Effect of Sterol Structure on Acyl Chain Ordering in Phosphatidylcholine Vesicles: A Deuterium Nuclear Magnetic Resonance and Electron Spin Resonance Study†

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ABSTRACT: Various sterols differing in the planarity of the α face of the tetracyclic ring were used to study the critical contact regions in the sterol molecule with adjacent fatty acyl chains in the lipid bilayer. For this purpose we have selected 3α-methylcholesterol, cycloartenol, and lanosterol for comparison with cholesterol. These sterols differ in the location or spatial disposition of methyl groups projecting from the α plane of the sterol ring system. ^{2}H NMR spectroscopy of phosphatidylcholine dispersions containing cholesterol, cycloartenol, or lanosterol labeled in the 3α position with deuterium showed that despite structural and conformational differences, the average orientation of the various sterol nuclei was parallel to the long axis of the fatty acyl chains. Spin-label studies with stearic acids having nitroxide groups at C5, C7, C12, and C16 showed that lanosterol, in contrast to cholesterol and cycloartenol, markedly perturbed fatty acyl chain ordering in the region of C12. Less effected were the polymethylene segments around C5 and C7. Differences in the behavior of lanosterol and cycloartenol had previously been attributed to an alteration in the spatial disposition of the C14α-methyl group of cycloartenol resulting from the planar and nonplanar conformation of the respective sterol ring systems [Dahl, C. E., Dahl, J. S., & Bloch, K. (1980) *Biochem. Biophys. Res. Commun. 92*, 221–228]. An axial methyl group at C3 of the cholesterol nucleus specifically perturbed fatty acyl chain ordering in the region of C5 but less so in regions closer to the bilayer interior (C7 and C12). None of the sterols showed significant acyl chain ordering in the C16 region which is adjacent to the flexible isooctyl side chain. Results of the studies emphasize the importance of a planar sterol α face for optimal packing of lipid molecules in the bilayer plane and may explain why sterols having the cholestane ring system are the sterols that function optimally in biological membranes.

Cholesterol, a major constitutent of all mammalian plasma membranes, is essential for cell growth and survival (Chen et al., 1974). At the physiological or biochemical level, sterol membrane function is not well understood. It has, however, been demonstrated that changes in cholesterol concentration alter the physical state of membranes and thereby modulate a variety of essential cellular processes (Frye & Edelin, 1970; Wiley & Cooper, 1975; Edelman, 1976; Heiniger et al., 1976). The various physiological responses are believed to be related in some manner to membrane fluidity which is controlled by the dynamic interactions between the cholesterol molecule and the fatty acyl chains of the phospholipids.

Studies in this laboratory have focused on the biological significance of the modulation of bilayer dynamics brought about by various alkyl-substituted sterols and stanols representative of the intermediates in cholesterol biosynthesis from lanosterol. This trimethylcholestane precursor of cholesterol is synthesized in mammalian cells by the oxidative cyclization of squalene (Tchen & Bloch, 1957). As such, lanosterol does not seem to perform any physiological role nor does it normally accumulate intracellularly as an end product. Rather, it is a transient intermediate that undergoes three sequential oxidative demethylation steps by membrane-bound enzymes to yield cholesterol (Bloch, 1965). In particular, it has been stressed that selective biological demethylation of lanosterol by producing a planar sterol α face affords a structure optimal for membrane function by maximizing van der Waals interactions between the sterol and the fatty acyl chains of phospholipids (Dahl, C. E., et al., 1980a,b; Dahl, J. S., et al., 1980). Recent findings have led to the further postulate that cholesterol may serve a dual role in biological membranes. Apart from modulating the physical state, cholesterol may function more specifically in localized or microdomains of the membrane to regulate specific biochemical processes (Dahl, J. S., et al., 1980, 1981).

