra obtained with the sequence presented in Fig. 1 *A* of (*A*) POPC; (*B*) POPC/cholesterol = 7:3 (mol/mol); and (*C*) POPC/ergosterol = 7:3 (mol/mol). All lipids were 50% by weight in D_2O. Temperature, 25°C. The cross-polarization contact time was 10 ms.

sponse is also an echo at $t' = \tau$, but whose decay as a function of the interpulse separation, τ, can be described for at least 50% of the magnetization decay, by the function:

$$E(\tau) = 1 - M_{2(\text{inter,av})}\tau^2/2 \qquad (5)$$

where $M_{2(\text{inter,av})}$ is the contribution to the second moment from the inter-pair dipolar interactions averaged over different membrane orientations and by fast (on the NMR time scale) molecular motions, as discussed extensively in the work of Boden and colleagues.

In our experiment (Urbina et al., 1995) protons are allowed to evolve under the influence of two phase-shifted $\pi/2$ pulses for a total period $t_1 = 2\tau$ (Fig. 1 *A*). At the ^{1}H dipolar echo maximum, ramped CP contact is established to transfer magnetization to directly coupled ^{13}C nuclei, which are then detected with high resolution, under MAS and with proton decoupling. By systematically varying τ, a 2D NMR spectrum is obtained which correlates the chemical shift of the different ^{13}C sites with the inter-proton pair dipolar spectrum of the directly attached protons. A spin-lock pulse previous to CP allows longitudinal proton spin exchange in the rotating frame, from which spin diffusion effects can be diagnosed. The inter-pair order parameters $S_{\text{ch(inter)}}$ can be calculated from the relationship (Janes et al., 1990; Boden et

al., 1975):

$$M_{2(inter,av)} = S^2_{ch(inter)} \cdot M_{2(inter)} \quad (6)$$

where $M_{2(inter)}$ is the inter-pair second moment for a saturated, all-*trans* hydrocarbon chain, $5.5 \times 10^9\ s^{-2}$ (Bloom et al., 1978).

The present experiment is conceptually closely related to 2D-wideline separation (WISE) NMR (Schmidt-Rohr et al., 1992) proposed by Spiess and co-workers. However, in the latter method the total ^1H dipolar spectrum is obtained for each carbon site. There, although valuable qualitative information has been obtained in a variety of polymer (Schmidt-Rohr et al., 1992) and lipid membrane (Hong et al., 1995a) systems, no detailed quantitative analysis is possible. In the new experiment, the dipolar echo selection before CP contact allows one, in the case of loosely coupled spin pairs, to obtain only the inter-proton pair dipolar spectrum (Boden and Mortimer, 1973; Boden et al., 1974; Janes et al., 1990; Boden et al., 1975) from which quantitative order parameter information can be obtained via Eq. 6.

Phospholipid/sterol systems

The assumption of "loosely coupled" proton spin pairs is expected to apply to the polymethylene chains and headgroups of phospholipid bilayer membranes, particularly in their liquid crystalline phases, since the rapid flexing and twisting of these parts of the lipid molecules will reduce the interpair dipolar interactions, compared with their intrapair counterparts. Experimental evidence in favor of this assumption is provided by the Gaussian decay of the ^1H dipolar echoes as a function of the interpulse separation, τ, in phosphatidyl-choline and cardiolipin membranes in excess water (Janes et al., 1990), as well as that of the ^{13}C resonance intensities of the lipid chains in dipolar-echo edited cross-polarization spectra of all the lipid systems studied in this work (Fig. 2; see also Montez et al., 1993; Urbina et al., 1995). As can be seen in Fig. 2, the decay of the ^1H dipolar echo for proton spin pairs directly attached to ^{13}C sites of the acyl chains in DMPC can be fitted very well to Gaussian functions for τ values of at least 60 μsec, which for the C5-C11 methylene groups corresponds to ~80% of the echo decay. Similar behavior has been observed for all other lipid phases studied, both lamellar and hexagonal (data not shown).

We show in Fig. 3 the 2D-NMR spectra of DMPC (*A*), DMPC/cholesterol (*B*), and DMPC/ergosterol (*C*) obtained with the pulse sequence shown in Fig. 1 *A*. The motionally averaged inter-pair second moments M_2(inter, av) were obtained from the widths of the slices of the 2D spectra (Eq. 3), as well as from plots of the intensities of the individual ^{13}C resonances of the 1D spectra as a function of the interpulse separation (Fig. 2) using Eq. 2. Typical results are shown in Table 1, and the corresponding inter-proton pair order parameters, calculated using Eq. 6, are plotted as a function of the methylene segment position (identified by its chemical shift; Forbes et al., 1988a) in Fig. 4. The inter-proton pair order parameters are to be distinguished from their intra-pair analogs (which provide information equivalent to that obtained from quadrupolar splittings in ^2H-NMR or the C-H dipolar splittings in PDLF or DROSS spectra), because the inter-pair values are influenced by both whole chain flexing and twisting motions, as well as by local motions of the methylene segment during the NMR time scale (Boden et al., 1975). They do, however, provide detailed information on the type and degree of motion of the different parts of the phospholipid molecules, as shown in Fig. 4. These "order profiles" are in good qualitative agreement with those obtained from (intra-pair) order parameters derived from ^2H-NMR of specifically labeled DMPC mol-

TABLE 2 Motionally averaged interpair second moments, $M_{2(inter,av)}$ ($\times 10^{-9}\ s^{-2}$) for POPC and POPC/sterol systems at 25°C in excess D_2O

Carbon Site	POPC	POPC/ Cholesterol	POPC/ Ergosterol	POPC/ Lanosterol
Glycerol C2	0.488	0.816	0.726	0.520
Choline C-α	0.150	0.473	0.112	0.189
Choline C-β	0.139	0.433	0.127	0.175
Choline C-γ	0.128	0.452	0.895	0.700
Chain C2	0.886	1.161	1.190	0.988
Chain C3	1.107	1.422	1.250	1.282
Chain C-5/C13	1.161	1.349	1.219	1.161
Chain C14	0.868	1.219	0.699	0.699
Chain C15	0.816	0.925	0.800	0.740
Chain C16	0.886	0.784	0.754	0.886

The motionally averaged interpair second moments were obtained from the decays of the intensities of individual resonances as a function of t_1 (Gaussian decays in all cases, Eq. 2), or from the widths of the slices in the 2D spectra at each ^{13}C resonance frequency (Eq. 3).

