Differential effects of cholesterol and lanosterol on artificial membranes

(glucose permeability/¹³C nuclear magnetic resonance spectroscopy)

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ABSTRACT   The effects of cholesterol, 4,4-dimethylcho-lesterol, and lanosterol (4,4′,14α-trimethyl-Δ⁸,²⁴-cholestadiene-3β-ol) on some properties of lecithin vesicles have been compared. Unlike cholesterol, lanosterol retards the exit of trapped glucose from phospholipid vesicles only slightly. The ¹³C nuclear magnetic resonance spectrum of cholesterol/lecithin vesicles shows no resonances attributable to the sterol. By contrast, several resonances attributable to quaternary carbon atoms or methyl groups are seen in the ¹³C nuclear magnetic resonance spectrum of lanosterol/lecithin vesicles, indicating that lanosterol is much less immobilized than cholesterol. Because the membrane behavior of 4,4-dimethylcholesterol is closely similar to that of cholesterol, it is concluded that the axial 14-α-methyl group is responsible for the lessened membrane immobilization of lanosterol. The results emphasize the importance of a planar sterol α-face for interaction with phospholipid acyl chains.

A hypothesis on the evolution of sterols has recently been presented (1). Because the prebiotic atmosphere is assumed to have been essentially anaerobic and because oxygen is an obligatory electron acceptor in the contemporary biosynthesis of sterols, it was suggested that any chemical evolution of the sterol pathway, if it did indeed occur, must have stopped at the stage of squalene. Only when the terrestrial atmosphere turned aerobic and after the arrival of aerobic cells could squalene be oxidized to squalene oxide and then cyclized to lanosterol. However, intracellular lanosterol is rapidly metabolized to cholesterol, and cells that terminate sterol biosynthesis at the lanosterol stage are not known. We suggest that the apparently compulsory metabolic conversion of lanosterol to cholesterol can be explained on the basis of current models (1–6) for cholesterol-containing membranes. According to these models the planar α-face of cholesterol and related cholestane derivatives interacts hydrophobically with the phospholipid fatty acyl chains in the membrane bilayer. As space-filling models show, the axial methyl group at C14 of lanosterol obstructs these interactions (1). On the other hand, according to such models, the two methyl substituents at C4 of lanosterol do not interfere sterically with sterol-fatty acyl chain contacts. We provide here some evidence supporting the above hypothesis. Two types of experiments were performed. The effect of sterols on artificial membranes was examined by determining the exit of trapped glucose and independently by ¹³C nuclear magnetic resonance (NMR) spectroscopy.

EXPERIMENTAL

Phosphatidylcholine (PtdCho) was purified from egg yolk by a slight modification of the procedure of Singleton et al. (7).

Cholesterol (Sigma) was dried at 56° at 0.5 mm Hg before use. [1-¹⁴C]Glucose (specific activity, 4.86 mCi/mmol) was from New England Nuclear. Lanosterol was purified according to the procedure of Bloch and Urech (8). 4,4-Dimethylcholesterol was prepared as described by Woodward et al. (9). All sterols were checked for purity by thin-layer and gas chromatography and were found to be >97% pure. For measuring glucose exit, vesicles were prepared as follows. The desired concentration of sterol and 20 μmol of PtdCho in chloroform were mixed and evaporated to dryness under reduced pressure, and the residue was dried in a dessicator for 30 min. Then, 2 ml of 1 mM [1-¹⁴C]glucose (2 × 10⁶ cpm) in 10 mM NaCl was added and the solution was sonicated in a Branson sonicator (microtip) at 4° for 30 min under nitrogen. The sonicated solution was then centrifuged at 105,000 × g for 30 min at 4° to separate vesicles from the larger multilamellar structures. The supernatant was checked for oxidation of unsaturated fatty acids (10); no oxidation was detected. The solution was then passed through a column of Sephadex G-50 medium (15 ml) preequilibrated with 1 mM unlabeled glucose in 10 mM NaCl. Vesicles containing trapped [¹⁴C]glucose were eluted in the void volume (3 ml); 1 ml of this solution was dialyzed (duplicate) against 10 ml of 1 mM glucose in 10 mM NaCl for 1 hr at 37°. The amount of [¹⁴C]glucose inside the dialysis bag before and after dialysis was assayed in a liquid scintillation counter. The data are expressed as percent glucose released per hour = 100 [1 − (G_a/G_b)] in which G_a is the amount of [1-¹⁴C]glucose retained in vesicles after incubation for 1 hr, and G_b is the amount present before incubation (11).