In order to define the critical contact regions of the sterol molecule in the lipid bilayer more precisely, we have now employed deuterium nuclear magnetic resonance (^{2}H NMR)[1] and electron spin resonance (ESR) spectroscopy to investigate the interaction of cholesterol and the alkyl derivatives 3α-methylcholesterol, cycloartenol, and lanosterol (Figure 1) with phospholipid fatty acyl chains. Molecular models show that the axial 3α-methyl group projects from the α plane formed by the trans-fused tetracyclic ring system. For that reason one would predict that this substituent will diminish attractive van der Waals contacts between the sterol α face and the proximal end of the fatty acyl chains of phospholipids.

Lanosterol and cycloartenol are isomeric, cycloartenol containing a 9,19-cyclopropane ring instead of the angular methyl group at C10, but they are otherwise identical. The 14α-methyl group of lanosterol projects from the planar α face (Figure 1). By contrast, the sterol ring system of cycloartenol is bent, forced into a nonplanar conformation by the presence of the cyclopropyl group on the β face of the molecule. It is apparent from stereomodels that in the curvilinear conformation of the cycloartenol ring system, the 14α-methyl group no longer protrudes from the plane of the α face but becomes embedded in an arc or belt of axial α-face H atoms. Consequently, the 14α-methyl group of cycloartenol unlike that of lanosterol might be expected to promote or at least not interfere with phospholipid–fatty acyl chain contacts. In line with this expectation, we have reported that cycloartenol shows

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[1] Abbreviations used: ^{2}H NMR, deuterium nuclear magnetic resonance; ESR, electron spin resonance; *S*, order parameter; NS, nitroxylstearic acid.

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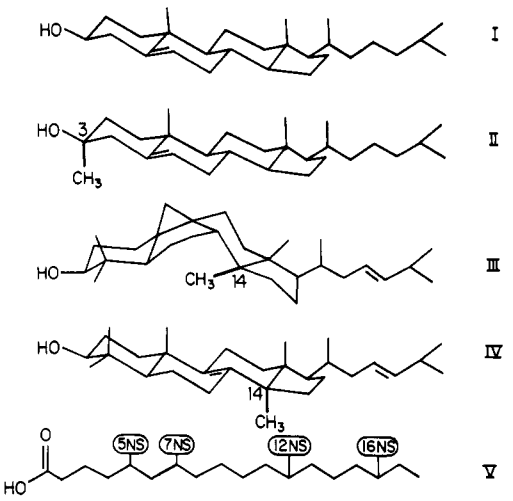

FIGURE 1: Diagrammatic representation of the positional relationship of sterol and fatty acid spin-labels in phosphatidylcholine vesicles containing either cholesterol (I), 3α-methylcholesterol (II), cycloartenol (III), lanosterol (IV), or 5-, 7-, 12-, or 16-nitroxylstearic acid (V). Axial methyl groups on the α face at C3 or C14 are indicated. As shown previously in schematic presentation (Dahl, C. E., et al., 1980b), the 14α-methyl group protrudes from the plane of axial hydrogens in lanosterol (IV) but not in cycloartenol (III).

an ability intermediate between those of lanosterol and cholesterol to increase the microviscosity of lecithin vesicles, to serve as a growth factor for the sterol auxotroph *Mycoplasma capricolum* (Dahl, C. E., et al., 1980b) or for the yeast mutant GL-7 (Buttke & Bloch, 1980), and to raise the microviscosity of *M. capricolum* membranes (Dahl, C. E., et al., 1980b).

We now show that (1) cholesterol, lanosterol, and cycloartenol, despite structural and conformational differences, all align themselves in parallel with the extended acyl chains of the phospholipid molecule and (2) that the location and spatial disposition of methyl groups at the α face in the region of C3 and C14 determine the effectiveness of van der Waals contacts between the sterol ring system and the various fatty acyl chain segments of phospholipids.

