Molecular organization of cholesterol in polyunsaturated phospholipid membranes: a solid state ^{2}H NMR investigation

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Abstract We compared the molecular organization of equimolar [3α-$^2H_1$]cholesterol in 18:0-18:1PC (1-stearoyl-2-oleoylphosphatidylcholine), 18:0-22:6PC (1-stearoyl-2-docosahexaenoylphosphatidylcholine), 18:0-20:4PC (1-stearoyl-2-arachidonylphosphatidylcholine) and 20:4-20:4PC (1,2-diarachidonylphosphatidylcholine) bilayers by solid state ^{2}H NMR. Essentially identical quadrupolar splittings ($\Delta\nu_r = 45 \pm 1$ kHz) corresponding to the same molecular orientation characterized by tilt angle $\alpha_0 = 16 \pm 1°$ were measured in 18:0-18:1PC, 18:0-22:6PC and 18:0-20:4PC. A profound difference in molecular interaction with dipolyunsaturated 20:4-20:4PC, in contrast, is indicated for the sterol. Specifically, the tilt angle $\alpha_0 = 22 \pm 1°$ (derived from $\Delta\nu_r = 37 \pm 1$ kHz) is greater and its membrane intercalation is only 15 mol%.

Key words: Cholesterol; Phospholipid membrane; Polyunsaturated acyl chain; Solid state ^{2}H NMR; Molecular orientation; Cholesterol solubility

1. Introduction

Of the native lipids comprising biological membranes, none possesses membrane-altering properties as does cholesterol [1]. Broadly speaking, the rigid steroid moiety disrupts acyl chain packing in the organized gel state and in the fluid liquid crystalline state limits acyl chain disorder. The transition between these states typically is depressed in temperature, broadened and eventually abolished at high (>20 mol%) cholesterol concentration. The changes have been mapped in phase diagrams which agree globally, if not in detail, on the basis of a variety of experimental methods [2]. A feature of the diagrams constructed by ^{2}H NMR, which for homoacid saturated and heteroacid saturated-monounsaturated PCs closely resemble [3,4], is the formation of a liquid ordered *lo* phase in which there is rapid reorientation but high conformational order over a wide range of temperatures for cholesterol concentrations >25 mol%. This same phase was identified by ^{13}C NMR for homoacid saturated PCs of varying chain length [5]. The molecular organization of cholesterol within homoacid saturated and heteroacid saturated-monounsaturated PC bilayers also appears similar as gleaned by ^{2}H NMR [6–11]. The steroid moiety reorients rapidly about its long molecular axis, the orientation of which fluctuates slightly relative to the bilayer normal. To describe the motion about the molecular axis, models involving axial diffusion through small or discrete jumps have been discussed [12]. Hydrogen bonding between the 3β-hydroxyl of cholesterol and the *sn*-2 carbonyl of phospholipids has been suggested from ^{13}C NMR chemical shifts [13], although differing opinions exist on the likelihood [14].

Much less is known about the interaction of cholesterol with polyunsaturated phospholipids, which are found at high levels in certain membranes. An example is the rod outer segment, wherein dipolyunsaturated phospholipids have been isolated [15]. Because rotational isomerization about the multiple *cis* double bonds is not permitted, it is hypothesized that conformational constraints imposed upon the acyl chain interfere with insertion of the rigid ring structure of cholesterol into the bilayer. This notion is lent credence by the reduction in condensation of average molecular area induced by cholesterol in unsaturated PC monolayers which contain double bonds in the upper portion of the acyl chain, corresponding to the depth into which cholesterol penetrates the bilayer [16]. The results of other techniques confirm that the effects of cholesterol on molecular order and dynamics [17], interfacial elasticity [18] and temperature of the gel to liquid crystalline phase transition [19] differ with highly polyunsaturated lipids. Specifically, the response is diminished.

No report on the molecular organization of cholesterol in polyunsaturated membranes has appeared. We address the issue here. Our solid state ^{2}H NMR investigation compares the ordering of equimolar [3α-$^2H_1$]cholesterol in 18:0-18:1PC, 18:0-22:6PC, 18:0-20:4PC and 20:4-20:4PC. For the first time a major distinction between the interaction of cholesterol with polyunsaturated versus saturated acyl chains is unequivocally identified.

