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Structural and Dynamical Details of Cholesterol–Lipid Interaction As Revealed by Deuterium NMR†

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ABSTRACT: Deuterium nuclear magnetic resonance of specifically deuterated α- and β-cholesterol and 1-myristoyl-2-(deuteriomyristoyl)-*sn*-glycero-3-phosphocholine (molar ratio 3:7, respectively, dispersed in water) was used to differentiate between pure angular fluctuations (segmental order parameter, S_α) and average orientations (geometrical parameter, S_γ) of C–^{2}H bonds with respect to the normal to the bilayer. Hence, the orientation of cholesterol within the lipid bilayer was determined. In addition, the side chain of β-cholesterol has been found to be as ordered as the condensed ring structure. Such information allows a quantitation of the disordering–ordering effect of cholesterol in biological membranes: through the quasi temperature independence of its "wobbling", β-cholesterol allows motion of the lipid acyl chains below T_c (the transition temperature of the pure lipid) and inhibits them above T_c. Cholesterol thus acts as a *regulatory* agent by maintaining the bilayer membrane in a liquid-crystalline state where the motions are axially symmetric and the local order high. The α-isomer of cholesterol has a disordering–ordering action similar to that found for β-cholesterol. However, α-cholesterol is less efficient in this regulatory role than is the β-isomer, especially at high temperatures. Moreover, α-cholesterol is inclined with respect to the bilayer normal, at physiological temperatures. In the presence of either α- or β-cholesterol, the lipid local order parameter manifests a step in its temperature dependence about T_c, indicating that even in mixed systems the lipid retains a "memory" of its phase transition. A loss of axially symmetric shapes of the deuterium powder spectra was observed at low temperatures [5 °C for β-cholesterol–1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and 15 °C for α-cholesterol–DMPC], indicative of restricted axial motion. For a given bilayer depth and at elevated temperatures, the degree of ordering of cholesterol is higher than that of DMPC; this demonstrates that in mixed systems, measurement of the properties of one component does not necessarily yield also the properties of the others. The latter should be considered in all types of probe experiments used in membrane systems.

Cholesterol is a major component of plasma membranes, where it is often equimolar in concentration with the phospholipids (Gomperts, 1977). It has amphipathic properties due to the presence of a hydroxyl group and a hydrophobic body (the steroid skeleton and the aliphatic tail attached at C-17). It is expected that cholesterol will orient itself within a lipid bilayer with its long axis normal to the membrane surface in order to maximize the hydrophilic and hydrophobic interactions (Figure 1).

Although the dynamics and conformational properties of cholesterol-containing membranes have been extensively investigated by a wide variety of techniques such as electron spin resonance (ESR)[1] (Schreier-Muccillo et al., 1973; Neal et al., 1976) or ^{2}H NMR (Stockton & Smith, 1976; Brown & Seelig, 1978; Oldfield et al., 1978; Taylor et al., 1981, 1982), there is considerable lack of detail at the molecular level. Most of the above studies concluded that cholesterol has a "condensing" effect on the lipid fatty acyl chains at temperatures above that of the gel to liquid-crystalline phase transition, T_c, and a disordering action below T_c. In some cases, it was reported that the gel to liquid-crystalline phase transition of the lipid was removed by addition of high amounts of cholesterol (Davis et al., 1979).

Several modes of action have been postulated. One of them uses the ordering–disordering properties to suggest that the sterol molecule controls the membrane "fluidity" (Madden et al., 1979) whereas another alternative invokes a direct cholesterol–protein or cholesterol–lipid interaction without modification of the so-called "viscosity" of the membranes (Dahl et al., 1981). The present study of cholesterol–lipid interactions at the *molecular level* provides more insight than the less direct or less accurate methods used previously to formulate the above models. It also provides a basis for the analysis of cholesterol–polyene antibiotic–lipid interactions (Dufourc & Smith, 1984).

^{2}H NMR is a reliable technique to obtain molecular information on the flexibility and dynamics of lipid molecules (Davis, 1983; Smith, 1984). Through the synthesis of specifically deuterated DMPC or α- and β-cholesterols (Figure

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[1] Abbreviations: ESR, electron spin resonance; ^{2}H NMR, deuterium nuclear magnetic resonance; DSC, differential scanning calorimetry; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; egg PC, egg phosphatidylcholine; THF, tetrahydrofuran; TLC, thin-layer chromatography.

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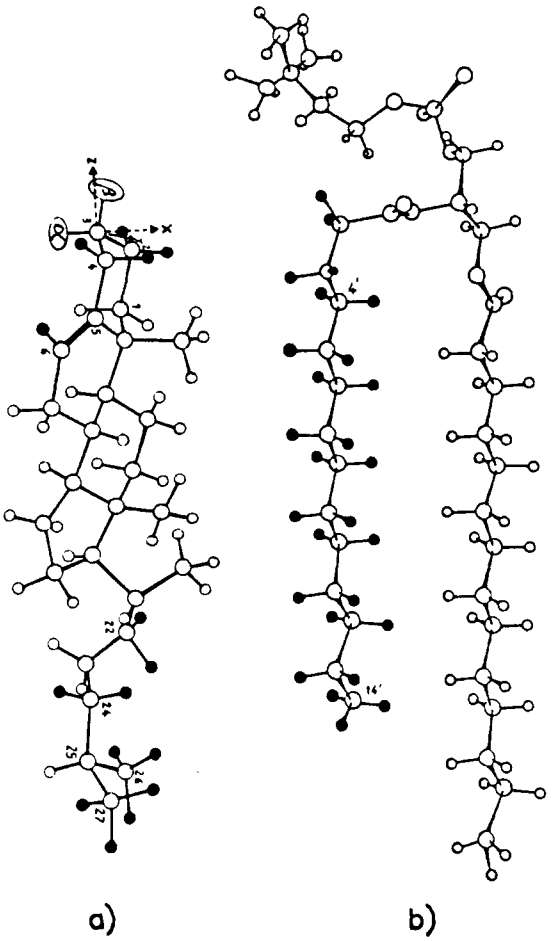

FIGURE 1: Structures of cholesterol (a) and DMPC (b): α = H, β = OH, β-cholesterol; α = OH, β = H, α-cholesterol. The filled circles represent the available deuterium labels. The sterol fixed-axis system (x, y, z) has its origin at C-3.

1), the cholesterol–lipid interaction was studied from the viewpoints of both lipid and cholesterol, at various depths in the bilayer.

Materials and Methods

Specifically deuterated DMPC was synthesized according to published procedures (Dufourc et al., 1983; Perly et al., 1983). Specifically deuterated myristic acids (4,4-$^2H_2$ and 4,4,14,14,14-$^2H_5$) were generously provided by Dr. H. C. Jarrell. [14,14,14-$^2H_3$]Myristic acid was purchased from Larodan Lipids, Sweden. β-[22,22-$^2H_2$]Cholesterol and β-[24,24-$^2H_2$]cholesterol were synthesized according to E. J. Parish and S. Chitrakorn (unpublished results). β-[26,26,26,27,27,27-$^2H_6$]Cholesterol was kindly provided by Dr. M. Taylor, Xerox Research Center, Mississauga, Canada. The α- and β-isomers of Δ^5-[2,2,3,4,4,6-$^2H_6$]cholesten-3-ol were synthesized by modification of a reported procedure (Gruenke & Craig, 1979), as described below.

Δ^4-Cholesten-3-one was prepared from cholesterol (Aldrich, Milwaukee, WI) according to Fieser (1963). Sodium hydride (NaH) (80 mg, 3.3 mmol) was dispersed in 10 mL of monodeuterated methyl alcohol (MeO^{2}H) and added to Δ^4-cholesten-3-one (5 g, 13 mmol). The resulting mixture was stirred and refluxed overnight under dry nitrogen (N_2), the solvent evaporated under dry N_2, and 10 mL of fresh MeO^{2}H added. The reaction was further refluxed 10 h and this cycle repeated. The reaction mixture was cooled and 2.5 mL of deuterated water (^2H_2O) added. The resultant mixture was treated with sodium sulfate (Na_2SO_4) and filtered in a hot funnel. The yellowish solution was dried. The residue was washed twice with CCl_4, and a second crop of exchanged Δ^4-cholesten-3-one was obtained by evaporation of CCl_4. Both crops were dissolved in CCl_4 for ^{1}H NMR. The two proton spectra were identical. Deuterated and nondeuterated compounds gave the same spectrum, except in the region 2.25–2.60 ppm (C-2 protons) and at 5.57 ppm (vinylic hydrogen at C-4) where the deuterated compounds showed no resonances. This loss is characteristic of deuterium incorporation at the C-2 and C-4 positions of the sterol body. Both crops were then poured together and evaporated to dryness to yield 3.5 g of Δ^4-[2,2,4,6,6-$^2H_5$]cholesten-3-one (70%).