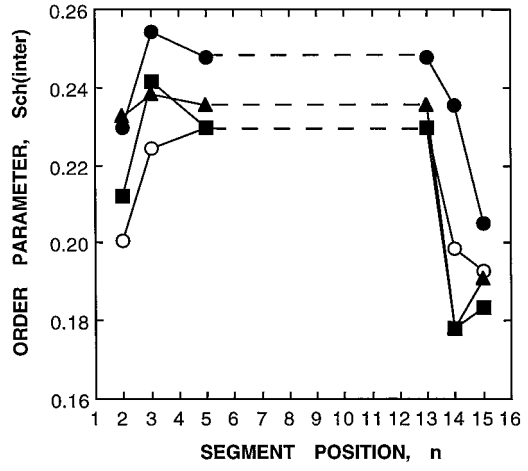

FIGURE 6 Inter-proton pair order parameters for POPC (—○—), POPC/CHOL (—●—), POPC/ERG (—▲—), and POPC/LAN (—■—) systems as a function of the segment position in the acyl chains. The order parameters were calculated from the values of $M_{2(inter,\ av)}$ using Eq. 6. Positions 5–13 were not resolved and a common value was assigned. However, the lineshape in the ^1H dimension for those segments could be fitted very well by a single Gaussian function ($R^2 \geq 0.99$).

TABLE 3 Motionally averaged interpair second moments, $M_{2(inter,av)}$ ($\times 10^{-9}$ s^{-2}) for POPC/Cholesterol with and without a 10-ms spin-mixing pulse, at 25°C, in excess D_2O

Carbon Site	− Spin Mixing	+ Spin Mixing
Glycerol C2	0.975	0.511
Choline C-α	0.515	0.345
Choline C-β	0.506	0.333
Choline C-γ	0.605	0.676
Chain C2	1.121	1.036
Chain C3	1.354	1.127
Chain C-5/C13	1.329	1.115
Chain C14	1.326	1.114
Chain C15	1.147	0.932
Chain C16	1.130	0.858

The motionally averaged interpair second moments were obtained from the decay of the intensities of individual resonances as a function of t_1 (Gaussian decays in all cases, Eq. 2), or from the width of the slices in the 2D spectra at each ^{13}C resonance frequency (Eq. 3).

ecules (Seelig, 1977; Urbina et al., 1995), including the anomalous value of the order parameters for positions 2 and 3 of the acyl chains, due to the different geometrical arrangement of these segments for the sn-2 chain, the high value of the order parameter for segments C5-C11 (which corresponds to the "plateau" region observed using ^2H-NMR) and the rapid decrease of order toward the ends of the chains, located at the center of the bilayer. The relative ordering effects were ergosterol>cholesterol≫lanosterol. These results also agree well with previous studies on these systems using both ^{13}C- and ^1H-$T_{1\rho}$ and ^{13}C-^1H cross-polarization methods (Urbina et al., 1995).

The 2D spectra for POPC/sterols are shown in Fig. 5 and the corresponding results for $M_{2(inter,av)}$ and $S_{ch(inter)}$ are presented in Table 2 and Fig. 6, respectively. It can be seen in this system that while the shape of the order profile is similar to that observed for DMPC (Fig. 4), the values of the inter-pair order parameters are significantly lower than for the saturated system, and the differences between the individual sterols are less pronounced. These results are again in good qualitative agreement with the limited data available for specifically labeled unsaturated fatty acid chains in model (Seelig and Seelig, 1977; Seelig and Browning, 1978; Seelig and Waespe-Sarcevic, 1978; Baeziger et al., 1991) and biological (Rance et al., 1980; Gally et al., 1979) membrane systems. It can also be noted from Tables 1 and 2 and Figs. 5 and 6 that in the unsaturated phospholipid membranes the ordering effectiveness of cholesterol and ergosterol are *reversed* when compared with the DMPC/sterol systems, being cholesterol > ergosterol ≫ lanosterol. Again, this observation agrees with the results of previous ^2H-NMR studies with perdeuterated molecules (Urbina et al., 1995), where it was argued that the relatively bulky and rigid side chain of ergosterol (see below) may limit the capacity of this molecule to order the unsaturated phospholipid acyl chains. These results also support the conclusions of previous studies using ^2H-NMR and ESR of spin labeled lipids, which concluded that ergosterol and lanosterol were much less effective than cholesterol in ordering natural and artificial phospholipid membranes having unsaturated acyl chains (Yeagle, 1985; Huang et al., 1991; Urbina et al., 1995; Semer and Gerelinter, 1979; Urbina et al., 1988).