2. Materials and methods

2.1. Materials

Avanti Polar Lipids (Pelham, AL, USA) was the source of 18:0-18:1PC, 18:0-22:6, 18:0-20:4PC and 20:4-20:4PC. [3α-$^2H_1$]-cholesterol deuterated at the 3α position was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) or was a gift from E. Oldfield [6]. Deuterium depleted water was purchased from Isotec Inc. (Miamisburg, OH, USA).

2.2. Sample preparation

Because of the sensitivity of polyunsaturated phospholipids to oxidation, all sample preparations occurred under nitrogen atmosphere

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Abbreviations: ^{2}H NMR, deuterium nuclear magnetic resonance; 18:0-18:1PC, 1-stearoyl-2-oleoylphosphatidylcholine; 18:0-22:6PC, 1-stearoyl-2-docosahexaenoylphosphatidylcholine; 18:0-20:4PC, 1-stearoyl-2-arachidonylphosphatidylcholine; 20:4-20:4PC, 1,2-diarachidonylphosphatidylcholine; 16:0-16:0PC, 1,2-dipalmitoylphosphatidylcholine; 14:0-14:0PC, 1,2-dimyristoylphosphatidylcholine; 22:6-22:6PC, 1,2-didocosahexaenoylphosphatidylcholine; 16:0-20:4PC, 1-palmitoyl-2-arachidonylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; ESR, electron spin resonance; MAS, magic angle spinning

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in a glovebox. Water or buffer used was thoroughly degassed. Exposure to light was also minimized. Stock solutions of 50–100 mg phospholipid in chloroform were combined with [3α-^{2}H$_1$]cholesterol in 1:1 mol ratio. The antioxidant BHT was added in methanol (1:250 BHT relative to lipid). The initial drying of the organic solution under nitrogen stream was followed by 12 h of vacuum pumping to remove residual solvent. After exhaustive mixing with 50% by weight 50 mM Tris buffer and adjusting the pH to 7.5, six lyophilizations with deuterium depleted water were performed to reduce naturally abundant ^2H_2O. The resultant samples consisting of an aqueous multilamellar dispersion were transferred to 5 mm NMR tubes and stored at −20°C. Prior to experimentation they were equilibrated to room temperature for 1 h. All samples were verified to be in lamellar phase via ^{31}P NMR.

2.3. NMR spectroscopy

^{2}H NMR powder pattern spectra were recorded on a homebuilt spectrometer operating at 27.6 MHz [20]. Two probes, one homebuilt and the other constructed by Cryomagnet Systems, Inc. (Indianapolis, IN, USA), with 5 mm transverse mounted coils were utilized. The spectra were collected with a phase alternated quadrupolar echo sequence $(90°_x–\tau_2–90°_y–\text{acquire–delay})_n$ [21]. Unless stated otherwise, spectral parameters were: 90° pulse width = 2.1–3.4 μs; separation between pulses τ_2 = 75 μs; delay time between pulse sequences = 0.075 s; sweep width = ± 500 kHz; data set = 1 K and number of transients = 10 000–100 000. To increase signal to noise, experiments were conducted on resonance and the 'out of phase' channel was zeroed before analysis. The consequent spectra are reflected about the central resonant frequency.

Additional ^{2}H NMR spectra were acquired at 76.7 MHz on another homebuilt spectrometer in the laboratory of R.J. Wittebort [22]. Spectral parameters were comparable except that the delay time between repetition of the pulse sequence was increased to 15 s to facilitate acquisition of a signal from solid cholesterol which has a spin lattice relaxation time T_1 = 4.3 s [23].

