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PREPARATION AND STRUCTURAL STUDIES OF CHOLESTEROL BILAYERS

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Bilayers consisting, in their hydrophobic core, entirely of cholesterol can be constructed if a hydrophilic molecular anchor is supplied. *O*-Methoxyethoxyethoxyethylcholesterol and cholesterol sulfate form multilayered liposomes in water. With equimolar cholesterol added, cholesterol sulfate, cholesterolphosphocholine, and *O*-methoxyethoxyethoxyethylcholesterol form small unilamellar liposomes on prolonged sonication. The dimensions of cholesterol-cholesterolphosphocholine vesicles are comparable to those of phospholipid vesicles. ^{13}C-NMR spectra suggest that the centers of the bilayers are liquid. The permeability of the cholesterol-cholesterolphosphocholine bilayer against glycerol is lower than that of dipalmitoylphosphatidylcholine-cholesterol bilayer; the activation energy of permeation is two times larger, an indicaiton of a higher degree of structural organization in the 'hydrogen belts' of the cholesterol-cholesterolphosphocholine bilayer.

Introduction

Phospholipid and cholesterol in 1 : 1 molar mixture form stable bilayers; metastable bilayers may accept as much as four moles of cholesterol per mole phospholipid [1,2]. We find that bilayers can be constructed which consist entirely of cholesterol in their core, provided a stabilizing hydrophilic anchor is supplied. Substituents on the cholesterol-3-*O* position such as methoxyethoxyethoxyethyl (PEG, for poly(ethylene glycol)), sulfate (SO_4), or phosphocholine (PC) are effective for this purpose. Not every cholesterol molecule need carry an anchor; and equimolar amount of cholesterol itself can be accommodated in the bilayer. We have prepared such membranes in the form of liposomes, either large multilamellar vesicles or small unilamellar vesicles. For the unilamellar cholesterol/cholesterol-PC liposomes we have determined physical dimensions.

Materials and Methods

Cholesterol-SO_4 (cholesterol sulfate, potassium salt) was purchased from ICN, Cleveland, OH. We synthesized cholesterol-PEG (3-*O*-methoxyethoxyethoxyethylcholesterol) [3] and cholesterol-PC (cholesterolphosphocholine) [4]. Liposomes were prepared in the conventional manner [5] by sonication of lipid films under 2 ml water with a Branson ultrasonic homogenizer with microtip, at 30 W and at 20°C (Table I), with 10 min sonication for multilamellar, 4 h for unilamellar liposomes. For the isolation of cholesterol/cholesterol-PC unilamellar liposomes, sonicated (4 h) suspensions were centrifuged at $100000 \times g$ for 30 min (to remove residual large liposomes). The supernatant vesicles were subjected to gel exclusion chromatography on Sepharose 4B for determination of size [5]; internal volume was measured [6] with [^{14}C]sucrose; ratio

Abbreviations: cholesterol-PC, cholesterol 3-*O*-phosphocholine; cholesterol-SO_4, cholesterol 3-*O*-sulfate, potassium salt; cholesterol-PEG, 3-*O*-methoxyethoxyethoxyethylcholesterol.

cholesterol/cholesterol-PC (Fig. 1) by measuring admixed [^{14}C]cholesterol radioactivity and phosphorus [7]. From the data, average physical parameters of the liposomes (Table II) were calculated as described before [7–11]. ^{13}C-NMR spectra were obtained as before [7]. The outside/inside ratio of cholesterol-PC in vesicles (Table II) was obtained from ^{31}P-NMR with ytterbium^{3+} as the reagent shifting the outside signals [7,12]. The permeability against glycerol (Fig. 2), and the activation energy of permeation, were determined according to conventional procedures [13–15].

Results and Discussion

Formation of vesicles

In Table I, the aqueous lipid dispersions are described according to their optical appearance: 'multilamellar liposomes' are milky, stable suspensions, 'unilamellar liposomes' are transparent suspensions. That these assignments are correct is shown by the isotonic swelling of the multilamellar liposomes (Fig. 1) and the characterization of the cholesterol/cholesterol-PC unilamellar liposomes (Table II).

TABLE I

PROPERTIES OF DISPERSIONS (LIPOSOMES) OF CHOLESTEROL DERIVATIVES, WITH AND WITHOUT CHOLESTEROL, IN WATER

100 μmol cholesterol derivative alone or +cholesterol (molar ratio 1:1) were dissolved in chloroform. The solvent was removed and the lipids sonicated in 2 ml water (10 min or 4 h). Multilamellar liposomes were milky dispersions that behaved as ideal osmometers (Fig. 1). Unilamellar liposomes were transparent, opalescent solutions.