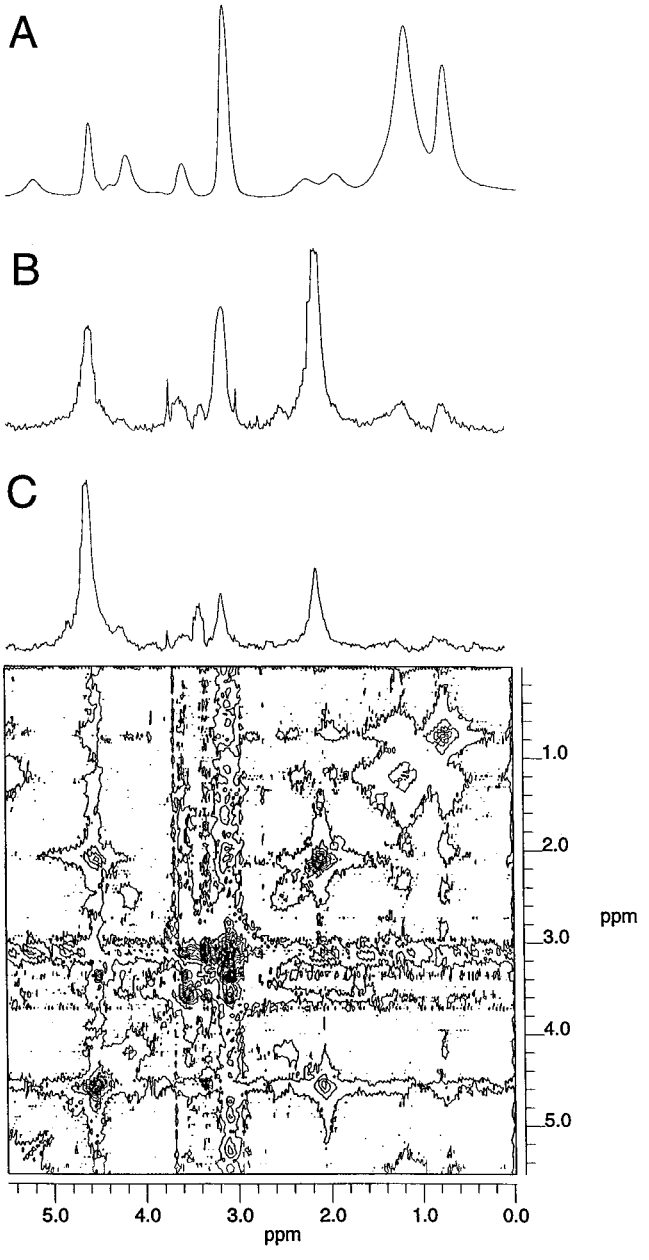

FIGURE 7 *Bottom:* 499.80 MHz ^1H ROESY spectra of POPC/CHOL = 7:3 (mol/mol) acquired with the pulse sequence shown in Fig. 1 *B* with 10 ms spin mixing. (*A*) Regular 1D-^1H-NMR spectrum; (*B*) and (*C*) cross-sections at 1.8–2.2 ppm (proton attached to C2 methylene gropus) and 4.6 ppm (water protons), respectively. For 1 ms mixing (not shown) no crosspeaks for the C2 protons were observed while for water the crosspeak intensities were sharply reduced. Lipid was 50% by weight in D_2O. Temperature, 25°C. Other experimental details are given in Materials and Methods.

We show in Table 3 the results for $M_{2(inter,av)}$ for the POPC/cholesterol system obtained from 2D-NMR spectra with and without the spin mixing step (a 10 ms proton spin lock, see Fig. 1). It can be seen that in the presence of the mixing pulse the apparent values of $M_{2(inter,av)}$ along the acyl chains fall to the values corresponding to the more mobile methylene segments in the absence of mixing, indicating the presence of spin diffusion within the polymethylene chain. These results also suggest spin exchange between the glycerol backbone and the choline headgroup. Similar results were obtained in all lipid systems investigated. Spin diffusion along the polymethylene chain was verified by ROESY experiments (Vega, 1988), carried out under conditions identical to those used in our 2D experiment. Fig. 7 shows that a spin lock pulse of 10 ms allowed efficient spin mixing between protons attached to the C2 segments of the acyl chains and those of the rest of the methylene and methyl groups, as well as with the choline headgroup protons. Strong spin mixing was also observed, as expected, between water and protons of the C2 segment and the choline headgroup, indicating efficient hydration of the carbonyl groups vicinal to C2. When the experiment was repeated with a spin lock pulse of 1 ms (not shown) no crosspeaks for the C2 protons were observed, while for water the crosspeak intensities were sharply reduced. Spin diffusion among the protons of the phospholipid acyl chains and between the chains and the headgroups in phospholipid liquid crystalline membranes has been suggested previously based on ^{13}C-detected ^{1}H $T_{1\rho}$ measurements (Urbina et al., 1995). These results demonstrated that ^{1}H rotating-frame relaxation rates in the different regions of these molecules were indistinguishable, despite large motional differences detected by ^{1}H-^{13}C cross-polarization rates and ^{13}C-$T_{1\rho}$ measurements. Similar conclusions have recently been reached using ^{1}H MAS NOESY NMR (Chen and Stark, 1996) and heteronuclear correlation spectroscopy (Hong et al., 1996), and related effects have also been reported in WISE NMR spectra of polymers.

The 2D experiment was also used to investigate directly the motions of the sterol molecules in the membrane. Fig. 8 presents the 2D-NMR spectrum for biosynthetically-^{13}C labeled ergosterol (65% labeled at positions 2, 4, 6, 8, 10, 11, 12, 14, 16, 20, 23, and 25) incorporated in [$^{2}H_{31}$]-POPC (3:7 = mol/mol ratio). The values of M_2(inter, av) for the protons attached to the labeled carbon atoms (indicated in parentheses in Fig. 7) map out the type and extent of motion of the different parts of the molecule. It can be seen that the motion of the steroid nucleus *and* the aliphatic side chain are highly restricted, a result consistent with those of previous ^{2}H-NMR studies of cholesterol specifically deuterated at both the steroid ring and side chain (Dufourc et al., 1984). Experiments in which the spin mixing step is varied indicate