2.4. Theory

The steroid ring system of cholesterol is inflexible. Its motion within a membrane consists of fast rotational diffusion about a molecular axis which 'wobbles' about the bilayer normal. The spectrum which ensues for [3α-^{2}H$_1$]cholesterol incorporated into the phospholipid bilayer of an aqueous multilamellar dispersion is a powder pattern consisting of a superposition of doublets from the random orientational distribution of membranes [6,9,24]. Two peaks dominate the powder pattern with splitting

$$\Delta\nu_r = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)|S_{CD}| \quad (1)$$

where (e^2qQ/h) = 170 kHz is the static quadrupolar coupling constant and S_{CD} is the order parameter describing the angular fluctuations of the C-^{2}H bond relative to the bilayer normal. The order parameter may be separated into

$$S_{CD} = S_\alpha S_\gamma. \quad (2)$$

The former term S_α is the molecular order parameter of the steroid moiety, while the latter term

$$S_\gamma = \frac{1}{2}(3\cos^2\gamma - 1) \quad (3)$$

is a geometrical factor governed by the angle γ between the C-^{2}H bond and the molecular axis. Fig. 1 illustrates the disposition of angles and axes. On the basis of earlier work [7,11], a value of γ = 79° may be assigned equivalent to $S_\gamma = -0.445$. The molecular order parameter is defined by

$$S_\alpha = \frac{1}{2}\langle 3\cos^2\alpha - 1\rangle \quad (4)$$

where α is the instantaneous angle between the steroid axis and the bilayer normal (Fig. 1), and the angular brackets designate a time average. Assuming an axially symmetric Gaussian distribution describes α, a most probable value or tilt angle α_0 [6,9,25] may be derived via numerical integration of

$$S_\alpha = \frac{\frac{1}{2}\int_0^\pi \sin(\alpha)\exp(-\alpha^2/2\alpha_0^2)(3\cos^2\alpha - 1)d\alpha}{\int_0^\pi \sin(\alpha)\exp(-\alpha^2/2\alpha_0^2)d\alpha}. \quad (5)$$

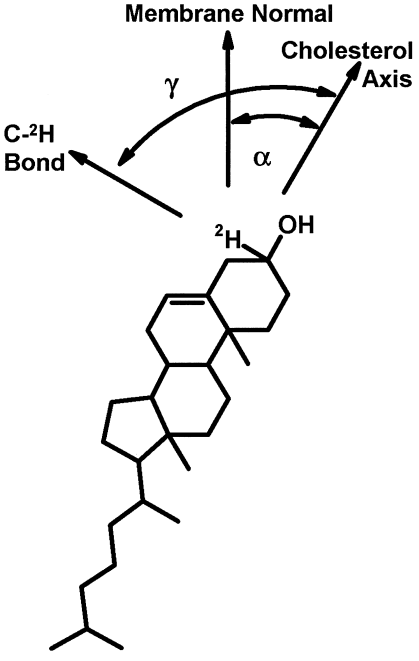

Fig. 1. Angle and axis designation in cholesterol.

3. Results

^{2}H NMR spectra collected for 50 wt% multilamellar dispersions of 18:0-18:1PC/50 mol% [3α-^{2}H$_1$]cholesterol and of 18:0-22:6PC/50 mol% [3α-^{2}H$_1$]cholesterol in 50 mM Tris (pH 7.5) at 20°C are shown in Fig. 2. They are powder patterns from the labelled cholesterol and are symptomatic of the anisotropy of molecular reorientation in membranes. A spike at the central frequency is due to residual ^2H_2O. As can be seen, the respective splittings $\Delta\nu_r = 46 \pm 1$ kHz and $\Delta\nu_r = 45 \pm 1$ kHz are nearly identical for [3α-^{2}H$_1$]cholesterol in 18:0-18:1PC and 18:0-22:6PC bilayers.

To elucidate the orientation of membrane incorporated sterol, the order parameter S_{CD} of the 3α C-^{2}H bond was calculated from the residual quadrupolar splitting $\Delta\nu_r$ (Eq. 1). The molecular order parameter S_α was then determined from S_{CD} (Eq. 2) and matched with numerically integrated values (Eq. 5) to extract a tilt angle α_0. The values of the various quantities obtained for equimolar [3α-^{2}H$_1$]cholesterol in 18:0-18:1PC and 18:0-22:6PC bilayers are included in Fig. 1. They demonstrate that the rigid steroid moiety adopts the same tilt angle $\alpha_0 = 16 \pm 1°$ in both samples. This agreement of α_0 between membranes where the *sn*-1 chain is saturated (18:0) suggests that the insertion of cholesterol is remarkably insensitive to polyunsaturation (22:6 versus 18:1) in the *sn*-2 chain.