Materials and Methods

Chemicals. Cholesterol (Sigma Chemical Co.) was recrystallized from ethanol and dried in vacuo. Lanosterol (Sigma) was purified according to established procedures (Bloch & Urech, 1958). Cycloartenol was purified from *Strychnos nux-vomica* seed fat by the procedure of Bentley et al., (1953). The seed extract was kindly supplied by the S. Penick Co. Cholesterol-3α-d_1 was synthesized from cholest-5-en-3-one (Fieser, 1963) by reduction with $LiAlD_4$ in diethyl ether according to the procedure of Rosenfeld et al. (1954). Lanosterol-3α-d_1 and cycloartenol-3α-d_1 were prepared similarly from the corresponding 3-ketones. 3α-Methylcholesterol was synthesized from cholest-5-en-3-one by reduction with methylmagnesium bromide by the procedure of Barton et al. (1956). Phosphatidylcholine vesicles (1.29 μmol) containing the desired amount of different sterols and spin-labeled fatty acids (1×10^{-4} M) were prepared by cosonication as described previously (Dahl, C. E., et al., 1980a,b). All sterols were checked before use by gas–liquid chromatography and found to be >98% pure. The spin-labeled fatty acids (5-, 7-, 12-, and 16-nitroxylstearates) were purchased from Syva Associates; egg yolk phosphatidylcholine was from Avanti.

Deuterium Nuclear Magnetic Resonance. The ^2H NMR experiments were performed at 41.6 MHz (6.8 T) with a Buker HX270 spectrophotometer (NMR Facility for Biomolecular Research at the Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology). The pulse width was 30 μs, and the repetition rate was 40 pulses/s.

Phosphatidylcholine–sterol mixtures were prepared by dissolving the phospholipid and sterol in chloroform (1:1 molar ratio). The chloroform was removed by a stream of argon gas, and the sample was placed under vacuum for 12 h to remove residual solvent. The phosphatidylcholine–sterol mixture was then dispersed in deuterium-depleted water (Aldrich) with the aid of a vortex mixer for 3–5 min at 37 °C.

Electron Spin Resonance. The ESR spectra were recorded at 25 °C on a Varian E-line 9.5-GHz spectrometer equipped with a Varian temperature controller. The microwave power was held constant at 5 mW to avoid signal saturation. In all measurements, the field sweep was 100 G, and the modulation amplitude was less than 1.0 G to avoid modulation broadening. Vesicle suspensions prepared by cosonication of phosphatidylcholine, sterol, and spin-labeled fatty acids were placed in a 1.0-mm quartz capillary which was inserted in a standard Varian 9.5-GHz quartz tube, and the ESR spectra were recorded (Dahl & Levine, 1978). Two parameters can be measured directly from the ESR spectra: the splittings between the outer hyperfine peaks ($2T_{\|}$) and the splittings between the inner hyperfine peaks ($2T_{\perp}$). From these, one can calculate the order parameter S, which is a measure of the mean angular deviation of the fatty acyl chain at the position of the nitroxide group from the bilayer normal (Hubbel & McConnell, 1971).

Results and Discussion

Evidence based on a variety of spectroscopic techniques has been interpreted as showing that cholesterol entering the membrane aligns in parallel with the extended acyl chains of phospholipid, the sterol hydroxyl group pointing toward the water–bilayer interface and the isooctyl side chain toward the bilayer interior (Rothman & Engelman, 1972). Some current, more explicit membrane models specify hydrogen bonding between the sterol hydroxyl function and some polar element of the phospholipid head group at the membrane–water interface (Brockerhoff, 1974). Regardless of these still controversial details, there appears to be general agreement that hydrophobic interactions between the sterol nucleus and the proximal segment of the phospholipid acyl chains are primarily responsible for modulating the physical state (fluidity) of biomembranes.

In order to determine whether α-face planarity of the tetracyclic sterol ring system is mandatory for optimal packing of lipid molecules in the plane of the lipid bilayer, we selected for comparison with cholesterol three sterols differing in the location or spatial disposition of methyl groups projecting from the α plane. Our first series of experiments using 3α-*d* sterols were designed to establish whether cholesterol, cycloartenol, and lanosterol despite structural differences undergo the same angular fluctuations in the lipid bilayer and orient themselves parallel to the long axis of fatty acyl chains. If the sterol molecule aligns in parallel with the extended acyl chains, the sterol hydroxyl group pointing toward the water–bilayer interface, then the 3α C–D bond may form an angle of approximately 90° with the long axis of the fatty acyl chains as well as the sterol ring system.