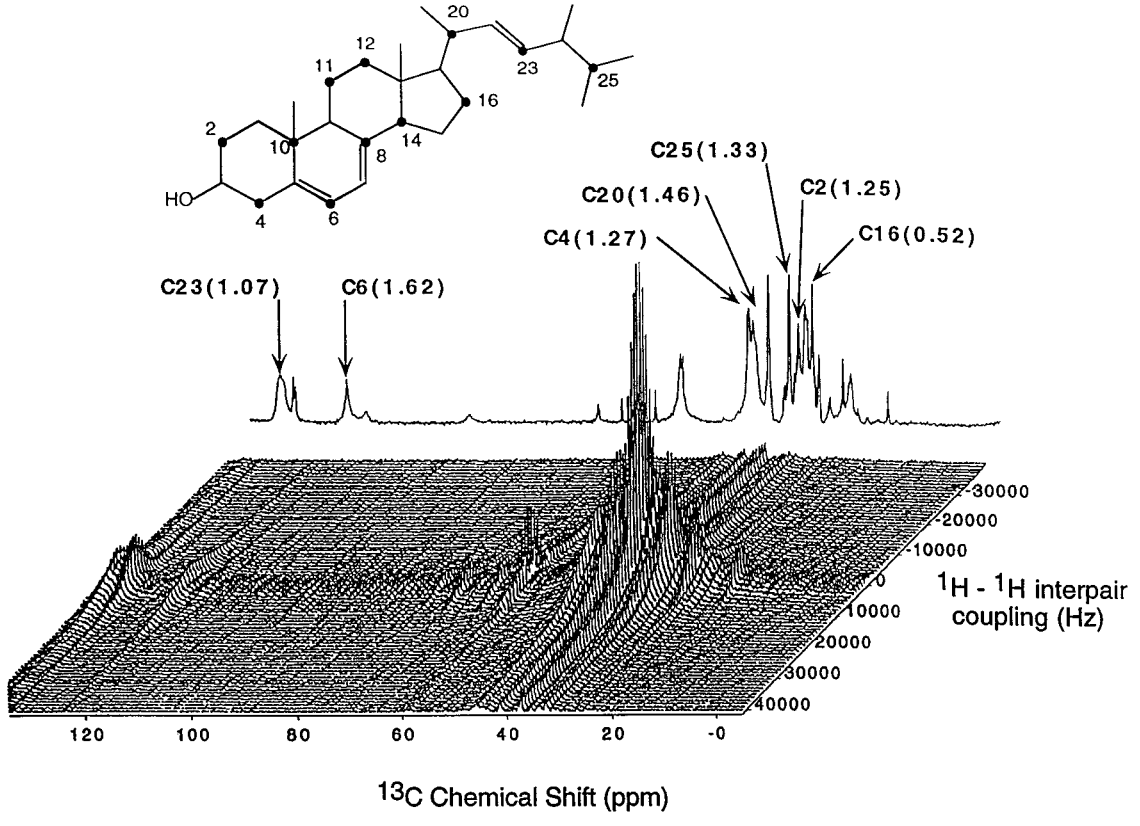

FIGURE 8 125.69 MHz 1D-, ^{13}C-, and 2D-NMR spectra obtained with the sequence presented in Fig. 1 *A* of [2,4,6,8,10,11,12,14,16,20,23,25-$^{13}C_{12}$]-ergosterol/d_{31}-POPC (3:7 mol ratio), 50% by weight in D_2O. Selected resonance peaks from labeled ^{13}C nuclei are indicated and the values of $M_{2(inter,av)}$ ($\times 10^{-9}$ s^{-2}) are given in parentheses. Temperature, 25°C. The cross-polarization contact time was 10 ms.