For ¹³C NMR spectroscopy, vesicles were prepared from colyophilized phospholipid and sterol by sonication in D₂O containing 10 mM NaCl/10 mM EDTA with a Heat Systems W 350 sonifier for 20 min. ¹³C NMR spectra were obtained in 10-mm tubes at 25.15 M Hz with a JEOL PS 100/EC100 Fourier transform spectrometer. Protons were noise decoupled. Spectra were obtained with 90° pulses at 1-sec intervals.

RESULTS

It is well known that, when added to phospholipids in the liquid-crystalline state, cholesterol reduces the mobility of fatty acyl chains in the bilayer, thereby increasing microviscosity (3, 5). Likewise, cholesterol will reduce the permeability of membranes to the hydrophilic solutes glucose and glycerol. This effect of cholesterol and of structurally modified sterols on solute permeability of lecithin vesicles has been studied extensively by van Deenen et al. (12). In similar experiments we examined the rate of exit of trapped glucose from single bilayer

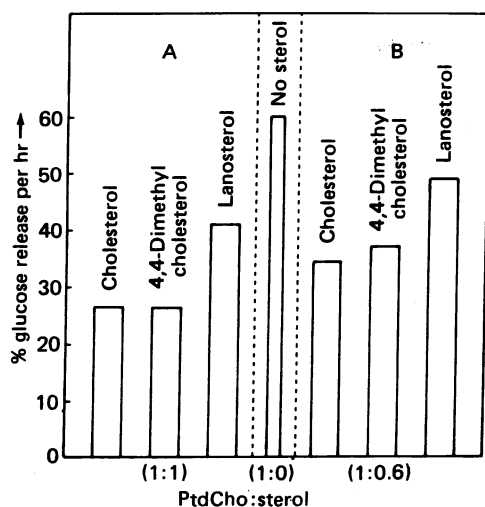Abbreviations: NMR, nuclear magnetic resonance; PtdCho, phosphatidylcholine.

FIG. 1. Percent glucose release per hr at 37° from PtdCho (PC):sterol vesicles at molar ratio 1:1 (A) and 1:0.6 (B).

FIG. 2. ^{13}C NMR spectrum of egg PtdCho vesicles in 100 mM NaCl/10 mM EDTA in D_2O, at 25 MHz and 23°.

vesicles in the presence of cholesterol, lanosterol, or 4,4-dimethylcholesterol. When the molar ratio of PtdCho to sterol was either 1:1 or 1:0.6, the permeability of vesicles containing lanosterol was significantly greater than that of vesicles containing cholesterol (Fig. 1). On the other hand, vesicles containing 4,4-dimethylcholesterol behaved more like those containing cholesterol.[‡] It should be noted that, at PtdCho/sterol molar ratios above 1:0.5, all the sterols tested behaved similarly (results not shown)—i.e., they failed to decrease glucose permeability significantly. At the lower sterol concentrations, PtdCho–PtdCho interactions may be the predominating determinants of membrane fluidity.

The effect of the same sterols on lecithin vesicles was also monitored by ^{13}C NMR. The spectrum of PtdCho vesicles is given in Fig. 2. Fig. 3 presents the spectra of PtdCho/lanosterol vesicles and PtdCho/cholesterol vesicles in a molar ratio of 1:0.5 and of lanosterol in $CDCl_3$, respectively. Resonance assignments are given in Fig. 3C according to the carbon numbering scheme for lanosterol. Each spectrum required about 24 hr to obtain.