The quadrupolar splitting value for a rigid body (i.e., the sterol tetracyclic ring system) rotating around its long axis where the C–D bond angle is 90° with respect to this axis has been calculated to be 63.8 kHz (Gally et al., 1976; Oldfield et al., 1978). A reduction of this value indicates that motional averaging of the electric quadrupole interaction of the deu-

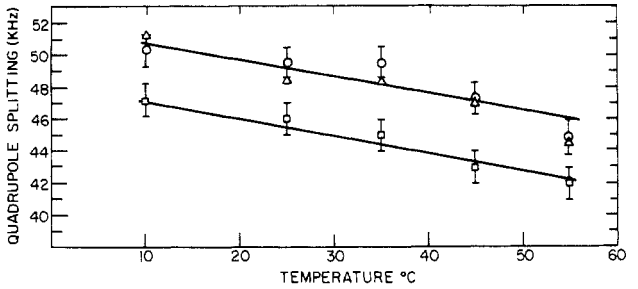

FIGURE 2: Quadrupole splitting values for cholesterol-3α-d_1 (○), lanosterol-3α-d_1 (△), or cycloartenol-3α-d_1 (□) in egg phosphatidylcholine at a 1:1 molar ratio as a function of temperature.

terium nucleus is occurring by rapid fluctuations in the angular orientation of the sterol in the lipid bilayer. The tetracyclic ring systems of cholesterol, lanosterol, and cycloartenol all are conformationally rigid. Although some torsional oscillations may occur about the C2–C3 and C3–C4 carbon–carbon bonds of the A ring, they will be of minimum amplitude and the constraints of the rigid sterol nucleus will prohibit internal reorientation of the 3α C–D bond in the A ring of the sterol nucleus. Thus, the observed quadrupolar splitting of all three sterols must be caused by the orientation or angle of tilt of the sterol in the bilayer. The quadrupolar splitting values for cholesterol, cycloartenol, and lanosterol and their temperature dependence (Figure 2) show that all three sterols are oriented in the bilayer in a similar manner. The quadrupolar splitting value for cholesterol is approximately 50 kHz. This is the same value observed by Gally et al. (1976) under comparable conditions and compares favorably with the results of Oldfield et al. (1978) for cholesterol-containing dimyristoylphosphatidylcholine at 23 °C. Significantly, lanosterol containing a bulky methyl group protruding from the plane of the α face gave the same values as cholesterol. For cycloartenol the values were slightly lower but the temperature dependence was the same. We attribute the slightly lower quadrupolar splitting values for cycloartenol to the bent, nonplanar conformation of the tetracyclic ring. This changes the angle of orientation of the 3α C–D bond in cycloartenol approximately 2–3° with respect to the angle of the 3α C–D bonds in cholesterol and lanosterol as seen in the respective Dreiding stereomodels. While the ^{2}H NMR experiments indicate that in all three cases the average orientation of the sterol nucleus is opposite to the long axis of the fatty acyl chains, these data do not make a statement with respect to the magnitude of the attractive van der Waals contacts between the two lipid components.

In order to determine the influence of alkyl group orientation and ring conformation on sterol–fatty acyl chain contacts, we have employed ESR spectroscopy using stearic acid having a spin-label nitroxide at the 5, 7, 12, or 16 positions of the fatty acid chain (Figure 1).

The various fatty acid spin-labels were incorporated into the lipid vesicles by cosonication. This provided paramagnetic reporter groups at different depths in the bilayer. The influence of various concentrations of cholesterol, cycloartenol, and lanosterol on the ordering of the fatty acyl chains of the phospholipid is shown in Figure 3 and of 3α-methylcholesterol in Figure 4. It is seen that cholesterol and cycloartenol order the acyl chains to the same extent. For these two sterols the slope of the ordering effect is similar for 5-NS, 7-NS, and 12-NS and much smaller for 16-NS.[2] Lanosterol, on the other

[2] Cycloartanol which differs from cycloartenol by the absence of a Δ^{24} double bond in the isooctyl side chain gave the same results.