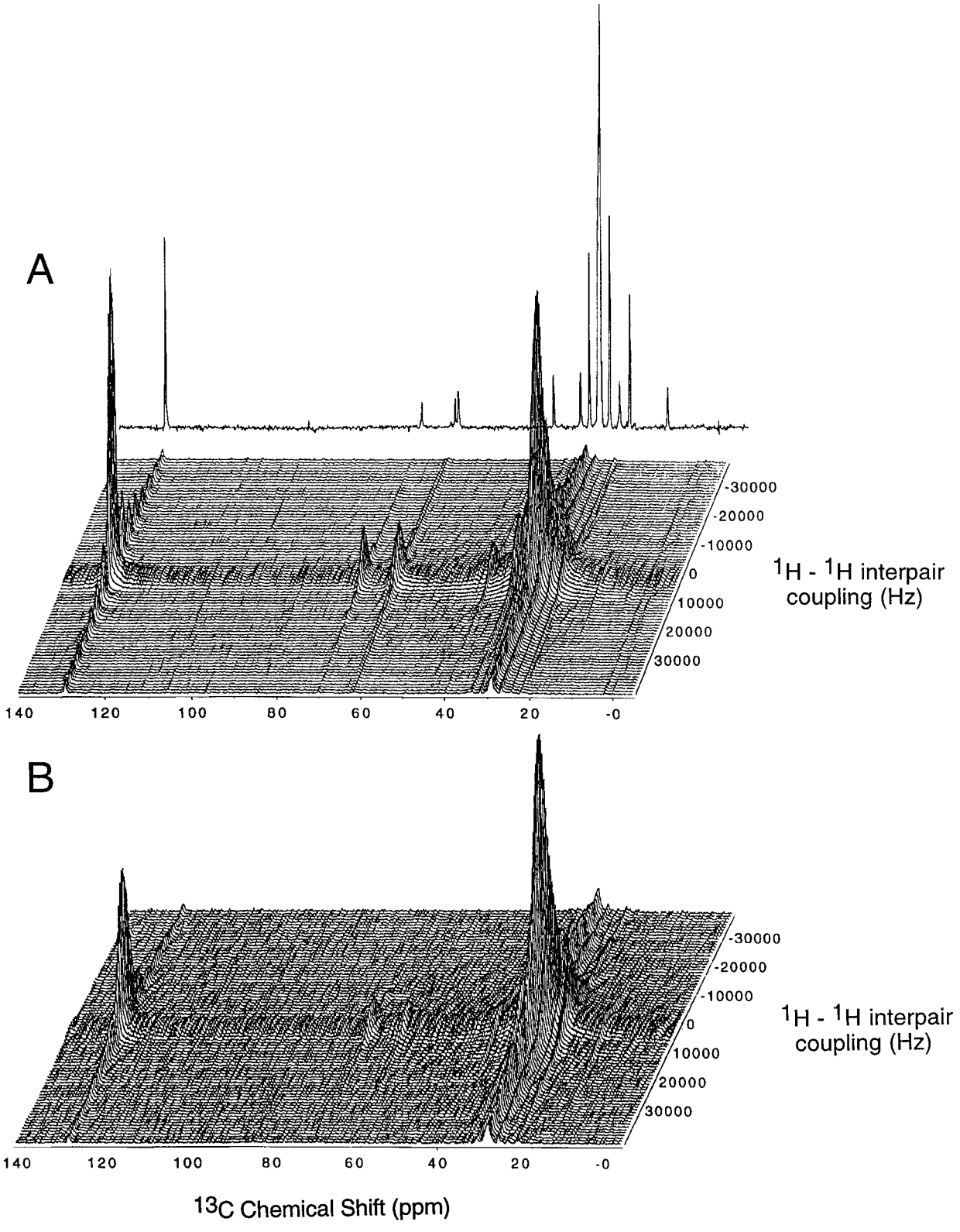

FIGURE 9 125.69 MHz 2D-NMR spectra obtained with the sequence presented in Fig. 1 *A* of (*A*) DOPE at 25°C (hexagonal H_{II} phase), and (*B*) DOPE at 4°C (lamellar L_α phase). Lipids were 50% by weight in D_2O. The cross-polarization contact time was 10 ms in (*A*) and 2 ms in (*B*).

strong spin exchange amongst the protons inside the sterol nucleus, as well as between the ring and the side chain (data not shown). Taken together, these results strongly support the idea that membrane sterol molecules, including ergosterol with its unsaturated side chain, should be viewed as rigid structures all along their molecular lengths.

Comparison of order and dynamics in lamellar and hexagonal lipid phases

The ability of several biological lipids to form non-lamellar phases under close to physiological conditions when isolated (lipid polymorphism) has attracted much interest (Tilcock, 1986; Gruner et al., 1985; Cullis et al., 1986; Cullis and De Kruijff, 1979), because the forces responsible for these phenomena seem to be important for the structure and activity of many membrane-bound proteins (Navarro et al., 1984; Jensen and Schutzbach, 1989; Bogdanov and Dowhan, 1995; Bogdanov et al., 1996). Among the lipids which exhibit such polymorphic behavior, phosphatidylethanolamine is of particular interest because it is the second most abundant phospholipid in eukaryotic cells, and by far the single most abundant lipid species in bacterial membranes. Although several groups have compared lipid order profiles between lamellar and hexagonal (H_{II}) phases (Thurmond et al., 1993; Lafleur et al., 1990; Eriksson et al., 1991), they have used perdeuterated acyl chains and a "smoothing" procedure that may not always provide an accurate estimate of actual profiles (Urbina et al., 1995). We therefore studied the order profiles of the lipid chains in dioleoyl-*sn*-phosphatidyl-ethanolamine (DOPE), which undergoes a lamellar to hexagonal (H_{II}) phase transition at 8°C in excess water (Epand, 1985). Lipid phases were verified using ^{31}P-NMR, which showed the expected (Cullis et al., 1986; Thurmond et al., 1993) reversal in sign and a reduction by a factor of $-½$ of the axially symmetric chemical shift anisotropy pattern when going from the lamellar to the reverse hexagonal phase. Fig. 9 shows the 2D-NMR spectra obtained with the pulse sequence of Fig. 1 in unlabeled DOPE at 4°C (lamellar, L_α phase) and 25°C (hexagonal, H_{II} phase), and Table 4 gives the values of $M_{2(inter,\ av)}$ for different protons attached to carbon sites in the acyl chains and headgroups in the two phases. It can be seen that, when compared with phosphatidyl-cholines (Tables 1 and 2), the length of the plateau region for the acyl chains in the lamellar phase of DOPE are significantly longer, despite the presence of the Δ-9,10 double bond in both chains. Although there are very limited data on PE's specifically deuterated in the acyl chains (Thurmond et al., 1991; Blume et al., 1982; Marsh et al., 1983), these results clearly indicate that the value and length of the order parameter plateau in dimyristoyl-*sn*-phosphatidyl-ethanolamine (DMPE) are significantly higher than the corresponding values in DMPC at the same reduced temperature, a result which agrees with molecular dynamics simulations on dilauroyl-*sn*-phosphatidyl-ethanolamine (Damodaran et al., 1992). Thus, our results with the unsaturated PE agree with the notion of a tighter molecular packing in PE lamellar phases, most probably due to the reduced repulsive forces between the smaller phosphoethanolamine headgroups, consistent with the higher L_β to L_α transition temperatures for phosphatidylethanolamines when compared with phosphatidylcholines. Going to the hexagonal phase, we observed a similar shape in the order profile but *less* than the expected reduction (1:4, see Eq. 6) in the values of $M_{2(inter,av)}$, due to geometric averaging introduced in the hexagonal phase (Thurmond et al., 1993; Lafleur et al., 1990). This indicates a more constrained packing for the di-unsaturated phospholipid molecules in the high temperature (lamellar) phase, particularly in the acyl chain region, an effect which is not observed in the ^{31}P-NMR data, or solely intra-pair couplings. This finding supports the idea that a key element in the formation of the inverted hexagonal phases is the presence of strong lateral and/or curvature stress in lipid species with such polymorphic behavior (Tilcock, 1986; Gruner et al., 1985; Thurmond et al., 1993).