Composition	Preparation of liposomes	
	10-min sonication	4-h sonication
Cholesterol-PC	gel	gel
Cholesterol-PC +cholesterol	multilamellar	unilamellar
Cholesterol-PEG	multilamellar	multilamellar
Cholesterol-PEG +cholesterol	multilamellar	unilamellar
Cholesterol-SO_4	insoluble	multilamellar
Cholesterol-SO_4 +cholesterol	multilamellar	unilamellar

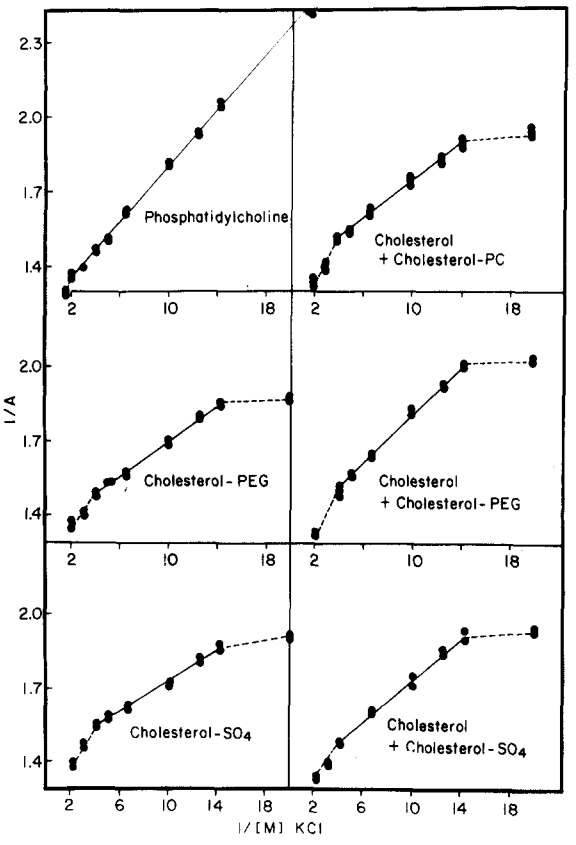

Fig. 1. Isotonic swelling in aqueous KCl of large liposomes. Liposomes were prepared in 0.1 M aqueous KCl. 20 μl were injected into 2 ml aqueous KCl of varying concentration, and the absorbance was measured after 30 min.

CholesterolPC alone at 0.05 M swells with water to give a gel. At 5 mM concentration it forms a micellar solution. No liposomes are formed, probably because the phosphocholine group is too bulky to be accommodated in the homomolecular bilayer. Cholesterol-PEG forms multilamellar liposomes very readily; cholesterol-SO_4 needs long sonication. Cholesterol-PEG alone, and cholesterol-SO_4 alone, do not form monolamellar vesicles, probably because the strong curvature of the inner layer of small vesicles does not allow accommodation of the head groups of the molecules.

Together with equimolar cholesterol, all three cholesterol derivatives form first multilamellar, then, after prolonged sonication, monolamellar liposomes. Cholesterol/cholesterol-SO_4 vesicles are

TABLE II

PHYSICAL PARAMETERS OF UNILAMELLAR CHOLESTEROL/CHOLESTEROL-PC VESICLES

Parameters of phosphatidylcholine vesicles are shown for comparison.

	Cholesterol/cholesterol-PC (1:1)	Phosphatidylcholine (egg) [a]
Vesicle wt.	$4.63 \cdot 10^6$	$1.88 \cdot 10^6$
No. of molecules		
Total	9868	2448
Outside	6832	1658
Inside	3036	790
Outside/inside phosphorus ratio	2.25	2.1
Inclusion volume (p/mol)	0.42	0.25 [b]
Ext. radius (nm)	13.7	9.9
Interior radius (nm)	9.4	6.2
Thickness (nm)		
Bilayer	4.3	3.7
Outer monolayer	2.6	2.1
Inner monolayer	1.7	1.6
Area (nm^2), head group		
Outer monolayer	0.69 [c]	0.74
Inner monolayer	0.73 [c]	0.61
Area (nm^2), intramembrane boundary		
Outer monolayer	0.45 [c]	0.46
Inner monolayer	1.02 [c]	0.97
Av. volume of lipid complex (nm^3)	1.49 [c]	1.25

[a] Huang and Mason [8].
[b] Zumbuehl and Weder [29].
[c] Values are for assumed 1:1 cholesterol-cholesterol-PC complex.

formed least, cholesterol/cholesterol-PEG vesicles most easily.

Osmotic properties of multilamellar liposomes

Fig. 1 shows that all multilamellar liposomes of Table I behave as osmometers, i.e., the vesicles are closed [13]. The osmotic swelling of dipalmitoylphosphatidylcholine liposomes is also shown. It can be seen that all cholesterol bilayers are somewhat less elastic than the phospholipid bilayer; they collapse, or leak, at lower pressure differentials.