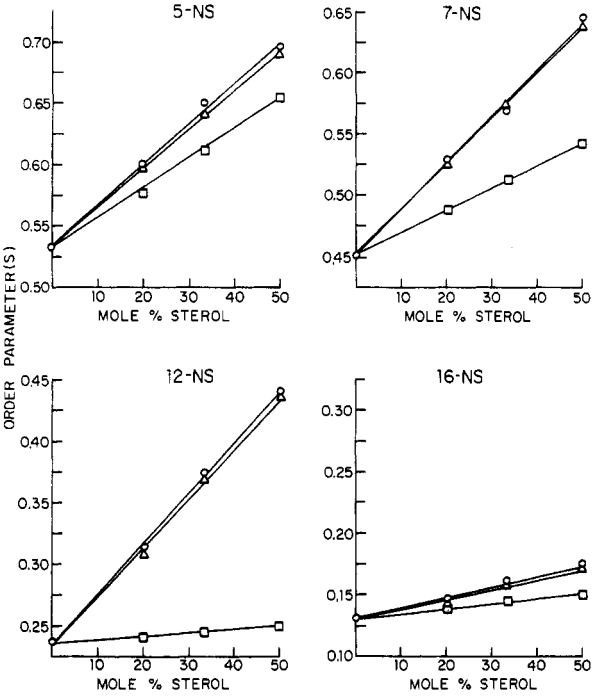

FIGURE 3: Effect of sterol structure on the order parameter (S) at 25 °C of 5-, 7-, 12-, or 16-nitroxylstearic acid in egg phosphatidylcholine vesicles containing cholesterol (○), cycloartenol (△), or lanosterol (□) at increasing concentrations.

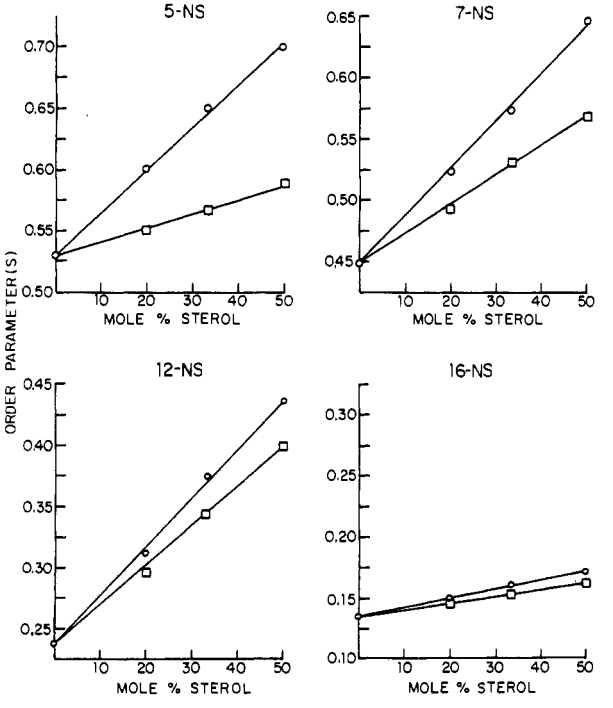

FIGURE 4: Effect of sterol structure on the order parameter (S) at 25 °C of 5-, 7-, 12-, or 16-nitroxylstearic acid in egg phosphatidylcholine vesicles containing cholesterol (○) or 3α-methylcholesterol (□) at increasing concentrations.

hand, has a significantly smaller ordering effect at C5 and C7 and almost none at C12 and C16.

Interestingly, the differences between the lanosterol values and those shown by cholesterol and cycloartenol are least for 5-NS, greater for 7-NS, and most pronounced for 12-NS. These differences can be rationalized in terms of the molecular structures of the sterols and their effects on van der Waals contacts. If the sterols are aligned in parallel with fatty acyl chains as specified above, the tetracyclic nucleus will extend

(ring D edge) to a position opposite to carbon 12 of the fatty acyl chains. This will place the 14α-methyl group of lanosterol at an intramembrane position closer to C12 than to any other of the spin-labeled fatty acid carbons, accounting for the minimal effect of lanosterol on 12-NS chain ordering.[3]

With 3α-methylcholesterol (Figure 4), a markedly different pattern is observed. The ordering effect is smallest on 5-NS, significantly greater on 7-NS, and only slightly less than the effect of cholesterol on 12-NS. Both cholesterol and the 3α-methyl derivative show essentially the same weak ordering effect on 16-NS.