The Δ^4-cholesten-3-one was then mixed with 4 mL of isopropenyl acetate and two drops of concentrated sulfuric acid (H_2SO_4) added. The reaction mixture was stirred and refluxed for 1 h under N_2. Anhydrous sodium acetate (0.3 g) was added to the solution and the mixture extracted with $CHCl_3$. The chloroform was evaporated to yield 3.2 g of yellow crystals of enol acetate. The enol acetate was dissolved in 40 mL of THF, and a solution of 3.2 g of $NaBH_4$ in 25 mL of MeO^{2}H containing 2 mL of H_2O was added dropwise with gentle stirring. The reaction mixture was refluxed 1 h and cooled, 40 mL of concentrated HCl was added, and the mixture was quickly poured into 150 mL of H_2O. The aqueous mixture was extracted 3 times with 100 mL of methylene chloride (CH_2Cl_2). The subsequent CH_2Cl_2 fractions were combined, and the solution was evaporated to yield 2.9 g of yellowish crystals.

The reaction mixture was diluted in 40 mL of hexanes/ether (95:5 v/v), warmed for dissolution, and poured onto a 4-cm diameter chromatographic column (250 g of silicic acid, Bio-Sil A 100–200 mesh, packed in hexanes). Compounds were eluted stepwise with 1 L of hexanes/ether (95:5 v/v), 2.5 L of hexanes/ether (90:10 v/v), and 7.5 L of hexanes/ether (85:15 v/v) at a flow rate of 5 mL/min.

Products were characterized by TLC on silica gel plates (Merck). The developing system was hexane/ether/acetic acid (50:50:1 v/v). The sterols were visualized as red on a white background by means of a sterol spray comprising concentrated H_2SO_4/glacial CH_3COOH (50:50) diluted once in water (Kates, 1972). The eluted compounds were identified by comparison with standard samples of Δ^5-α-cholesten-3-ol and Δ^5-β-cholesten-3-ol [Schwarz/Mann (New York, NY) and Sigma Chemical Co. (St. Louis, MO), respectively].

Deuterated α-cholesterol was obtained from the column with 4.5 L of elution, whereas the β-cholesterol appeared after 6 L. Recrystallization from ethanol yielded 120 mg and 1.1 g of the α- and β-isomers of deuterated cholesterol, respectively ($\sim$25% yield based on the undeuterated Δ^4-cholesten-3-one as starting material). The melting points of the α- and β-isomers were 136–138 and 147–149 °C, respectively. Mass spectra of the two isomers revealed incorporation of 4.5 deuterons in each case. Using mass spectral data, and high-resolution deuterium NMR data, we estimated the percentage of incorporation to be $\sim$85% at the C-2, C-4, and C-6 positions and 20% at C-3.

DMPC and cholesterol (α or β) (7:3 molar ratio, respectively) were dissolved in methanol/chloroform (1:2 v/v). The solvent was removed under vacuum at −10 °C. The resulting residue was dispersed in 5 mL of water and lyophilized overnight. The solids appeared as a fluffy white powder which was hydrated with excess deuterium-depleted water (Aldrich Chemical Co., Milwaukee, WI) on a vortex mixer; samples were freeze–thawed and mixed until they were homogeneous and gave reproducible ^{2}H NMR spectra.

Table I: Deuterium Quadrupolar Splittings of the DMPC–β-Cholesterol System,[a] Plateau Region

labeled positions	temp (°C)											
	5	10	15	20	25	30	35	40	45	50	55	65
β-cholesterol[b]												
6-^{2}H		4.0	3.8	3.6	3.4	3.2	3.2	3.2	2.7	2.5	2.4	2.2
3-^{2}H		52.2	52.2	52.2	51.5	51.2	51.0	50.8	48.8	48.3	48.1	46.4
2-^{2}H, eq		34.6	34.6	34.2	34.2	34.2	33.8	33.6	32.1	31.9	31.3	30.3
4-^{2}H, eq		32.2	32.2	32.0	32.0	31.8	31.6	31.4	32.1	31.9	31.3	30.3
2,4-$^2H_2$, ax		49.4	49.0	48.6	48.2	48.0	47.4	47.0	46.0	45.2	44.6	43.4
DMPC[b]												
4′,4′-$^2H_2$	(54.7)[c]	54.2	53.2	52.5	50.8	48.8	46.9	44.4	42.1		37.9	

[a] Data from dePaked spectra, in kilohertz. [b] Accuracy of the splittings ~1–2%. [c] Estimated from the powder spectrum, accuracy ~5%.

NMR signals were acquired on a Bruker CXP-300 spectrometer and analyzed on a Nicolet 1280 computer as described elsewhere (Dufourc et al., 1983).

Results and Discussion

Theoretical Background. The lipid components of biological membranes are now considered as comprising mainly lamellar liquid-crystalline phases in which the bilayer normal is an axis of motional averaging. When the motions of the C–^{2}H segments are axially symmetric, one can relate the residual quadrupolar splittings, $\Delta\nu$, of the ^{2}H NMR powder spectrum to the orientational order parameter, $S_{C\text{-}^2H}$, of the C–^{2}H bond vector according to (Seelig, 1977; Davis, 1983)

$$\Delta\nu = \frac{3}{2}A_Q\frac{3\cos^2\theta' - 1}{2}S_{C\text{-}^2H} \qquad (1)$$

The static deuterium quadrupolar coupling constant (A_Q) (where $A_Q = e^2qQ/h$) is 170 kHz for aliphatic C–^{2}H bonds (Burnett & Muller, 1971) and 175 kHz for olefinic C–^{2}H bonds (Kowalewski et al., 1976). In eq 1, the notations introduced by Petersen & Chan (1977a,b) have been used with $S_{C\text{-}^2H} = S_\alpha S_\theta$ (with $\theta = \gamma$). S_α represents the segmental order parameter, i.e., the angular fluctuations *of* the axis of motion of the rigid subunit with respect to the bilayer normal, whereas S_θ relates the average orientation of a given C–^{2}H bond with respect to the axis of motion of the rigid subunit itself. The angle θ' is that between the bilayer normal and the magnetic field. For C–^{2}H bonds linked to a rigid structure such as the four rings of cholesterol, one can consider that the S_α values are identical. However, these bonds can give rise to different $S_{C\text{-}^2H}$ values, depending upon their geometrical orientations (different S_θ values) with respect to the axis of segmental motion (vide infra).

DMPC–β-Cholesterol Interactions in the Plateau Region of the Bilayer. To monitor the interactions near the top of the bilayer, ^{2}H NMR spectra of β-cholesterol–DMPC mixtures (3:7 molar ratio) were obtained; the deuterium was either on the 4′-carbon of the DMPC *sn*-2 chain or on the 2,2,3,4,4,6-carbon positions of the rigid steroid nucleus (the A and B rings). Spectra were recorded in the temperature range 0–65 °C. Above 10 °C, the spectra of both the lipid and cholesterol are characteristic of axially symmetric motion (Figure 2); increasing temperature leads to increased degrees of averaging of the quadrupolar interaction; i.e., the spectra reduce in width, while maintaining the same shape.

Below 10 °C, the spectra change in shape and increase in width as the temperature is lowered. Such spectral shapes have already been described (Seelig, 1977; Davis, 1983) or encountered in long hydrocarbon chains (Taylor et al., 1983) and are characteristic of a lack of axial symmetry. Since the electric field gradient is axially symmetric for C–^{2}H bonds (Seelig, 1977), the spectra must be due to axially asymmetric motion, probably around the long molecular axis. Approximate calculations to simulate these spectra led to an *apparent* asymmetry parameter of 0.2, at 0 °C.