Permeability

The bilayer tested was that of cholesterol/cholesterol-PC multilamellar liposomes; the permeant was glycerol. Fig. 2 shows the Arrhenius plot, also a plot for dipalmitoylphosphatidylcholine-cholesterol for comparison. This phosphoglyceride-cholesterol bilayer has an already low

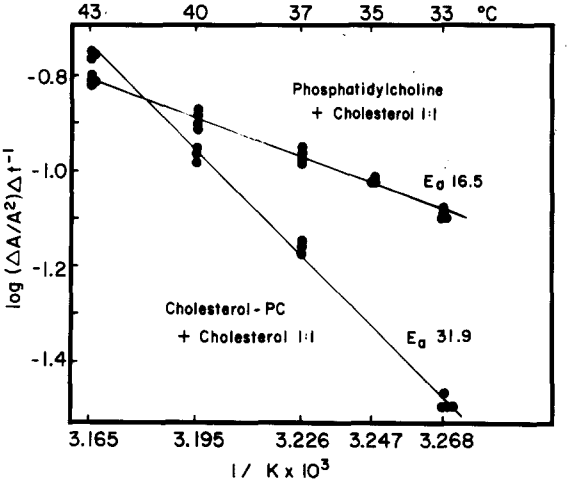

Fig. 2. Arrhenius plot of permeation of glycerol through cholesterol/cholesterol-PC bilayer (large liposomes). Permeation of glycerol through dipalmitoylphosphatidylcholine/cholesterol bilayer is shown for comparison. E_a, energy of activation of permeation.

permeability compared with bilayers containing unsaturated fatty acids, or no cholesterol [14]. The cholesterol bilayer is even less permeable. Nevertheless, it is not impermeable. How does a glycerol molecule pass through the bilayer? In phospholipid bilayers, a permeant may progress through pockets (kinks) created by conformational isomerizations of the fatty chains [16]. Such pocket formation is not possible in cholesterol bilayers; cholesterol nuclei must be pushed apart in their entirety by the permeant.

The activation energy, E_a, for the permeation of the cholesterol/cholesterol-PC bilayer is nearly 2-times larger than that for the phosphoacylglycerol-cholesterol bilayer. We found a similarly large E_a for the permeation of a lysophosphatidylcholine-cholesterol bilayer [7]. The effect is accounted for by a low groundstate of the entropy of activation [7,15]. E_a has been shown to be a measure of the energy of dehydration of the permeant [17–19]. This dehydration must take place before the permeant enters the lipid bilayer, in the region of the membrane which we like to call 'hydrogen belt' [20,21] because it consists of hydrogen bond acceptors (the CO groups of phospho- and sphingolipids) and hydrogen bond donors (the OH of cholesterol, sphingolipids, water, and possibly proteins). Therefore, we interpret the low groundstate of the entropy of activation of permeation as evidence for a high degree of order in the 'hydrogen belt', brought about by hydrogen bonding. Hydrogen bonding from hydroxycholesterol to the phosphate of cholesterol-PC might be considered; sterically, it is a possibility.

Composition and dimensions of cholesterol/-cholesterol-PC monolamellar vesicles

A mixture of cholesterol (with [^{14}C]cholesterol added) and cholesterol-PC in 1:1 molar ratio, sonicated for 4 h, was chromatographed by gel exclusion. Fig. 3 shows the elution profile of ^{14}C and phosphorus. Just as in the case of sonicated phosphatidylcholine [5] or lysophosphatidylcholine-cholesterol liposomes [7], a minor peak of residual multilamellar vesicles is eluted with the void column; then, unilamellar vesicles emerge in which the cholesterol/cholesterol-PC ratio is 1. From the retention coefficient, the inclusion volume, and the outside/inside ^{31}P ratio we calculate the physical parameters of the vesicles [7–11] (Table II). (it is assumed that the ratio cholesterol/cholesterol-PC is the same, 1, in outer and inner monolayer.) The parameters are compared to those of phosphatidylcholine unilamellar vesicles.

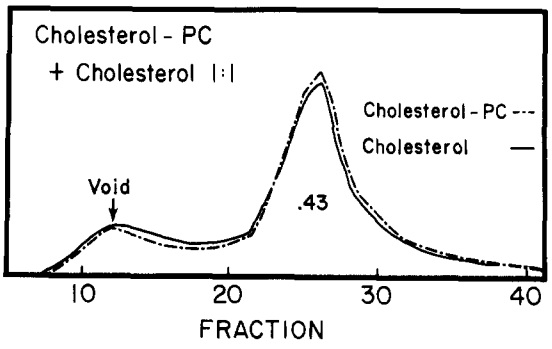

Fig. 3. Gel filtration chromatography on Sepharose 4B of sonicated cholesterol/cholesterol-PC vesicles. ------, phosphorus (from cholesterol-PC); ———, radioactivity (from [^{14}C]cholesterol).