A further test of the impact of polyunsaturation was accomplished by comparing 50 mol% [3α-^{2}H$_1$]cholesterol in heteroacid saturated-polyunsaturated 18:0-20:4PC and in homo-

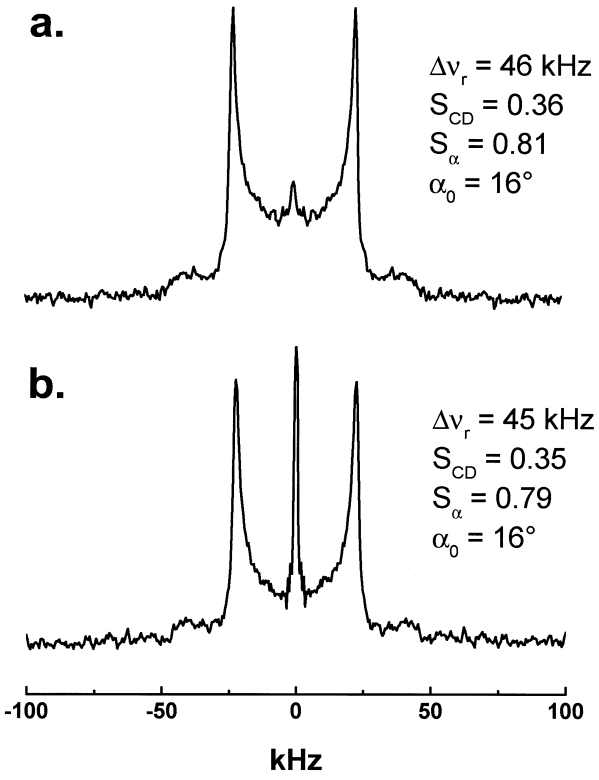

Fig. 2. ^{2}H NMR spectra at 20°C for 50 wt% aqueous multilamellar dispersions in 50 mM Tris (pH 7.5) of a: 18:0-18:1PC/50 mol% [3α-^{2}H$_1$]cholesterol and b: 18:0-22:6PC/50 mol% [3α-^{2}H$_1$]-cholesterol. Spectral parameters (repetition delay = 75 ms) are as described in Section 2. The insets list quadrupolar splitting $\Delta\nu_r$, C-^{2}H order parameter S_{CD}, molecular order parameter S_α and tilt angle α_0. Uncertainties are ±1 kHz for $\Delta\nu_r$, ±0.01 for S_{CD} and S_α, and ±1° for α_0.

acid dipolyunsaturated 20:4-20:4PC bilayers. Fig. 3 shows the ^{2}H NMR spectra recorded at 20°C for 50 wt% multilamellar dispersions in 50 mM Tris (pH 7.5). They are the characteristic powder patterns expected. The quadrupolar splitting $\Delta\nu_r = 44 \pm 1$ kHz and corresponding tilt angle $\alpha_0 = 16 \pm 1°$ for [3α-^{2}H$_1$]cholesterol in 18:0-22:4PC (Fig. 3a) are, within experimental uncertainty, the same as measured in 18:0-18:1PC and 18:0-22:6PC. In contrast, the powder pattern for [3α-^{2}H$_1$]cholesterol in 20:4-20:4PC is significantly narrower with quadrupolar splitting $\Delta\nu_r = 37 \pm 1$ kHz and associated tilt angle $\alpha_0 = 22 \pm 1°$ (Fig. 3b). Clearly the sterol is substantially more tilted with respect to the bilayer normal in 20:4-20:4PC containing polyunsaturated acyl chains at *sn*-1 and *sn*-2 positions than in the other PCs studied which have a saturated 18:0 *sn*-1 chain and *sn*-2 chain with different degrees of unsaturation.