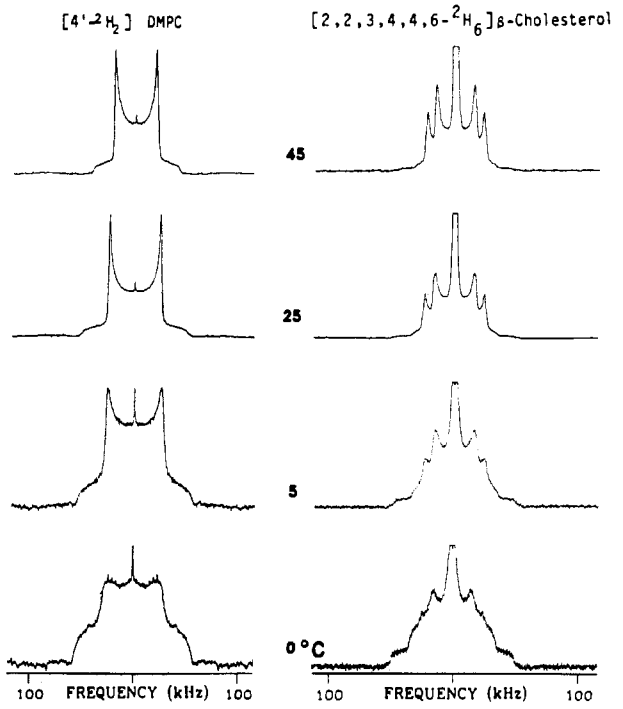

FIGURE 2: Temperature dependence of the ^{2}H NMR spectra of the β-cholesterol–DMPC (3:7 molar ratio) system. Experimental parameters: $\pi/2$ pulse length 4 μs; pulse spacing 60 μs; recycle time 50 ms for deuterated cholesterol and 100 ms for deuterated DMPC; spectral window 500 kHz; 18 000 accumulations.

It is interesting to notice that the ^{2}H powder spectra of both lipid and cholesterol lose their axially symmetric shapes at the same temperature, ca. 5 °C, indicating therefore that the DMPC–β-cholesterol system is uniform with respect to the type of motions involved in averaging the quadrupolar interactions, at low temperatures. It is also noteworthy that the existence of the axially symmetric liquid-crystalline phase of the lipid is extended 15–18 °C below the temperature of the gel–liquid-crystalline phase transition of pure DMPC, i.e., 23 °C.

Above 10 °C, the spectra of both lipid and β-cholesterol are characteristic of axial symmetry. The powder spectra were therefore dePaked (Bloom et al., 1981) to obtain a spectral simplification, i.e., only one macroscopic orientation ($\theta' = 90°$). Figure 3 shows an example for β-cholesterol at 25 °C; the measurement of quadrupolar splittings is far easier on the dePaked spectrum than on the powder pattern. This procedure was used for all spectra, above 10 °C, and the results are compiled in Table I. The assignments of the β-cholesterol deuterons were made on the basis of the calculations below and correspond also to those proposed by Taylor et al. (1981). The C–^{2}H bonds of the rigid sterol nucleus give very different quadrupolar splittings despite their undergoing the same an-

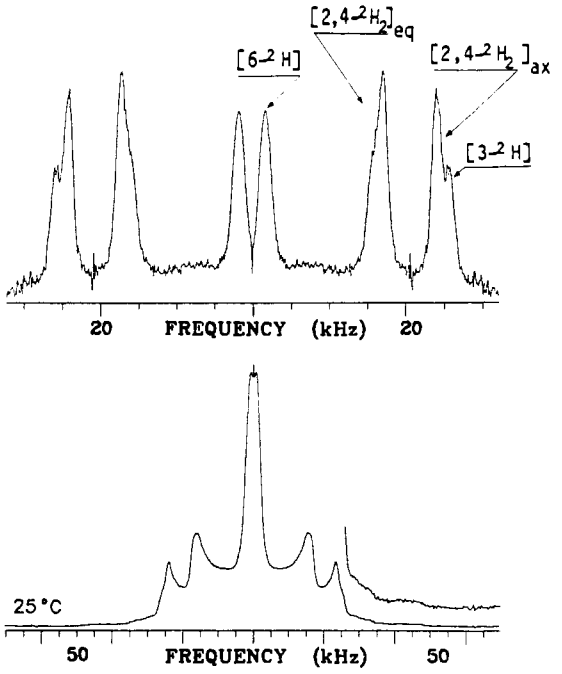

FIGURE 3: ^{2}H NMR spectrum (bottom) and dePaked spectrum (top) of β-[2,2,3,4,4,6-^{2}H$_6$]cholesterol in DMPC (3:7 ratio), at 25 °C; same experimental parameters as in Figure 2. Processing parameters were deconvolution on 800 points and three iterations.

gular fluctuations; i.e., they have the same segmental order parameter S_α (or S_{mol}). These different splittings are due to their different geometrical orientations with respect to the axis of segmental motion. This geometrical dependence can be accounted for and the pure angular fluctuations of the C–^{2}H bonds determined. At least two methods are available to determine S_{mol} (Dufourc et al., 1983). One of them uses the S_{C-^2H} order parameters, and the molecular atomic coordinates, to calculate the order matrix in an arbitrary axis system linked to the rigid unit. This matrix is diagonalized to axially symmetric form, reflecting the properties of the model membrane. The other method defines the orientation of the axis of motion as a function of two angles, β and γ, of the arbitrary molecule-fixed coordinate system and "searches" for these angles. The latter therefore gives S_{mol}, once the orientation of the axis of motional averaging is known. Each method has its particular drawbacks. The first requires, in the most general case, five ^{2}H NMR observables but gives a unique answer. The second does not require as many S_{C-^2H} values but may give rise to several answers. Whereas the first method allows a "blind" search, the second requires a hint to choose the correct solution. Due to the lack of symmetry of the cholesterol molecule, a matrix analysis requires the maximum of S_{C-^2H} order parameters, i.e., five. Although, theoretically, this method could be applied for the present set of data, the quadrupolar splittings are not different enough in magnitude to allow accurate calculations: the best solution led to a value of $S_{mol} \sim 1.2 \pm 0.4$. The second method was applied by using as a hint the fact that cholesterol must orient almost normal to the membrane surface in order to maximize the hydrophobic and hydrophilic interactions. This method, applied to cholesterol, is detailed in the Appendix. At 25 °C, the final result gave $S_{mol} = 0.80 \pm 0.03$ and an orientation of the cholesterol molecule within the bilayer such that the C_3–^{2}H bond vector makes an average angle $\theta = 84° \pm 2°$ with respect to the axis of segmental motion; i.e., the cholesterol molecule is indeed quasi-perpendicular to the membrane surface. This result agrees well with a value of $S_{mol} = 0.78$ estimated by Oldfield

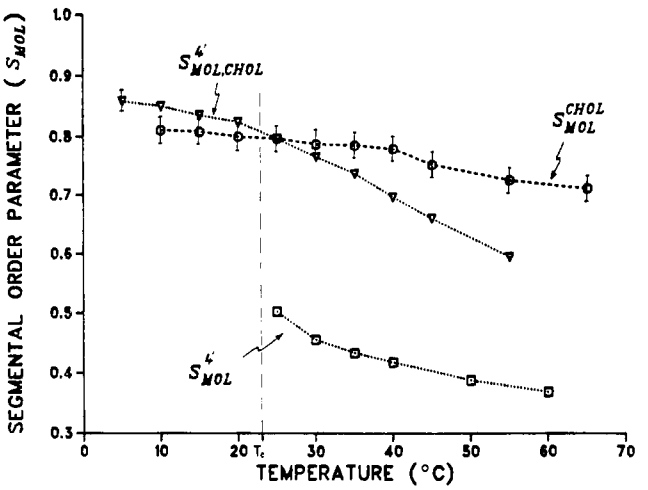

FIGURE 4: Temperature variation of the segmental order parameter, S_{mol}, of the β-cholesterol–DMPC (3:7) system. $S^{4'}_{mol,chol}$ is S_{mol} of DMPC at C-4′, $S^{4'}_{mol}$, but in the presence of sterol, and S^{chol}_{mol} is S_{mol} of the four rings of cholesterol in DMPC. The bars and symbols give an estimate of the error.

et al. (1978) at 23 °C for β-cholesterol in a similar system mixture, arbitrarily assuming $\theta(C_3–^2H) = 90°$. Our findings for the β-cholesterol–DMPC (3:7) system also correlate well with those obtained by Taylor et al. (1981) [$S_{mol} \simeq 0.87$, $\theta(C_3–^2H) = 79° \pm 2°$] for β-cholesterol in egg PC (1:1) at 25 °C.