The results in Figures 3 and 4 are in full accord with space-filling models constructed along the lines proposed by Rothman & Engelman (1972) for cholesterol–phospholipid bilayers. Inherent in this model is the prediction that the greater the number of van der Waals contacts, the more restricted the number of conformations accessible to the proximal or upper segment of the fatty acyl chains. Increases in order parameter signify reduced conformational freedom. These increases are clearly greatest for cholesterol regardless of the spin-labeled position. By contrast, the 14α-methyl group of lanosterol obstructs the α-face approach to the proximal segment of fatty acyl chains (van der Waals contacts have an r^{-6} distance dependence). With lanosterol, therefore, the opportunity for trans–gauche rotation, i.e. flexibility of fatty acyl chains, is enhanced with the overall result that the polymethylene segment available for attractive van der Waals contacts is reduced.

It is generally assumed that fatty acyl chains in the fully extended all-trans conformation maximize van der Waals contacts, causing minimal fluidity in the bilayer. This assumption may have to be modified in light of the apparently anomalous membrane behavior of cycloartenol which is essentially indistinguishable from that of cholesterol. The apparent contradiction arises from the nonplanar conformation of cycloartenol which is not compatible with interactions involving fatty acyl chains in the all-trans conformation.

We have previously argued (Dahl, C. E., et al., 1980b) that the "cholesterol-like" membrane behavior of cycloartenol can be attributed to the burial or shielding of the 14α-methyl group in a curvilinear belt of six axial α-face hydrogen atoms. Ordering of the acyl chains at carbon atoms 5, 7, and 12, i.e., those which are adjacent to the sterol nucleus, is therefore possible in spite of its nonplanar conformation. Thus, it appears that lateral displacement of acyl chains may be tolerated and energetically allowed as long as surface contact with the sterol α face is maintained.

As for the behavior of lanosterol, the most striking but also predictable result is that the C12 spin-label, presumably nearest to the 14α-methyl group, displays little if any chain ordering. In addition it is noteworthy that the same methyl group of lanosterol not only abolishes contacts with nearby fatty acyl carbons (C12) but also diminishes interaction at more distant atoms (C5 and C7). This is perhaps to be expected from the rigidity of the tetracyclic ring system.

All four sterols tested exert their lowest chain ordering effects on C16 spin-labeled fatty acid. This is in line with expectations since C16 is opposite to the flexible isooctyl side chain, remote from the sterol nucleus.

The results for 3α-methylcholesterol shown in Figure 4 also support the hypothesis that an unobstructed planar α face of the sterol ring system is mandatory for optimal packing between the two lipid components. When the spin-label is at C5 in proximity to the projecting 3α-methyl group, van der Waals contacts are minimal and the lipid bilayer is most fluid. As the spin-label is moved down the alkyl chain from C7 to C12, increasing the distance from the projecting methyl group at C3, van der Waals contacts become more effective and acyl chain ordering occurs. Little acyl chain ordering is observed when the spin-label is at C16 as is the case with the other sterols.

In summary, the present experiments provide strong support for the hypothesis that the absence or biosynthetic removal of projecting axial methyl groups from the α face of the sterol ring system enhances cholesterol–phospholipid interactions and thereby favors the selection of sterols with a planar α face for function in biological membranes.

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[3] In a phospholipid bilayer above the transition temperature, the spin-labeled fatty acid is not likely to be in the all-trans conformation. If it were not, the effective chain length would be reduced perhaps by one or two methylene groups. This would place, for example, the nitroxide reporter group at C12 at intramembrane depths contiguous with the C14α position of the sterol nucleus.