Close inspection of the spectrum plotted in Fig. 3b for 20:4-20:4PC/[3α-^{2}H$_1$]cholesterol reveals a signal to noise ratio that is noticeably worse than in Fig. 3a for 18:0-20:4PC/[3α-^{2}H$_1$]cholesterol, or for 18:0-18:1PC/[3α-^{2}H$_1$]cholesterol and 18:0-22:6PC/[3α-^{2}H$_1$]cholesterol (Fig. 2a and b). A discrepancy in neither amount of sample nor number of acquisitions is responsible. The loss of signal intensity, instead, reflects a major distinction in the interaction of cholesterol with the dipolyunsaturated membrane. Our ^{2}H NMR experiments were designed to observe the signal from [3α-^{2}H$_1$]cholesterol incorporated within the membrane, for which the spin lattice relaxation time $T_1 \sim 3$ ms is short (Brzustowicz and Wassall, unpublished). The delay time of 75 ms between repetition of the quadrupolar echo sequence employed in collecting Figs. 2 and 3 ensures complete relaxation and detection of this spectral component. However, a more slowly relaxing spectral component would be attenuated and not seen if T_1 were sufficiently long.

Fig. 4a is the spectrum acquired with a delay time of 15 s between sequences for the 20:4-20:4PC/[3α-^{2}H$_1$]cholesterol sample at 20°C. There are two components. A motionally narrowed powder pattern with splitting $\Delta\nu_r = 37$ kHz, equivalent to the membrane incorporated cholesterol identified in Fig. 3b, is superposed upon a broad powder pattern with splitting $\Delta\nu_r = 115$ kHz. On the basis of comparison with the spectrum for solid [3α-^{2}H$_1$]cholesterol shown in Fig. 4b, this latter spectral component is assigned to solid-like cholesterol for which the spin lattice relaxation time $T_1 = 4.3$ s is relatively long [23]. Although observable with a 15 s repetition delay, the faster repetition delay of 0.075 s used to obtain Fig. 3b would prohibit observation. A control spectrum collected for 18:0-20:4PC/50 mol% [3α-^{2}H$_1$]cholesterol with 15 s between sequences is presented in Fig. 4c as a confirmation of our

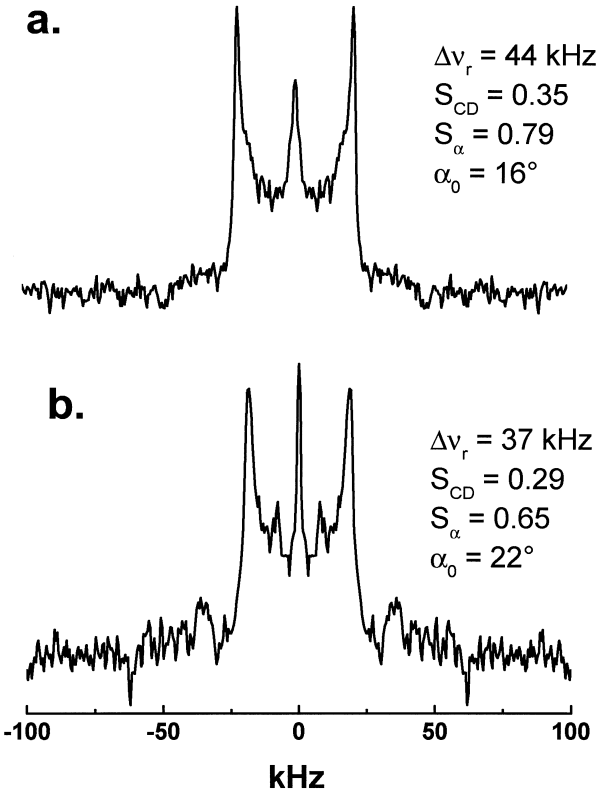

Fig. 3. ^{2}H NMR spectra at 20°C for 50 wt% aqueous multilamellar dispersions in 50 mM Tris (pH 7.5) of a: 18:0-20:4PC/50 mol% [3α-^{2}H$_1$]cholesterol and b: 20:4-20:4PC/50 mol% [3α-^{2}H$_1$]-cholesterol. Spectral parameters (repetition delay = 75 ms) are as described in Section 2. The insets list quadrupolar splitting $\Delta\nu_r$, C-^{2}H order parameter S_{CD}, molecular order parameter S_α and tilt angle α_0. Uncertainties are ±1 kHz for $\Delta\nu_r$, ±0.01 for S_{CD} and S_α, and ±1° for α_0.