These calculations were performed for all spectra obtained between 10 and 65 °C. The resultant S_{mol} values are plotted as a function of temperature in Figure 4. In this figure, we also report S_{mol} for the lipid labeled at C-4′, in the presence and absence of 30 mol % β-cholesterol. Since the average orientation of a chain methylene C–^{2}H bond with respect to the axis of segmental motion can be taken to be 90°, i.e., the C–^{2}H bond angular reorientations are equiprobable around $\theta = 90°$, $S_{mol} = |2S_{C-^2H}|$ for the lipid at C-4′ (Seelig & Niederberger, 1974). The first noticeable feature on Figure 4 is the virtual temperature independence of the β-cholesterol segmental order parameter, S^{chol}_{mol}, between 10 and 40 °C; even at 65 °C, the S^{chol}_{mol} value differs by less than 10% from its value at 10 °C. The segmental order parameter of the lipid at C-4′ in the presence of β-cholesterol, $S^{4'}_{mol,chol}$, changes rather drastically over the same temperature range. Below $T_c = 23$ °C, the temperature of the gel to liquid-crystal phase transition of the pure lipid, the change in $S^{4'}_{mol,chol}$ with temperature is subtle whereas above 23 °C the drop in $S^{4'}_{mol,chol}$ with increasing temperature is rather marked. Despite this apparent memory of the pure lipid phase transition, it is difficult to conclude, on the basis of the available data, that there is indeed a phase transition occurring at ca. 23 °C. One can nevertheless notice that near T_c, the gel to liquid-crystalline phase transition temperature of the pure lipid, the temperature dependence of the lipid chain flexibility changes remarkably. Below T_c the amplitudes of the angular fluctuations of the C–^{2}H segments at C-4′ are almost the same as those of the cholesterol body. It should be recalled that below T_c the lipid chains would not show any C–^{2}H angular fluctuations at all if β-cholesterol were not present (Davis, 1979). One may therefore interpret the "fluidizing" effect as a disordering effect: β-cholesterol through its virtually temperature-independent wobbling forces the lipid chains to fluctuate even below 23 °C, since it fluctuates itself. Since between 10 and 23 °C $S^{chol}_{mol} \sim S^{4'}_{mol,chol}$, it appears therefore that the cholesterol controls, through its own motions, the motions of the entire system, in this temperature range. Above ~23 °C, $S^{4'}_{mol,chol}$ behaves similarly to $S^{4'}_{mol}$ (segmental

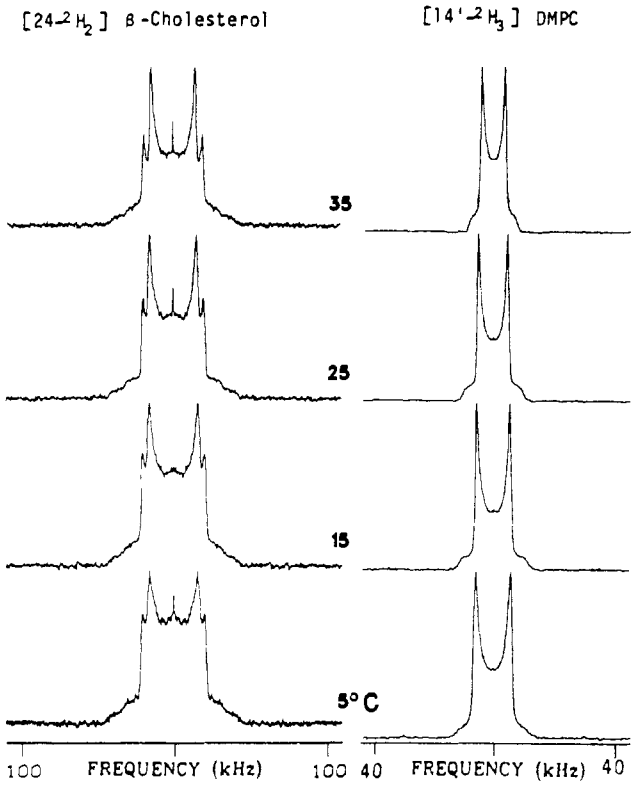

FIGURE 5: Temperature dependence of ^2H NMR spectra of the β-cholesterol–DMPC system at the indicated labeled positions. The same experimental parameters as in Figure 2 were used, except for a recycle time of 100 ms for deuterated sterol and 1 s for [14′,14′,14′-$^2H_3$]DMPC; a 250-kHz spectral window was used for deuterated DMPC.

order parameter at C-4′ in the absence of cholesterol) with increasing temperature, except that the $S^{4'}_{mol,chol}$ profile is shifted toward higher values of the segmental order parameter. It is interesting to notice that at high temperatures S^{chol}_{mol} is markedly higher than $S^{4'}_{mol,chol}$. The system is thus characterized by two kinds of angular fluctuations, i.e., those of the lipid and those of β-cholesterol. This point is rather important, and we would like to emphasize that measuring the segmental order parameter of β-cholesterol at 65 °C would not give information about the flexibility of the lipid chains. One would measure the property of the probe in the system rather than that of the system itself.

DMPC–β-Cholesterol Interactions in the Region of the Cholesterol Alkyl Chain. The amplitude of the motions near the center of the bilayer was monitored by spectra from both DMPC and β-cholesterol molecules (7:3 molar mixture). The deuterium was on either the 14′-position of the DMPC *sn*-2 chain or the 22-, 24-, 26-, and 27-positions of the β-cholesterol alkyl tail. Figure 5 shows the temperature variation of the spectra of both [14′,14′,14′-$^2H_3$]DMPC and β-[24,24-$^2H_2$]-cholesterol, whereas Figure 6 compares the deuterium powder patterns of the β-cholesterol tail at positions 22, 24, 26, and 27, at 25 °C. Several interesting features can be noticed in Figure 6. First, multiple powder patterns are observed for positions near the end of the tail, i.e., positions 24,24-$^2H_2$ and 26,26,26,27,27,27-$^2H_6$. The inequivalence of the terminal methyl groups of β-cholesterol (Figure 6d) has already been demonstrated in egg PC by Taylor et al. (1982). These authors observed that the degree of inequivalence diminished progressively as the concentration of β-cholesterol increased; at 30 mol % β-cholesterol, the two methyl groups were essentially equivalent. This might appear to be the case also in Figure 6d; however, the high signal to noise ratio allows us to distinguish two shoulders (i.e., two $\theta' = 0°$ orientations), indicated by arrows. It is worthwhile mentioning that the frequency of the spectrometer was carefully set in the center of the powder pattern such that no distortion was induced by folding the spectrum about its center in order to increase the signal to noise ratio by $2^{1/2}$. The dePaking procedure was tried on this spectrum, but the two components were still not resolved; this is simply due to the fact that the difference in quadrupolar splitting is of the order of the individual component line widths.

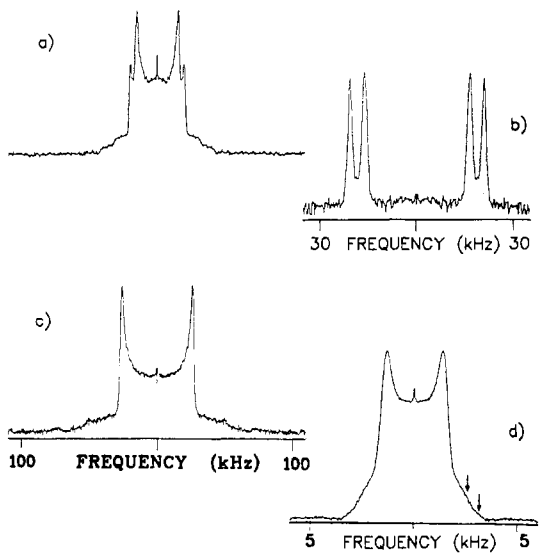

FIGURE 6: ^2H NMR spectra of β-cholesterol in DMPC (3:7) at 25 °C: (a) β-[24,24-$^2H_2$]cholesterol; (b) same as (a), dePaked; (c) β-[22,22-$^2H_2$]cholesterol; (d) β-[26,26,26,27,27,27-$^2H_6$]cholesterol.