^{13}C-NMR spectra

The spectrum of cholesterol/cholesterol-PC sonicated liposomes (Fig. 4) shows a prominent choline $N(CH_3)_3$ signal at 56.3 ppm with a linewidth of 11 Hz, also two smaller peaks at 60.9 ppm ($\underline{C}H_2CH_2N$) and 67.6 ppm (CH_2N). The effective spin-spin relaxation time T_2^* of the $N(CH_3)_3$ peak is 29 ms. It has been found that while the spin-lattice relaxation times, T_1, of phos-

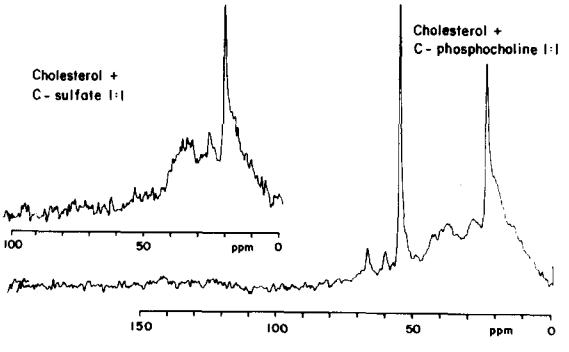

Fig. 4. ^{13}C-NMR spectrum of cholesterol/cholesterolphosphocholine (1:1 molar) and cholesterol/cholesterol sulfate vesicles, sonicated in ^2H_2O.

pholipids are not much influenced by the state of aggregation or packing of the lipid molecules, the spin-spin relaxation times T_2 (for membrane lipid carbons T_2 = approx. $T_2^\star$ [22]) are quite sensitive: they decrease as the particular molecular region becomes immobilized with increasing aggregation or tighter packing of the membrane (e.g., by addition of cholesterol) [22–24]. The $T_2^\star$ of the $N(CH_3)_3$ carbons in Fig. 4 is very close to the corresponding $T_2^\star$ for phosphatidylcholine [23] or lysophosphatidylcholine-cholesterol [7] and shows that the choline group is equally mobile in these three bilayers.

Cholesterol nucleus and side chain give no, or very poorly resolved, ^{13}C-NMR signals when cholesterol is part of a phospholipid-cholesterol bilayer [22]. In sonicated lysophospholipid-cholesterol vesicles, however, a signal appears at 23.3 ppm [7] with an intensity of two-thirds of that of the $N(CH)_3)_3$ signal, i.e., corresponding to two carbons, the side chain methyl groups C-26 and C-27 of cholesterol (22.8 ppm in chloroform). The same signal is seen in the cholesterol bilayers of Fig. 4. (The upfield shoulder on the peak may be the side chain C-21 signal.) The $T_2^\star$, approx. 18 ms, for the C-26 and C-27 methyl is much smaller in the vesicles than in chloroform solution of cholesterol ($T_2^\star \geqslant 80$ ms); this indicates a loss of mobility of the side chain in the cholesterol bilayer as compared to monomolecularly dissolved cholesterol. However, it must be considered that the $T_2^\star$ of the end methyl groups, in the vesicles, is very similar to that of the mobile $N(CH_3)_3$; that all other cholesterol resonances (which are very sharp in chloroform solution) are suppressed (i.e., have very short $T_2^\star$); and that in phosphatidylcholine-cholesterol vesicles the signal of the C-26 and C-27 methyl groups is also suppressed. The obvious conclusion is that the end methyl groups are quite mobile in cholesterol bilayer and lysophosphatidylcholine-cholesterol bilayer, in which all lipid molecules are wedge-shaped; but not in the phosphatidylcholine-cholesterol bilayer in which the inversely wedge-shaped phosphatidylcholine causes tighter packing in the middle of the bilayer. Thus, the ^{13}C-NMR spectra of the vesicles suggest strongly that the innermost region of the cholesterol bilayer is liquid.

Conlcusion

Cholesterol bilayers might be useful for the construction of liposomes of very low permeability combined with resistance to lipolytic enzymes; or for studies on cholesterol-cholesterol associations, as they might occur early in atherogenesis [25] or in other pathological conditions [26]; or for studies on cholesterol-protein associations such as recently reported [27,28]. Cholesterol bilayers might also be valuable as control bilayers, free of charged or hydrogen-bonding groups, in studies of phospholipid-protein interaction in membranes.

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