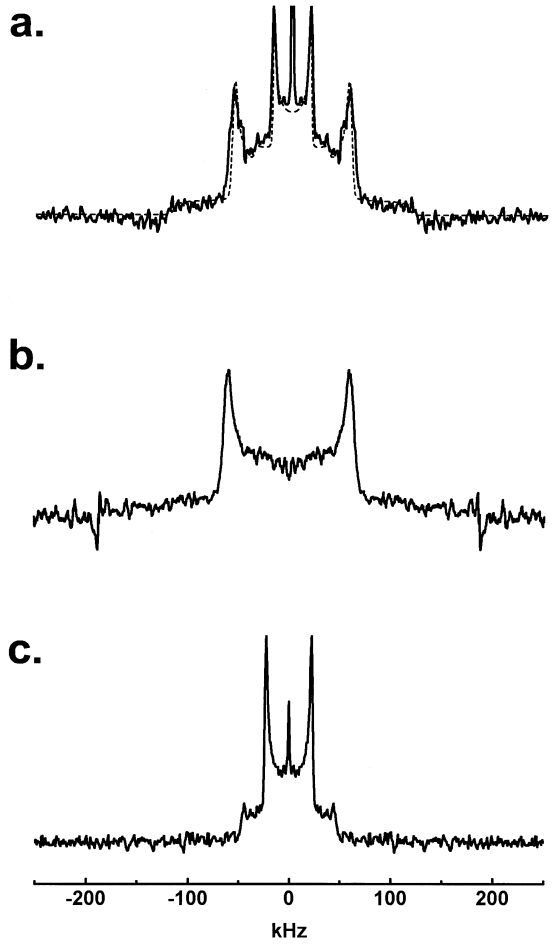

Fig. 4. ^{2}H NMR spectra at 20°C. a: 50 wt% aqueous multilamellar dispersion in 50 mM Tris (pH 7.5) of 20:4-20:4PC/50 mol% [3α-^{2}H$_1$]cholesterol; b: [3α-^{2}H$_1$]cholesterol as a powder; and c: 50 wt% aqueous multilamellar dispersion in 50 mM Tris (pH 7.5) of 18:0-20:4PC/50 mol% [3α-^{2}H$_1$]cholesterol. The dashed line in a is a simulation composed of a superposition of two powder patterns with quadrupolar splittings 37 kHz and 115 kHz in, respectively, relative integrated intensity 30:70. Spectral parameters (repetition delay = 15 s) are as described in Section 2.

interpretation. The spectrum corresponds to complete incorporation of 50 mol% cholesterol into the membrane of 18:0-20:4PC. In contrast to Fig. 4a, a broad component due to solid cholesterol is not seen despite the long delay time that would guarantee its detection. There is only a single narrow powder pattern characterized by a splitting $\Delta\nu_r = 45$ kHz that agrees with the spectrum recorded with a short delay for the same system (Fig. 3a).

A spectrum composed of a superposition of powder patterns from spectral components with quadrupolar splittings corresponding to membrane incorporated and solid cholesterol is simulated as the dashed line included in Fig. 4a. As can be seen, it reproduces the experimental data for 20:4-20:4PC/50 mol% [3α-^{2}H$_1$]cholesterol. The relative integrated intensities of the two respective components are 30:70, which establishes that only 15 mol% cholesterol is intercalated into the dipolyunsaturated bilayer while the remaining cholesterol exists as solid.