The two deuterons at C-24 are also inequivalent but have their respective powder patterns well separated from each other (Figure 6a); the dePaked spectrum (Figure 6b) shows that the two pairs of resonances have the same area, within experimental error. Furthermore, there was no change in the relative areas of the dePaked lines for position 24 over the temperature range 5–65 °C, thus ruling out the possibility of two long-lived conformations. More likely, it would appear that the two deuterons at C-24 have different average orientations with respect to the axis of segmental motion. Such a case has already been encountered (Engel & Cowburn, 1981a,b; Dufourc et al., 1983) and is a quite general situation for the two deuterons at the C-2′ *sn*-2 position in phospholipid bilayers (Seelig, 1977). The method used to calculate the average orientation of the rigid sterol moiety was used here to calculate the order and average orientation of the two deuterons at C-24, with respect to the axis of segmental motion. Although not very accurate due to the use of only two splittings, this method led to two main solutions: $S_{mol} = 0.66 \pm 0.04$, $\theta_1 = 88° \pm 4°$, and $\theta_2 = 74° \pm 4°$; $S_{mol} = 0.66 \pm 0.04$, $\theta_1 = 36° \pm 4°$, and $\theta_2 = 73° \pm 4°$, where S_{mol} is the segmental order parameter of the methylene unit at C-24 and θ_1 and θ_2 are the average angles made by the two deuterons with respect to the axis of segmental motion. It is interesting to notice that both solutions give the same order parameter with different orientations of the 24,24-$^2H_2$-labeled segment: one of them is such that the deuterons are almost at 90° with respect to the axis of motion (similar to methylene segments in a lipid fatty acyl chain) whereas the other would involve the normal to the ^2H–C-24–^2H plane almost perpendicular to the axis of motion. Although a choice between these two solutions is rather difficult to make, the orientation involving an extended cholesterol side chain conformation seems the more plausible, on the basis of the following.

The two deuterons at C-22 exhibit a single powder pattern; i.e., they possess the same average orientation with respect to the axis of segmental motion. Assuming that this orientation is 90°, the segmental order parameter at C-22 will be equal to $|2S_{C-^2H}|$ which leads to $S_{mol} = 0.83 \pm 0.01$, at 25 °C. This value agrees well with the molecular order parameter found in the previous section for the rigid steroid structure, i.e., $S_{mol} = 0.80 \pm 0.03$, at the same temperature. This indicates that one can consider the cholesterol acyl chain, up to C-22, as rigid as the four-ring structure; i.e., the rigid cholesterol cylinder-like body can be extended up to C-22. Moreover, one can also consider that all the cholesterol side chain is highly ordered since at C-24, two carbons from the end of the chain, the segmental order parameter is equal to 0.66. These findings confirm unambiguously the earlier hypothesis that the β-cholesterol side chain was rigid (Darke et al., 1972; Shimshik & McConnell, 1973).

With regard to the low-temperature spectra, one notices in Figure 5 that the β-[24,24-$^2H_2$]cholesterol spectrum begins to show small features indicative of axial asymmetry at 5 °C, as was found for the rigid cholesterol nucleus. Conversely, and in contrast to [4′,4′-$^2H_2$]DMPC at low temperatures (see previous section), the terminal methyl group of DMPC does not show these features. However, it is difficult to compare the motions of methylene and methyl groups. It is indeed well-known that the methyl unit provides an additional averaging of the quadrupolar interaction through rotation about its C_3 axis. It is then possible that when the motions approach axial asymmetry for the methylene units, the additional motion of the methyl group preserves the axial symmetry. This is of course an oversimplified view of the methyl motions. The line shapes of the [14′,14′,14′-$^2H_3$]DMPC spectra (which are not well understood) at lower temperatures suggest that a qualitative and quantitative description of these motions would require a detailed line-shape analysis beyond the scope of the present study.

The hypothesis that only the cholesterol rigid nucleus determines the fluidizing–condensing effects in lipid–cholesterol mixtures (Rothman & Engelman, 1972) can be investigated by comparing the temperature response of the cholesterol tail positions with respect to that of the lipid positions at the same approximate depth in the membrane, i.e., near the center of the bilayer. The only available variable temperature data to date are the quadrupolar splittings of [24,24-$^2H_2$]cholesterol and of [14′,14′,14′-$^2H_3$]DMPC. As mentioned earlier, it is difficult to compare methylene and methyl motions; however, Stockton et al. (1976) proposed to relate the motions of these two groups with respect to the same axis of motion, i.e., the bilayer normal. To do so, $S_{mol}^{CH_3}$ can be defined as (Stockton et al., 1976)

$$S_{mol}^{CH_3} = |6S_{C-^2H_3}| \quad (2)$$

In the following qualitative analysis, we shall use eq 2, but we point out that due to the additional averaging brought by the methyl free rotation, $S_{mol}^{CH_3}$ might be underestimated. Figure 7 shows the segmental order parameter of the lipid position 14′ in the presence of β-cholesterol, $S_{mol,chol}^{14'}$, and that of β-cholesterol at C-24, S_{mol}^{24}, in the temperature range 5–65 °C. Also shown are data for lipid positions C-12′ and C-13′ (E. J. Dufourc et al., unpublished results), for the same system at 25 °C, in order to compare the magnitude of S_{mol} for lipid and cholesterol methylene units. The similarity in profile between Figure 7 and Figure 4 (plateau region) is striking: the S_{mol}^{24} shows almost the same temperature insensitivity as S_{mol}^{chol}, whereas the lipid at C-14′ exhibits the same phase transition memory around 23 °C as was observed for the

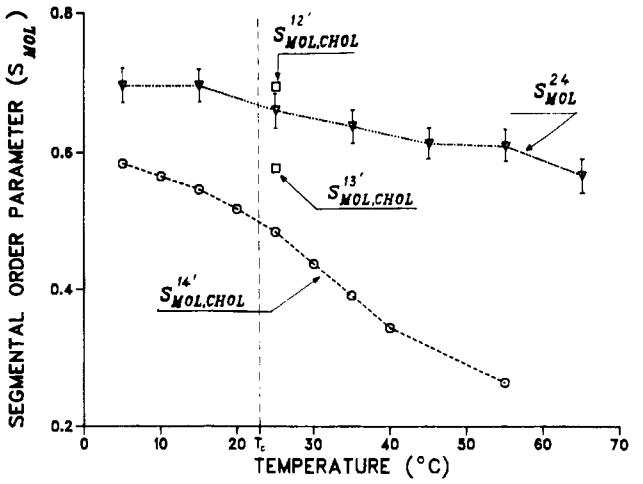

FIGURE 7: Temperature variation of the segmental order parameter, S_{mol}, of the β-cholesterol–DMPC model membrane system: S_{mol}^{24} is S_{mol} of sterol labeled at C-24, in DMPC; $S_{mol,chol}^{14'}$ is S_{mol} of DMPC labeled at C-14′, in the presence of cholesterol; $S_{mol,chol}^{12'}$ and $S_{mol,chol}^{13'}$ are the corresponding data for C-12′ and C-13′, respectively.

$S_{mol,chol}^{4'}$ in Figure 4. This leads to the conclusion that although the amplitudes of the C–^{2}H angular fluctuations are different, their temperature responses are identical for all lipid and cholesterol positions, respectively. The marked difference in magnitude, at 25 °C, between the $S_{mol,chol}^{14'}$ and S_{mol}^{24} values may be accounted for by the fact that the C–$^2H_3$ lipid unit undergoes additional motional averaging than does the C–$^2H_2$ unit of the cholesterol tail, at C-24. The segmental order parameter at positions 12′ and 13′ shows that indeed, around 25 °C, the amplitudes of the angular fluctuations of the C–$^2H_2$ lipid units are of the same order of magnitude as those of the cholesterol side chain methylene units. The role of cholesterol, as discussed for the plateau region, can therefore be generalized throughout the membrane bilayer. In addition, due to the relative rigidity of its side chain, the cholesterol molecule can be considered as an almost rigid cylinder, with a molecular order parameter of 0.7–0.8, at 30 mol % DMPC. It would be interesting to investigate whether the rigidity of the cholesterol side chain is dependent on cholesterol concentration.