The ^{13}C NMR spectra of sterol-containing vesicles revealed striking differences between the behavior of cholesterol and of lanosterol in phospholipid bilayers. For cholesterol, no ^{13}C resonances were observed because the molecule is strongly immobilized by its interaction with the phospholipids (13). Immobilization of a molecule leads to broad linewidths, in this case too broad to be observed. For vesicles containing lanosterol, it was possible to discern resonances not arising from the phospholipid and, by comparison to the ^{13}C NMR spectrum of lanosterol in $CDCl_3$ (Fig. 3C), the sterol resonances seen in the vesicle spectra can be assigned. A distinct resonance appeared on the downfield side of the phospholipid double bond resonance (I in Fig. 3A), which arises from carbon atoms C8 and C9 of the lanosterol double bond. Between the N-methyl resonance and the hydrocarbon chain methylene envelope arising from the phospholipid, two broad resonances appeared that can be assigned to C14 and C13 of lanosterol (II and III, respectively, in Fig. 3A). Additional signals appeared just upfield of resonance III but cannot be definitely assigned. Two more

resonances appeared between the penultimate and terminal carbon resonances of the phospholipid hydrocarbon chain (IV and V). IV most likely arises from C21 and C19 and V most probably from C18 or C30. In both cases, the two carbon resonances were too close to be distinguished once they were broadened by the phospholipid environment. The spectrum of 4,4-dimethylcholesterol in PtdCho vesicles did not show any distinct sterol resonances that can be clearly assigned. Thus, it is not possible to describe the motion of this sterol except that its behavior is intermediate between that of lanosterol and of cholesterol, with a greater similarity to the latter.

The observed carbon nuclei in the spectrum of lanosterol in

FIG. 3. ^{13}C NMR spectra of egg PtdCho/lanosterol (1:0.5) vesicles (A) and egg PtdCho/cholesterol (1:0.5) vesicles (B) in 100 mM NaCl/10 mM EDTA in D_2O, and lanosterol in $CDCl_3$ (C) at 25 MHz and 23°. (Inset) Difference, A − B.

[‡] Qualitatively similar results have been obtained with mixed dispersions of sterols and egg lecithin obtained manually (liposomes). With these multilamellar preparations, the percentage of trapped glucose released per hr was 5% for cholesterol, 10% for 4,4-dimethylcholesterol, 43% for lanosterol, and 50% for sterol-free liposomes (H. K. Lin, A. K. Lala, and K. Bloch, unpublished data).
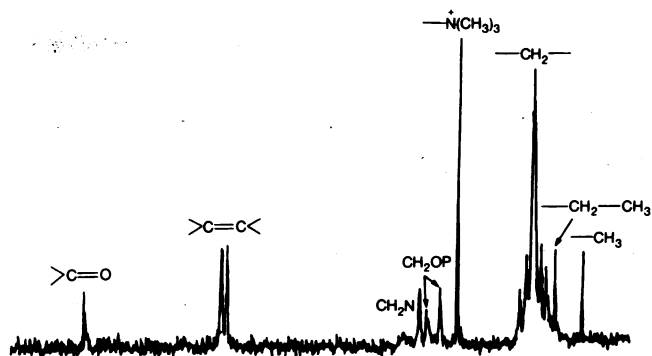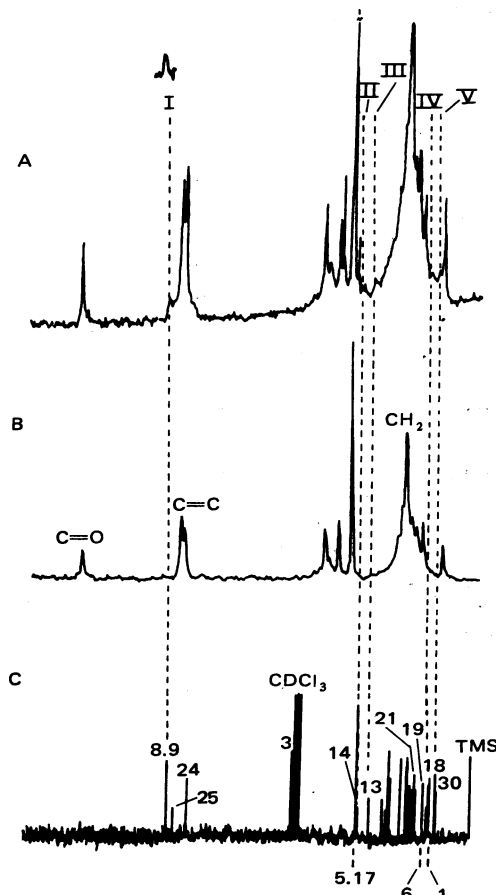
PtdCho vesicles are all either quaternary carbons or methyl groups, which is reasonable because the nonprotonated carbons would be expected to be least broadened by immobilization, and methyl groups can undergo internal rotation to average some of the dipolar broadening. Broadening effects are much more pronounced for protonated carbons.