4. Discussion

The solid state ^{2}H NMR spectra recorded for 18:0-18:1PC/50 mol% [3α-^{2}H$_1$]cholesterol (Fig. 2a), 18:0-22:6PC/50 mol% [3α-^{2}H$_1$]cholesterol (Fig. 2b) and 18:0-20:4PC/50 mol% [3α-^{2}H$_1$]cholesterol (Fig. 3a) membranes have essentially identical quadrupolar splitting $\Delta\nu_r = 45 \pm 1$ kHz. Closely similar orientation of cholesterol in each membrane is implied, and illustrated by the common tilt angle $\alpha_0 = 16 \pm 1°$ derived assuming a weighted Gaussian distribution describes the 'wobbling' of the rapidly rotating steroid moiety relative to the bilayer normal. This finding, which applies to the PCs studied here containing a saturated 18:0 *sn*-1 chain and an unsaturated *sn*-2 chain with one, four or six double bonds, suggests that the molecular organization of cholesterol within heteroacid PC membranes is unaffected by the number of double bonds in the *sn*-2 chain.

A comparable picture is apparent from a review of previously published ^{2}H NMR results. Quadrupolar splitting $\Delta\nu_r = 45.0$ kHz and tilt angle $\alpha_0 = 16°$ were reported at 24°C and 60°C for 16:0-16:0PC/50 mol% [3α-^{2}H$_1$]cholesterol liposomes [9]. The same residual splitting $\Delta\nu_r = 45$ kHz was measured at 30°C for equimolar [3α-^{2}H$_1$]cholesterol in egg PC [26], which is a heterogeneous mixture of PCs with predominantly saturated 16:0 and 18:0 *sn*-1 chains and unsaturated 18:1 and 18:2 *sn*-2 chains. In 1:1 mol ratio 14:0-14:0PC/[3α-^{2}H$_1$]cholesterol bilayers at 23°C and 60°C, respectively, splittings of approx. 49 kHz and 47 kHz were observed [6].

Two interpretations for the lack of response to *sn*-2 chain polyunsaturation shown by our ^{2}H NMR data on the molecular ordering of [3α-^{2}H$_1$]cholesterol in heteroacid PCs come to mind. Either polyunsaturation has no effect upon membrane incorporation of cholesterol, or the sterol preferentially associates with the saturated *sn*-1 chain thereby minimizing contact with the polyunsaturated *sn*-2 chain. The ^{2}H NMR spectra shown for [3α-^{2}H$_1$]cholesterol in dipolyunsaturated 20:4-20:4PC (Fig. 3b and 4a), where making contact with saturated chains is eliminated, resolve this question. They definitively establish a markedly different interaction of cholesterol with the polyunsaturated chain. The solubility within the 20:4-20:4PC membrane is greatly reduced, as indicated by the poor signal to noise ratio of Fig. 3b. Confirmation is provided in Fig. 4a by the detection of a broad spectral component due to solid cholesterol in addition to the narrow component representing membrane intercalated cholesterol. A solubility of 15 mol% in the dipolyunsaturated bilayer is estimated on the basis of integrated intensity, whereas in excess of 50 mol% is the figure generally accepted for PC membranes [27,28].

The quadrupolar splitting $\Delta\nu_r = 37 \pm 1$ kHz measured for membrane incorporated [3α-^{2}H$_1$]cholesterol in 20:4-20:4PC, moreover, corresponds to tilt angle $\alpha_0 = 22 \pm 1°$ as opposed to $\alpha_0 = 16 \pm 1°$ calculated from the splitting $\Delta\nu_r = 45 \pm 1$ kHz in 18:0-20:4PC. The 6° increase in tilt may indicate that the depth to which cholesterol inserts is restricted by the presence of multiple double bonds in both chains, although it cannot be ruled out that the change merely reflects the lower concentration of cholesterol incorporated within 20:4-20:4PC membranes. Greater molecular ordering of the sterol with increased bilayer content is the consensus of ^{2}H NMR and

ESR work which, with the exception of the current investigation, is yet to include dipolyunsaturated phospholipids [26,29].