With respect to the terminal methyl units at C-26 and C-27, the occurrence of such small quadrupolar splittings is not fully understood. Stockton et al. (1976) have used $S_{mol} = 18S_{C-^2H}$ for the three choline methyl groups, arguing that their S_{C-^2H} could be related to the instantaneous axis of motion by three geometric transformations. According to these authors, one will find, using Table II, $S_{mol}^{26,27} \sim 0.38$–$0.44$, which is in good agreement with the value found for $S_{mol,chol}^{14'}$, at 25 °C. However, this calculation does not account for either the inequivalence of C-26 and C-27 or the additional motional averaging due to the free methyl rotation. Although $S_{mol,chol}^{14'} \sim S_{mol}^{26,27}$, the value of S_{mol} for methyl groups might simply be peculiar to methyl motions and not segmental chain fluctuations. The deuteration at C-25 of the β-cholesterol side chain should give additional useful information.

α-Cholesterol–DMPC System. A recent study of the interaction of β-thiocholesterol with egg PC (Parkes et al., 1982) concluded that the β-hydroxy group of cholesterol determines, through its hydrogen bonding with phospholipids, the solubility of sterol and the membrane ordering properties. The α-isomer of cholesterol (epicholesterol, see Figure 1) is thus expected to exhibit weak hydrogen bonding with lipids, a limited solubility (Demel et al., 1972), and little ordering effect on the phospholipid membranes. The results presented below are an attempt to clarify this assertion and to provide an explanation

Table II: Deuterium Quadrupolar Splittings of the DMPC–β-Cholesterol System,[a] Tail Region

labeled positions	temp (°C)										
	5	10	15	20	25	30	35	40	45	55	65
β-cholesterol											
22,22-$^2H_2$					53.1						
24-^2H	(33.2)[d]		33.2		32.2		30.3		27.8	25.4	23.7
24-^2H	(42.0)[d]		42.0		41.0		39.8		38.1	36.1	34.4
26,26,26-$^2H_3$					2.7[c]						
27,27,27-$^2H_3$					3.1[c]						
DMPC[b]											
12′,12′-$^2H_2$[e]					44.3						
13′,13′-$^2H_2$[e]					36.8						
14′,14′,14′-$^2H_3$	12.4	12.0	11.6	11.0	10.3	9.3	8.3	7.3		5.6	

[a] From dePaked spectra, in kilohertz. [b] Accuracy of the splittings ~1–2%. [c] Estimated from the shoulders of the powder spectrum. [d] Estimated from the powder spectrum, since this latter spectrum showed a nonsymmetrical shape (see text), accuracy ~5%. [e] From [*sn*-2-$^2H_{27}$]DMPC–β-cholesterol (7:3) (E. J. Dufourc et al., unpublished results).

Table III: Deuterium Quadrupolar Splittings of the DMPC–α-Cholesterol System,[a] Plateau Region

labeled positions	temp (°C)									
	10	20	25	30	35	40	45	50	55	65
α-cholesterol[b]										
6-^2H		5.0	5.8	6.0		7.8		9.4	9.8	10.2
2-^2H, ax		53.5	54.7	54.1		52.2		49.3	48.8	46.6
2-^2H, eq		26.5	24.9	24.2		20.5		15.1	15.1	13.1
4-^2H, ax		53.5	54.7	54.1		52.2		49.3	48.8	46.6
4-^2H, eq		26.5	24.9	24.2		23.0		20.0	20.0	18.6
DMPC[b]										
4′,4′-$^2H_2$	(53.7)[c]	50.8	48.3	45.9	43.0		38.3		34.9	
14′,14′,14′-$^2H_3$	(12.0)[c]	10.3	9.3	8.3	7.3		5.9		5.1	

[a] Data from dePaked spectra, in kilohertz. [b] Accuracy of the splittings ~1–2%. [c] Estimation from the powder pattern, accuracy ~5%.

for the absence of α-cholesterol in natural membranes.

Deuterium spectra of α-cholesterol–DMPC (3:7) were obtained over the temperature range 10–65 °C. The ^2H labels were either on the lipid at positions C-4′ and C-14′ or on α-cholesterol at positions 2, 3, 4, and 6. Above 20 °C, the spectra are characteristic of axial symmetry, and around 15 °C, evidence of asymmetry appears. Whereas the β-cholesterol–DMPC system shows axially symmetric behavior for 15–18 °C below T_c, epicholesterol at the same concentration does so for only 5–8 °C below T_c. It can therefore be concluded that α-cholesterol is less efficient than β-cholesterol with respect to the fluidizing effect on DMPC. This will be emphasized further in the next section.

Figure 8 shows some corresponding spectra of α- and β-cholesterol. The utility of the dePaking procedure is well illustrated; individual lines can be resolved on a dePaked spectrum even when they are barely seen in the powder pattern, e.g., α-cholesterol at 65 °C. Quadrupolar splittings are reported in Table III. The assignment of the α-cholesterol splittings is based on the calculations described below and in the Appendix.

First, a qualitative comment can be made regarding the relative changes in both spectral width and shape for α- vs. β-cholesterol with increasing temperature. From 25 to 65 °C, all splittings of β-cholesterol decrease; this indicates that the molecular order parameter decreases without any change in the location of the motional averaging axis relative to the molecular framework. Over the same temperature range, certain labeled positions of α-cholesterol show a decrease in quadrupolar splitting, whereas others increase (Figure 8, top spectra). Thus, for α-cholesterol, both S_{mol} and the average orientation of the α-isomer within the bilayer membrane are changing with temperature.

For quantitation of these observations, calculations analogous to those carried out for β-cholesterol (vide supra) were undertaken for α-cholesterol (see Appendix for details). Unfortunately, it was not possible to obtain a unique solution,

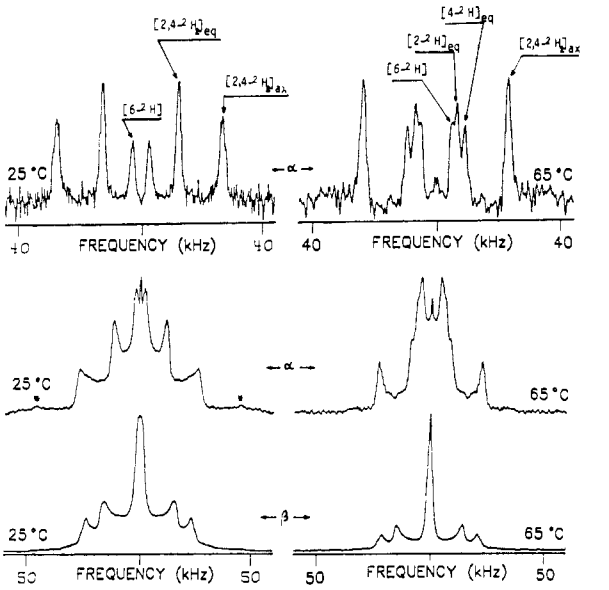

FIGURE 8: ^2H NMR spectra of α-[2,2,3,4,4,6-$^2H_6$]cholesterol (center) and β-cholesterol (bottom) in DMPC (3:7) at the indicated temperatures. The top spectra are the dePaked analogues of the central spectra. The experimental and processing parameters are the same as in Figure 3.

that is, to determine unambiguously the position of the axis of motion about which the four rings of α-cholesterol rotate. As mentioned in the Appendix, two solutions were found. The first solution directs the OH group (in α) toward the aqueous surface, whereas the second points the hydroxyl group toward the hydrophobic core (see sketches in Figure 9). To decide which of these solutions is correct, an additional experiment involving α-cholesterol labeled at C-3 would be needed. The average orientation of the axis of motion of the four rings of α-cholesterol in DMPC (3:7) predicts a quadrupolar splitting at C_3–^2H of ca. 40 kHz or ca. 100 kHz for the two possible solutions (see the Appendix). The compound is not presently