The fact that any lanosterol resonances could be observed in PtdCho vesicles sets this sterol apart from cholesterol and 4,4-dimethylcholesterol. Lanosterol must be considerably more mobile in the phospholipid bilayer than cholesterol. Subtraction of ^{13}C NMR spectra of cholesterol-containing vesicles from spectra of lanosterol-containing vesicles (both at a molar ratio of 1:0.5) for the region of peak I (see Fig. 3A inset) yielded a single resonance with a linewidth of about 60 Hz. This linewidth is not very different from the C5 resonance of cholesterol ester in human low density lipoprotein (14), an environment in which the sterol ring is considered to be relatively mobile. Neither is it much broader than the carbonyl resonance of the phospholipid, also a nonprotonated carbon atom.

It has been recently demonstrated (15–17), by studying the $^{31}P\{^1H\}$ nuclear Overhauser effect, that the preferred average conformation of the PtdCho head group in bilayers is parallel to the membrane surface, involving intermolecular interactions between the positively charged trimethylammonium group and the phosphate group of neighboring phospholipids. In PtdCho vesicles containing cholesterol in a molar ratio of 1:0.5, the interaction between the two neighboring lipids was not observed because introduction of cholesterol increased the distance between the neighboring phospholipids. At lower concentrations of cholesterol (<30 mol %) this effect was not predominant. Similar results were also obtained with lanosterol, suggesting that, although lanosterol is not hydrophobically interacting with fatty acyl chains in the bilayer as strongly as cholesterol, it does cause separation of phospholipid head groups, like cholesterol.

DISCUSSION

The results obtained by ^{13}C NMR spectroscopy and by measuring glucose permeability are mutually consistent, both indicating less immobilization of lanosterol than of cholesterol in phospholipid vesicles. On the other hand, 4,4-dimethylcholesterol affects membrane behavior in much the same way as cholesterol. For these reasons it may be concluded that it is the methyl group at C14 of lanosterol that is primarily responsible for disrupting interactions between sterol and phospholipid fatty acyl chains. As molecular models show, this methyl group (14α-axial) protrudes from the otherwise planar sterol α-face. Thus, our results strongly support the view that the planar α-face of the sterol ring region is an essential feature in the interaction between sterol and phospholipid.§

Membrane mobility controls not only transport processes but also the activity of some membrane-bound enzymes (18–21). Cholesterol may play a major role in this control, particularly in eukaryotic plasma membranes. It is therefore reasonable to speculate that metabolic demethylation of lanosterol resulted from evolutionary pressures leading to improved membrane function. Interestingly, the 14-methyl group of lanosterol is the first to be removed during the enzymatic conversion of lanosterol to cholesterol (22).

The advantages gained from elimination of the 4,4-dimethyl

groups are less apparent but appear to be real because 4,4-dimethyl sterols are not found in membranes in significant amounts. Judging from space-filling models, the 4,4-dimethyl substituent (4,β-axial and 4α-equatorial) does not interfere sterically with the sterol α-face–fatty acyl chain interactions. At this time we can offer no explanation for the apparently compulsory removal of these substituents in eukaryotic cells.

In support of the general hypothesis presented here, the sterol requirement of some biological systems may be cited. In insects that are nutritionally dependent on a sterol source such as cholesterol, neither lanosterol nor 4,4-dimethylcholesterol satisfies the sterol requirement (23). Anaerobic yeast, an artificial sterol auxotroph, grows well on cholesterol or ergosterol but only poorly on lanosterol (24). Finally, Chang *et al.* (25) have recently isolated a cholesterol-requiring mutant of Chinese hamster ovary cells defective in lanosterol demethylation. Such cells accumulate lanosterol; they are not viable and lyse rapidly.

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§ Also consistent with this model is the finding that 3α-methylcholestan-3β-ol, when incorporated into lecithin vesicles, has no effect on the exit rate of trapped glucose, (A. K. Lala and K. Bloch, unpublished data).