The location of the solid cholesterol we identify in 20:4-20:4PC cannot be determined from the spectrum in Fig. 4a. Preliminary optical, X-ray diffraction and DSC observations indicate monohydrate crystals residing external to the membrane (Brzustowicz, Caffrey, Cherezov, Stillwell, Williams and Wassall, unpublished). Solid cholesterol has been detected by solid state NMR methods in other membrane preparations under certain experimental conditions. In qualitative agreement with our result, formation of a separate crystalline phase was very recently inferred by ^{1}H NMR for much of the 50 mol% cholesterol added to dipolyunsaturated 22:6-22:6PC [30]. Most of the NMR signals from the sterol were too broad to be seen by MAS. An estimate of solubility was not made. A superposition of ^{2}H NMR powder patterns from bilayer incorporated and solid-like [2,2,3,4,6-$^{2}H_5$]cholesterol was observed in *A. laidlawii* B membranes exogeneously enriched with up to 41 mol% cholesterol [23]. The solid pool was solubilized into the bilayer pool by an increase in temperature or after lyophilization and rehydration at high temperature. Tight association of undefined nature with the membrane was concluded. Separate signals from cholesterol in monohydrate crystalline and bilayer incorporated forms were resolved by ^{13}C MAS NMR of 16:0-16:0PC with slight molar excess of the sterol [14]. The monohydrate crystals were deemed to be outside the bilayer on the basis of optical microscopy and DSC.

The lower limiting solubility identified here for cholesterol in 20:4-20:4PC offers some rationalization for the behavior of cholesterol containing polyunsaturated phospholipid membranes monitored by alternative biophysical techniques. It was observed via DSC that 20 mol% cholesterol removes the gel to liquid crystal phase transition in 16:0-20:4PC [31]. In contrast, addition of up to 50 mol% cholesterol has little impact upon the transition temperature or enthalpy for 20:4-20:4PC [19]. Analyzing fluorescence anisotropy data for DPH in a series of homoacid and heteroacid PCs of varying degree of unsaturation, appreciable slowing of rotational motion and restriction of orientational freedom for the fluorescent probe due to 30 mol% cholesterol was concluded in all bilayers except dipolyunsaturated 20:4-20:4PC and 22:6-22:6PC [17]. Elastic area compressibility moduli (C_s^{-1}) measured using a Langmuir film balance reveal that >30 mol% cholesterol diminishes the in-plane elasticity of 16:0-22:4PC much more than 20:4-20:4PC [18]. The experimental C_s^{-1} values for the dipolyunsaturated monolayer deviated only slightly from a simple calculation that summed the apportioned compressibility for each lipid, whereas more intimate mixing of the sterol in the heteroacid monolayer is implied by greater deviation from the simple additive model.

The relative affinity of cholesterol for saturated versus polyunsaturated acyl chains has been proposed as a mechanism for lateral separation within membranes into cholesterol rich and poor microdomains [16,17,32]. The models have the sterol segregating into regions that minimize and maximize contact with polyunsaturation and saturation, respectively. In favor of the basic premise behind these proposals, the insensitivity to *sn*-2 chain composition seen here for the molecular orientation of equimolar [3α-$^{2}H_1$]cholesterol in 18:0-18:1PC, 18:0-20:4PC and 18:0-22:6PC may be rationalized in terms of preferential association with saturated over polyunsaturated chains. The exclusion of [3α-$^{2}H_1$]cholesterol from 20:4-20:4PC demonstrated by our results, furthermore, gives direct support.

In conclusion, the molecular organization of [3α-$^{2}H_1$]-cholesterol within 20:4-20:4PC membranes is shown by our solid state ^{2}H NMR investigation to differ profoundly from within 18:0-18:1PC, 18:0-20:4PC or 18:0-22:6PC. Whereas the latter three systems exhibit virtually no dependence upon the level of *sn*-2 unsaturation, the spectra for the former system establish both reduced order and greatly diminished solubility accompanies substitution of polyunsaturation for saturation at the *sn*-1 position. This drastic modification to the behaviour of cholesterol in 20:4-20:4PC identified here has implications of tremendous importance to the interpretation of the effects of the sterol upon the properties of dipolyunsaturated bilayers.

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