TABLE 4 Motionally averaged interpair second moments, $M_{2(inter,av)}$ ($\times 10^{-9}$ s^{-2}) for DOPE at 4°C (L_α phase) and 25°C (H_{II} phase), in excess D_2O

Carbon Site	4°C	25°C
Glycerol-C2	0.361	0.256
Ethanolamine C-α	0.475	0.327
Chain C2	0.861	0.642
Chain C3	0.852	0.662
Chain C4–C7	0.909	0.708
Chain C9, C10	0.825	0.604
Chain C12–C15	0.922	0.723
Chain C16	0.950	0.780
Chain C17	0.802	0.680
Chain C18	0.640	0.650

The motionally averaged interpair second moments were obtained from the decay of the intensities of individual resonances as a function of t_1 (Gaussian decays in all cases, Eq. 2), or from the width of the slices in the 2D spectra at each ^{13}C resonance frequency (Eq. 3).

CONCLUSIONS

We have presented a simple and robust natural abundance ^{13}C 2D-NMR method to obtain detailed information on order and dynamics in systems containing loosely coupled proton spin pairs, such as lipid bilayers and other liquid crystals, and potentially in mobile polymers. The results are comparable to those obtained with more complex pulse sequences, or through the use of expensive and time-consuming specific isotopic labeling methods. The experiment confirms and extends the results of previous studies in these systems using ^{1}H dipolar echo decay spectroscopy (Janes et al., 1990) but provides increased spectral resolution, associated with the higher ^{13}C chemical shift dispersion. Our results show that the ordering effects of sterols in membranes, as evidenced from the inter-proton pair order parameters, depend both on the sterol structure as well as on the type of acyl chains esterified to the phospholipid, being ergosterol > cholesterol for saturated phosphatidylcholines but the reverse for the unsaturated lipid, POPC. They also demonstrate effective spin diffusion within the acyl chains, and between the glycerol backbone and the choline headgroup of phospholipids. We also report the first motional data on ergosterol in liquid crystalline phospholipid membranes. The results here indicate that motion of both the

sterol nucleus and the alkenyl side chain are highly restricted. Finally, we have obtained evidence which supports the notion that phosphatidylethanolamine lamellar phases are more ordered than the equivalent phases of phosphatidylcholines, and that in hexagonal phases, despite the increased translational freedom of the lipid molecules, there is highly constrained packing in the acyl chain region.

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REFERENCES

Auger, M. 1995. Solid state NMR study of protein structure. Methods based on the measurement of internuclear distances. *J. Chim. Phys.* 92:1751–1760.

Baeziger, J. E., H. C. Jarrell, R. J. Hill, and I. C. P. Smith. 1991. Average structural and motional properties of a diunsaturated acyl chain in a lipid bilayer: Effects of two cis-unsaturated double bonds. *Biochemistry.* 30:894–903.

Bloom, M., E. E. Burnell, A. L. MacKay, C. P. Nichol, M. I. Valic, and G. Weeks. 1978. Fatty acyl chain order in lecithin model membranes determined from proton magnetic resonance. *Biochemistry* 17: 5750–5762.

Blume, A., D. M. Rice, R. J. Witterbort, and R. G. Griffin. 1982. Molecular dynamics and conformation in the gel and liquid-crystalline phases of phosphatidylethanolamine bilayers. *Biochemistry* 21:6220–6230.

Boden, N., and M. Mortimer. 1973. An NMR "solid" echo experiment for the direct measurement of the dipolar interactions between spin-1/2 pairs. *Chem. Phys. Lett.* 21:538–540.

Boden, N., Y. K. Levine, M. Mortimer, and R. T. Squires. 1974. NMR "solid" echoes in systems of loosely coupled spin-1/2 pairs. *Phys. Lett.* 46A:329–330.

Boden, N., Y. K. Levine, D. Lightowlers, and R. T. Squires. 1975. NMR dipolar echoes in liquid crystals. *Chem. Phys. Lett.* 31:511–515.

Bogdanov, M., and W. Dowhan. 1995. Phosphatidyl-ethanolamine is required for in vivo function of the membrane-associated lactose permease of *Escherichia coli*. *J. Biol. Chem.* 270:732–739.

Bogdanov, M., J. Sun, H. R. Kaback, and W. Dowhan. 1996. A phospholipid acts as a chaperone in assembly of a membrane transport protein. *J. Biol. Chem.* 271:11615–11618.

Chen, Z.-j., and R. E. Stark. 1996. Evaluating spin diffusion in MAS-NOESY spectra of phospholipid multibilayers. *Solid State NMR.* 7:239–246.

Cross, T. A., and S. J. Opella. 1994. Solid-state NMR structural studies of peptides and proteins in membranes. *Curr. Opinion Struct. Biol.* 4:574–581.

Cullis, P. R., and B. De Kruijff. 1979. Lipid polymorphism and the functional roles of lipids in biological membranes. *Biochim. Biophys. Acta* 559:399–420.

Cullis, P. R., M. J. Hope, and C. P. S. Tilcock. 1986. Lipid polymorphism and the roles of lipids in membranes. *Chem. Phys. Lipids* 40:127–144.

Cushley, R. J., and J. D. Filipenko. 1976. ^{13}C Fourier transform n.m.r. XIII-reassignement of the ^{13}C spectrum of ergosterol. *Org. Magn. Res.* 8:308–309.

Damodaran, K. V., K. M. Merz Jr., and B. P. Gaber. 1992. Structure and dynamics of the dilauroylphosphatidylethanolamine lipid bilayer. *Biochemistry* 31:7656–7664.

Davis, J. H. 1983. The description of lipid conformation, order and dynamics by ^{2}H-NMR. *Chem. Phys. Lipids* 40:223–258.

Dufourc, E. J., E. J. Parish, S. Chitrakorn, and I. C. P. Smith. 1984. Structural and dynamical details of cholesterol-lipid interactions as revealed by deuterium NMR. *Biochemistry* 23:6062–6071.

Epand, R. N. 1985. High sensitivity differential scanning calorimetry of the bilayer to hexagonal phase transition of phosphatidylethanolamines. *Chem. Phys. Lipids* 36:387–393.