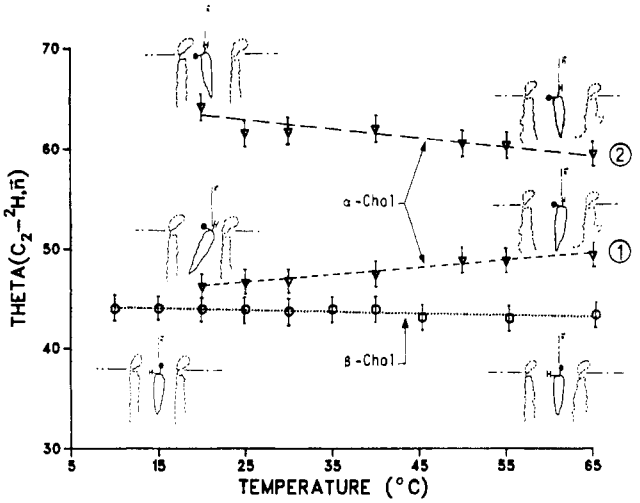

FIGURE 9: Temperature dependence of the angle $\theta(C_2-^2H_{eq}, \vec{n})$ of α- or β-cholesterol in DMPC (3:7). The filled circles in the molecular sketches represent the OH group in the α- or β-position.

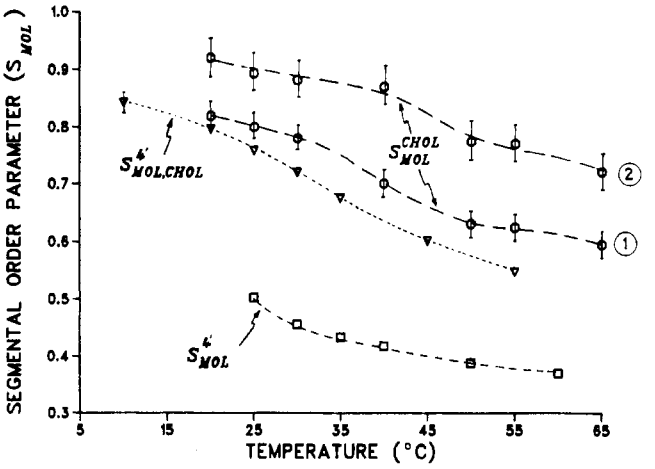

FIGURE 10: Temperature dependence of the segmental order parameter, S_{mol}, in the system α-cholesterol–DMPC (3:7): S^{chol}_{mol} is S_{mol} of the four rings of cholesterol in DMPC; $S^{4'}_{mol,chol}$ is that of DMPC labeled at C-4′, $S^{4'}_{mol}$, but in the presence of sterol.

available, and the low ^2H incorporation at C-3 (~20%) in α-[2,2,3,4,4,6-$^2H_6$]cholesterol does not allow unequivocal interpretation of the weak spectral features at ca. 94 kHz in Figure 8 (indicated by an asterisk in the spectrum of α-cholesterol at 25 °C) as an argument in favor of the second solution. In order to characterize the reorientation of α-cholesterol within the DMPC bilayer, it was chosen to follow the angular variation $[\theta(C_2-^2H_{eq})]$ of a particular C–^2H bond vector with respect to the axis about which the rigid region of cholesterol rotates (axis of segmental motion) as a function of temperature. Figure 9 reports such a variation for both α- and β-cholesterol. Whereas there is no change in orientation of β-cholesterol in DMPC from 10 to 65 °C, both solutions for α-cholesterol show that the $C_2-^2H_{eq}$ bond vector tends to reorient toward the magic angle with respect to the internal averaging axis, as the temperature increases. This agrees well with the observation that the $\Delta\nu_Q$ value for this bond decreases more rapidly than do the others of α-cholesterol when the temperature is increased (Table III and Figure 8). It is interesting to notice that at physiological temperatures β-cholesterol sits vertically in the bilayer membrane, whereas the α-isomer shows a marked tilt with respect to the membrane surface (see sketches in Figure 9). Such an orientation for α-cholesterol in DMPC can be understood by considering the hydrophilic and hydrophobic interactions as well as some simple mechanistic concepts. The hydroxyl group in β-cholesterol can be expected to coincide with the axis of inertia of the entire molecule whereas in the α-isomer (assuming to first order that α- and β-cholesterol have the same axis of inertia) the OH group is definitely not aligned along this axis. To maximize the hydrophobic and hydrophilic interactions, the β-isomer therefore sits vertically within the membrane, its OH group pointing toward the water surface. Such a situation satisfies both the hydrophilic–hydrophobic forces and the inertial equilibrium of the molecule and can thus be expected to be very stable: it is therefore not surprising that β-cholesterol does not change the location of its motional averaging axis, even when the amplitude of allowed motion increases (Figure 9). The α-isomer tends also to direct its OH group toward any hydrophilic region (the water surface or the C=O of the lipids), and to do so it must adopt a tilted orientation with respect to the bilayer. On the other hand, the inertia of the molecule tends to align α-cholesterol perpendicular to the surface, parallel to the principal axis of motion of the membrane, the bilayer normal. Therefore, the observed tilt would be the resultant of two constraints, the amphipathic interactions and the inertia of α-cholesterol, acting in different directions. At high temperatures, the amplitude of allowed motions increases, leading to a decrease of the lateral bilayer pressure; the hydrophobic and hydrophilic interactions are therefore less demanding, and the α-isomer will tend to align its axis of inertia toward the perpendicular to the bilayer surface (Figure 9).

The above analysis, although approximate, leads to two main conclusions. First, a small modification in the structure of cholesterol (OH α or β) leads to important modifications of molecular organization in bilayers. Second, the tilted position of α-cholesterol within the bilayer membrane could be the reason that this isomer of cholesterol is not present in natural membranes—it would disturb too greatly the parallel packing of the lipid chains.

The molecular order parameter of α-cholesterol (four-ring system) in DMPC, S^{chol}_{mol}, has been calculated by using the procedure described for β-cholesterol. The results are reported in Figure 10 together with the segmental order parameter of DMPC labeled at C-4′ with and without α-cholesterol, $S^{4'}_{mol,chol}$ and $S^{4'}_{mol}$, respectively. One notices first that α-cholesterol shows disordering–ordering properties similar to those observed for β-cholesterol (compare Figures 4 and 10). There is a greater temperature dependence of S_{mol} of α-cholesterol than was observed for the β-isomer. Since we cannot choose with confidence between the two sets of solutions for S^{chol}_{mol}, we must therefore postpone discussion of the relative amplitudes of angular fluctuations of α- or β-cholesterol. The temperature profiles of $S^{4'}_{mol,chol}$ (Figures 4 and 10) indicate that the lipids containing α- or β-cholesterol show almost the same segmental order parameter at low temperatures whereas above T_c $S^{4'}_{mol,chol}$ is significantly lower in the presence of the α-species than of the β-isomer. This seems to be general for all labeled positions since inspection of Tables II and III reveals that the quadrupolar splittings of C-14′-labeled DMPC containing α-cholesterol are always lower than those observed in the presence of the β-isomer, above T_c. To summarize the comparisons of the ordering capabilities of both isomers, the α-isomer is also able to regulate the motions of the lipid acyl chains but is less efficient in that role than β-cholesterol, especially at high temperatures. One also notices that the lipid mixed with 30 mol% α-cholesterol exhibits the memory of its phase transition temperature, as was observed with the DMPC–β-cholesterol system.

Table IV: Ordering and Orientation of α- and β-Cholesterol

(A) β-Cholesterol

parameter	temp (°C) 10	15	20	25	30	35	40	45	55	65
$S_{mol} \pm 0.03$	0.81	0.81	0.80	0.80	0.79	0.78_5	0.78	0.75	0.72_5	0.71
$\beta \pm 1°$	21	21	21	21	21.5	21	21	22	22	21.5
$\gamma \pm 3°$	108	107.5	106.5	106.5	106	106.5	105.5	106.5	104	106
$\theta(C_3\text{–}c, \vec{n})^a \pm 2°$	83.5	84	84	84	84	84	84.5	84	85	84
$\theta(C_2\text{–}b, \vec{n})^b \pm 2°$	44	44	44	44	43.5	44	44	43	43	43.5

(B) α-Cholesterol

parameter	temp (°C) 20	25	30	40	50	55	65
$S_{mol} \pm 0.03$	0.82	0.80	0.78	0.70	0.63	0.62_5	0.59_5
$\beta \pm 1°$	30	27.5	26.5	23	19	19	17.5
$\gamma \pm 3°$	150	148	147	141.5	134.5	134	130.5
$\theta(C_2\text{–}b, \vec{n}) \pm 2°$	46	46.5	46.5	47.5	49	49	49.5
$\theta(C_3\text{–}c, \vec{n}) \pm 2°$	64.5	67	68	72.5	77	77	79
$S_{mol} \pm 0.03$	0.92	0.89_5	0.88	0.87	0.77_5	0.77	0.72
$\beta \pm 1°$	4	4	4	4	5	5.5	6
$\gamma \pm 3°$	24.5	68	67	60.5	74.5	74.5	80.5
$\theta(C_2\text{–}b, \vec{n}) \pm 2°$	64	61.5	61.5	62	60.5	60.5	59.5
$\theta(C_3\text{–}c, \vec{n}) \pm 2°$	93.5	91.5	91.5	92	91.5	91.5	91

[a] Angle between the C_3–c bond vector and the axis of motion, $\vec{n}$, where c is axial. [b] Angle between the C_2–b bond vector and $\vec{n}$, where b is the equatorial deuteron at C-2.

Concluding Remarks. Deuterium solid-state NMR is able to distinguish the local order of various components embedded in model membranes. Such information allows a quantitation of the disordering–ordering effect of cholesterol: through the quasi temperature independence of its wobbling, β-cholesterol induces motion of the lipid acyl chains below T_c and inhibits them above T_c. Cholesterol thus acts as a *regulatory* agent by maintaining the bilayer membrane in a liquid-crystalline state where motions are axially symmetric and local order is high, possibly to stabilize the bilayer against external perturbations such as temperature jumps or high gradients of local pressure. In addition, the side chain of cholesterol has been found to be as ordered as the condensed ring structure. This presents a picture of cholesterol as a spinning and wobbling cylinder, moderating the angular fluctuations of the lipids, throughout the bilayer.

Analysis of the ^2H NMR observables of the DMPC–α-cholesterol system showed that, at physiological temperatures, the α-isomer of cholesterol is tilted with respect to the normal to the bilayer. This tilted configuration is proposed as one of the reasons that α-cholesterol does not occur in natural membranes, arguing that such a molecular tilt would disturb the parallel packing of the lipid chains and thus decrease membrane stability. With respect to the ordering effect of α-cholesterol on membrane lipids it can be concluded, from both the lipid and cholesterol viewpoints, that the α-isomer, if present in natural membranes, would have an action similar in kind but lesser in magnitude to that of β-cholesterol.

Finally, although there is no other indication that a phase transition occurs around 23 °C for DMPC–cholesterol, the segmental order parameter of DMPC shows a marked change in the slope of its temperature dependence around T_c, leading to the conclusion that the lipid has, even in mixed systems, a memory of its phase transition temperature. This memory was observed with both the α- and β-cholesterol–DMPC systems.

A change in shape of the deuterium powder spectra has been observed at low temperatures (5 °C for β-cholesterol–DMPC and 15 °C for α-cholesterol–DMPC) and attributed to a loss of axially symmetric motions.

The extraordinarily small deuterium quadrupolar splittings observed for the terminal methyl groups of the cholesterol side chain are not well understood and require further investigation.

Acknowledgments

We thank Dr. H. C. Jarrell for valuable discussions and a critical reading of the manuscript and Fred Cooper for mass spectrometric analyses.

Appendix

Since the method used herein has already been discussed elsewhere [see Appendix B in Dufourc et al. (1983)], we will only indicate the general idea and report the resulting calculation as used for α- and β-cholesterol.

A coordinate system $C(x, y, z)$ is attached to the cholesterol molecule as shown in Figure 1. The axis system is defined such that the x axis is colinear with the C_3-axial bond and the z axis belongs to the OH–C_3–H plane. The atomic coordinates of both the carbon and hydrogen atoms have been extracted from the fractional atomic coordinates of cholesteryl laurate (X-ray data; Sawsik & Craven, 1980).

The axis of segmental motion ($\vec{n}$) (aligned along the bilayer normal) is defined in the axis system C as

$$\vec{n} = \begin{pmatrix} \cos\gamma \sin\beta \\ \sin\gamma \sin\beta \\ \cos\beta \end{pmatrix}$$

The angle $\theta_{i,n}$ that an individual C–i bond vector makes with the axis of segmental motion can thus be defined

$$\cos\theta_{i,n} = \frac{l_i \cos\gamma \sin\beta + m_i \sin\gamma \sin\beta + n_i \cos\beta}{\|\overrightarrow{C\text{–}i}\| \cdot \|\vec{n}\|}$$

where l_i, m_i, and n_i are the direction cosines of the C–i bond vector obtained from the atomic coordinates of carbon and deuterium atoms in the C axis system.

The position of the axis of motion is then "sought" by varying the angles β and γ. One sees that for given values of β and γ one can obtain $P_2(\cos\theta_{i,n})$ terms [$P_2(\cos\theta_{i,n}) = {}^1/_2(3\cos^2\theta_{i,n} - 1)$] and compare them with the experimental values. The method of comparison has been described elsewhere [see Appendix B in Dufourc et al. (1983)]. Once the correct orientation of the axis of motion, $\vec{n}$, has been defined with respect to $C(x, y, z)$ by a certain value of the angles β and

γ, one knows the geometrical orientation of a given *C–i* bond with respect to the axis of motion; i.e., one knows S_θ (see Theoretical Background under Results and Discussion), and it is therefore straightforward to obtain the segmental order parameter S_α (also called S_{mol}). We emphasize that this analysis *assumes* that the angular fluctuations of cholesterol (α or β) can be defined, in the liquid-crystalline phase in which the motions have axial symmetry (spectral shapes axially symmetric), by an axially symmetric order matrix.

Table IV summarizes the results for β- and α-cholesterol. All possible combinations of *C–i* direction cosines and quadrupolar splittings were considered, leading to *one* main solution for β-cholesterol and *two* main solutions for α-cholesterol. Exchanging the quadrupolar splitting of the equatorial deuterons at C-2 and C-4 gave the same results for β-cholesterol (Table IVA) with, however, less accuracy.

Both solutions for α-cholesterol were obtained by only one combination of splittings and *C–i* bonds, for the lowest margin of error, leading therefore to the assignment shown in Figure 8. Whereas one obtains a unique solution for β-cholesterol, it is difficult to choose between the two possibilities for α-cholesterol. The first solution indicates that the hydroxyl group (in α) is pointing toward the hydrophilic medium, i.e., the water surface, whereas the other suggests that the OH group points slightly toward the hydrophobic core. On the basis of the available data, it is not possible to choose between the two solutions. However, from the calculated orientations, one can predict that the quadrupolar splitting arising from a β-deuteron (in α-cholesterol) will be about 40 kHz according to the first solution, and 100 kHz according to the second, at 25 °C. A single experiment with α-[3-^{2}H]cholesterol in DMPC (3:7) will thus give an unambiguous answer.

To calculate the position of the axis of motion of a methylene subunit (e.g., cholesterol tail), the fixed axis system $C(x, y, z)$ was defined such that the y axis bisects the ^{2}H–C–^{2}H plane and the z axis is normal to that plane.

Registry No. DMPC, 18194-24-6; α-Δ^5-[2,2,3,4,4,6-$^2H_6$]cholesten-3-ol, 92543-07-2; β-Δ^5-[2,2,3,4,4,6-$^2H_6$]cholesten-3-ol, 92543-08-3; α-cholesterol, 474-77-1; β-cholesterol, 57-88-5; Δ^4-cholesten-3-one, 601-57-0; Δ^4-[2,2,4,6,6-$^2H_5$]cholesten-3-one, 72560-60-2.

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