Eriksson, P.-O., L. Rilfors, A. Wieslander, G. Lundberg, and G. Lindblom. 1991. Order and dynamics of mixtures of membrane glucolipids from *Acholeplasma laidlawii* studied by ^{2}H-NMR. *Biochemistry* 30: 4916–4924.

Forbes, J., J. Bowers, L. Moran, X. Shan, E. Oldfield, and M. A. Moscarello. 1988a. Some new developments in solid-state nuclear magnetic resonance spectroscopic studies of lipids and biological membranes, including the effects of cholesterol in model and natural systems. *J. Chem. Soc. Faraday Transactions* 84:3821–3849.

Forbes, J., C. Husted, and E. Oldfield. 1988b. High field, High resolution proton "magic angle" sample-spinning nuclear magnetic spectroscopic studies of gel and liquid crystalline lipid bilayers and the effects of cholesterol. *J. Am. Chem. Soc.* 110:1059–1065.

Gally, H. U., G. Pluschke, P. Overath, and J. Seelig. 1979. Structure of *Escherichia coli* membranes. Phospholipid conformation in model membranes and cells as studied by deuterium magnetic resonance. *Biochemistry.* 18:5605–5610.

Gross, J. D., D. E. Warschawski, and R. G. Griffin. 1997. Dipolar recoupling in MAS NMR: A probe for segmental order in lipid bilayers. *J. Am. Chem. Soc.* 119:796–802.

Gruner, S., P. R. Cullis, M. J. Hope, and C. P. S. Tilcock. 1985. Lipid polymorphism: the molecular basis of nonbilayer phases. *Ann. Rev. Biophys. Biophys. Chem.* 14:211–238.

Hester, R. K., J. L. Ackerman, B. L. Neff, and J. S. Waugh. 1976. Separated local field spectra in NMR: determination of structure in solids. *Phys. Rev. Lett.* 36:1081–1083.

Hong, M., K. Schmidt-Rohr, and D. Nanz. 1995a. Study of phospholipid structure by ^{1}H,^{13}C and ^{31}P dipolar couplings from two-dimensional NMR. *Biophys. J.* 69:1939–1950.

Hong, M., K. Schmidt-Rohr, and A. Pines. 1995b. NMR measurement of signs and magnitudes of C-H dipolar couplings in lecithin. *J. Am. Chem. Soc.* 117:3310–3311.

Hong, M., K. Schmidt-Rohr, and H. Zimmermann. 1996. Conformational constraints on the headgroup and sn-2 chain of bilayer DMPC from NMR dipolar couplings. *Biochemistry* 35:8335–8341.

Huang, T.-H., A. J. DeSiervo, and Q.-X. Yang. 1991. Effect of cholesterol and lanosterol on the structure and dynamics of the cell membrane of *Mycoplasma capricolum*. Deuterium nuclear magnetic resonance study. *Biophys. J.* 59:691–702.

Janes, N., E. Rubin, and T. F. Taraschi. 1990. ^{1}H-NMR dipolar echo decay spectroscopy: A sensitive probe of membrane structure. *Biochemistry.* 29:8385–8388.

Jensen, J. W., and J. S. Schutzbach. 1989. Phospholipase-induced modulation of dolichyl-phosphomannose synthase activity. *Biochemistry.* 28: 851–855.

Lafleur, M., B. Fine, E. Sternin, P. R. Cullis, and M. Bloom. 1989. Smoothed orientational order profile of lipid bilayers by ^{2}H-nuclear magnetic resonance. *Biophys. J.* 56:1037–1041.

Lafleur, M., P. R. Cullis, B. Fine, and M. Bloom. 1990. Comparison of the orientational order of lipid chains in the Lα and H_{II} phases. *Biochemistry.* 29:8325–8333.

Le Guernevé, C., and M. Auger. 1995. New approach to study fast and slow motions in lipid bilayers: application to dimyristoylphosphatidylcholine-cholesterol interactions. *Biophys. J.* 68:1952–1959.

Marsh, D., A. Watts, and I. C. P. Smith. 1983. Dynamic structure and phase behavior of dimyristoylphosphatidylethanolamine bilayers studied by deuterium nuclear magnetic resonance. *Biochemistry.* 22:3023–3026.

Metz, G., X. Wu, and S. O. Smith. 1994. Ramped amplitude cross-polarization in magic angle spinning NMR. *J. Magn. Res.* A 110: 219–227.

Montez, B., E. Oldfield, J. A. Urbina, S. Pekerar, C. Husted, and J. Patterson. 1993. Editing of ^{13}C-NMR spectra of membranes. *Biochim. Biophys. Acta.* 1152:314–318.

Nakai, T., and T. Terao. 1992. Measurements of heteronuclear dipolar powder patterns due only to directly bonded couplings. *Mag. Res. Chem.* 30:42–44.

Navarro, J., M. Toivio-Kinnucan, and E. Racker. 1984. Effect of lipid composition on the calcium/adenosine 5′-triphosphate coupling ratio of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *Biochemistry.* 23:130–135.

Oldfield, E., D. Chapman, and W. Derbyshire. 1971a. Deuteron resonance: a novel approach to the study of hydrocarbon chain mobility in membrane systems. *FEBS Lett.* 16:102–104.

Oldfield, E., J. Marsden, and D. Chapman. 1971b. Proton NMR relaxation study of mobility in lipid water systems. *Chem. Phys. Lipids.* 7:1–8.

Oldfield, E., J. L. Bowers, and J. Forbes. 1987. High resolution proton and carbon-13 NMR of membranes: Why sonicate? *Biochemistry.* 26: 6919–6923.

Opella, S. J. 1994. Solid-state NMR structural studies of proteins. *Ann. Rev. Phys. Chem.* 45:659–683.

Pines, A., M. G. Gibby, and J. S. Waugh. 1972. Proton-enhanced nuclear induction spectroscopy. A method for high resolution NMR of dilute spins in solids. *J. Chem. Phys.* 56:1776–1777.

Pines, A., M. G. Gibby, and J. S. Waugh. 1973. Proton-enhanced NMR of dilute spins in solids. *J. Chem. Phys.* 59:569–590.

Powles, J. G., and J. H. Strange. 1963. Zero time resolution nuclear magnetic resonance transient in solids. *Proc. Phys. Soc.* 82:6–15.

Ramamoorthy, A., F. M. Marassi, M. Zasloff, and S. J. Opella. 1995. Three-dimensional solid state NMR spectroscopy of a peptide oriented in membrane bilayers. *J. Biomol. NMR.* 6:329–334.

Rance, M., K. R. Jeffrey, A. P. Tulloch, K. W. Butler, and I. C. P. Smith. 1980. Orientational order of unsaturated lipids in the membranes of *Acholeplasma laidlawii* as observed by ^{2}H-NMR. *Biochim. Biophys. Acta* 600:245–262.

Salmon, A., S. E. Dodd, G. D. Williams, J. M. Beach, and M. F. Brown. 1987. Configurational statistics of acyl chains in polyunsaturated lipid bilayers from ^{2}H-NMR. *J. Am. Chem. Soc.* 109:2600–2609.

Schaefer, J., and E. O. Stejskal. 1976. Carbon-13 nuclear magnetic resonance of polymers spinning at the magic angle. *J. Am. Chem. Soc.* 98:1031–1032.

Schmidt-Rohr, K., J. Clauss, and H. W. Spiess. 1992. Correlation of structure, mobility and morphological information in heterogeneous polymer materials by two-dimensional wideline separation NMR spectroscopy. *Macromolecules* 25:3273–3277.

Schmidt-Rohr, K., D. Nanz, L. Emsley, and A. Pines. 1994. NMR measurement of resolved heteronuclear dipole couplings in liquid crystals and lipids. *J. Phys. Chem.* 98:6668–6670.

Seelig, A., and J. Seelig. 1977. Effect of a single cis double bond on the structure of a phospholipid bilayer. *Biochemistry* 16:45–50.

Seelig, J. 1977. Deuterium magnetic resonance: theory and applications in lipid membranes. *Q. Rev. Biophys.* 10:353–418-287.

Seelig, J. 1978. ^{31}P nuclear magnetic resonance and the headgroup structure of phospholipids in membranes. *Biochim. Biophys. Acta.* 515: 105–140.

Seelig, J., and J. L. Browning. 1978. General features of lipid conformation in membranes. *FEBS Lett.* 92:41–44.

Seelig, J., and N. Waespe-Sarcevic. 1978. Molecular order in cis and trans unsaturated phospholipid bilayers. *Biochemistry.* 17:3310–3315.

Semer, R., and E. Gerelinter. 1979. A spin label study of the effects of sterols on egg lecithin bilayers. *Chem. Phys. Lipids.* 23:201–211.

Smith, R. L., and E. Oldfield. 1984. Dynamic structure of membranes by deuterium NMR. *Science.* 225:280–287.

Stohrer, J., G. Grobner, D. Reimer, K. Weisz, C. Mayer, and G. Kothe. 1991. Collective lipid motions in bilayer membranes studied by transverse deuteron spin relaxation. *J. Chem. Phys.* 95:672–678.

Thurmond, R. L., S. W. Dodd, and M. F. Brown. 1991. Molecular areas of phospholipids as determined by ^{2}H-NMR spectroscopy. Comparison of phosphatidylethanolamines and phosphatidylcholines. *Biophys. J.* 59: 108–113.

Thurmond, R. L., G. Lindblom, and M. F. Brown. 1993. Curvature, order and dynamics of lipid hexagonal phases studied by deuterium NMR spectroscopy. *Biochemistry.* 32:5394–5410.

Tilcock, C. P. S. 1986. Lipid polymorphism. *Chem. Phys. Lipids.* 40: 109–125.

Urbina, J. A., and J. S. Waugh. 1974. Proton-enhanced ^{13}C nuclear magnetic resonance of lipids and biomembranes. *Proc. Natl. Acad. Sci. USA.* 71:5062–5066.

Urbina, J. A., J. Vivas, H. Ramos, G. Larralde, Z. Aguilar, and L. Avilan. 1988. Alteration of lipid order profile and permeability of plasma membranes from *Trypanosoma cruzi* epimastigotes grown in the presence of ketoconazole. *Mol. Biochem. Parasitol.* 30:185–196.

Urbina, J. A., S. Pekerar, H.-b. Le, J. Patterson, B. Montez, and E. Oldfield. 1995. Molecular order and dynamics of phosphatidyl-choline membranes in the presence of cholesterol, ergosterol and lanosterol: a comparative study using ^{2}H, ^{13}C and ^{31}P nuclear magnetic resonance spectroscopy. *Biochim. Biophys. Acta* 1238:163–176.

Urbina, J. A., and E. Oldfield. 1998. Solid state nuclear magnetic resonance spectroscopic approaches to the study of the molecular organization of biological membranes. *In* Biomembrane Structures. D. Chapman and P. I. Haris, editors. IOS Press, Amsterdam. 113–133.

Vega, A. J. 1988. Heteronuclear chemical-shift correlations of silanol groups studied by two-dimensional cross-polarization/magic angle spinning NMR. *J. Am. Chem. Soc.* 110:1049–1054

Weisz, K., G. Grobner, C. Mayer, J. Stohrer, and G. Kothe. 1992. Deuteron nuclear magnetic resonance study of the dynamic organization of phospholipid/cholesterol bilayer membranes: molecular properties and viscoelastic behaviour. *Biochemistry.* 31:1100–1112.

Yeagle, P. L. 1985. Lanosterol and cholesterol have different effects on phospholipid acyl chain ordering. *Biochim. Biophys. Acta.* 815:33–36.