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EVIDENCE FOR STEREOSPECIFIC PHOSPHOLIPID-CHOLESTEROL INTERACTION IN LIPID BILAYERS

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Summary

The CH_2 proton NMR linewidths of *sn*-3 and *sn*-1 dipalmitoyl phosphatidylcholine respond differently to the addition of cholesterol to the lipid vesicles. This result points to a stereospecific phospholipid-cholesterol interaction in the "hydrogen belt" region.

Cholesterol, when added to a bilayer of phospholipids above their crystalline-liquid crystalline transition temperature, depresses its fluidity [1—3] and its permeability [4, 5]. The mobility of the fatty acid chains of the phospholipids decreases: proton NMR spectra show that the width of the $-CH_2-$ proton resonance band broadens and its height shrinks with increasing molar percentage of cholesterol [6, 7]. This effect is the result of a freezing of $-CH_2-$ motion. The consensus of present opinion is that cholesterol serves as a filler and sealer of biological membranes by inserting its polyannular disk into the cavities between the head sections of different phospholipid molecules, while the cholesterol side chain reaches toward the hydrophobic center of the membrane.

Recently, interest has arisen in those regions of lipid bilayers and biological membranes that we would like to call "hydrogen belts" [8, 9] because they contain neither hydrophobic nor strongly polar parts but consist of hydrogen bond acceptors (the CO groups of phosphoglycerides and sphingolipids) and hydrogen bond donors (the OH of cholesterol, sphingosine, α-hydroxy fatty acids, water, perhaps proteins). Based on steric and energetic arguments, and on the finding [10] that the β-oriented hydroxyl of cholesterol is essential for membrane closure, we have suggested cholesterol-OH-phospholipid-CO hydrogen bonding [8, 9]. Such bonding has also been suggested by Huang [11] on the basis of steric arguments. Evidence in support of the postulated hydrogen belt architecture is now accumulating [12—16].

An interaction between phospholipids and cholesterol in the hydrogen belt might possibly be influenced by the configuration around the asymmetric carbon of the phospholipid, the C-2 of glycerol, which is near to the hydrogen bond accepting ester CO groups. We have, therefore, measured the -CH_2- proton NMR spectra of vesicles of a natural *sn*-3-phosphatidylcholine and its antipode, *sn*-1-phosphatidylcholine, and their change on addition of cholesterol. The difference we observed was more obvious and striking than we had anticipated.

sn-3-Dipalmitoyl phosphatidylcholine, chromatographically pure, was bought from Sigma, St. Louis, Mo. The antipode was prepared from D,L-dipalmitoyl phosphatidylcholine (Sigma) by treatment with phospholipase A_2 (lyophilized *Crotalus atrox* venom) [17] which hydrolyzes only the natural *sn*-3 compound. The products were isolated by chromatography: silicic acid, 1 g per 20 mg lipid, pre-eluted with chloroform/methanol (4 : 1, v/v), elution of the *sn*-1-phosphatidylcholine with chloroform/methanol (1 : 1, v/v), 6 g D,L lipid yielded 2.9 g *sn*-1 lipid. The product was pure as judged by thin-layer chromatography, and yielded no further lysolipid and fatty acid on renewed treatment with the phospholipase.

For preparation of liposome vesicles, chloroform solutions of the phospholipids (with the required cholesterol) were evaporated in vacuum (1 mmHg, 70°C) for 1 h, with frequent flushing with N_2, and then dissolved in ^2H_2O. The liposome emulsion was then sonicated for 10 min [6] with a Bronson sonifier, microtip, 30 W. Vesicle preparation and NMR measurements were carried out at 45°C (transition temperature of the phospholipid, 41°C). High resolution proton magnetic resonance spectra were obtained with a Varian XL100 (100 MHz) instrument with a Nicolet Fourier transform accessory, and recorded on a sweep-width of 1000 Hz. The resonances for -$N(CH_3)_3$, -CH_2-, and terminal -CH_3, represented as δ values (ppm) are in agreement with those reported [6, 7]. Peak widths at half-height were measured after triangulation and expressed in Hertz (Table I). The NMR results given here were essentially duplicated in separate experiments with a Jeol PFT-100 instrument.

To ascertain that the *sn*-1 phospholipid takes cholesterol into its bilayers, just as the *sn*-3 phospholipids do, we prepared hand-shaken liposomes of *sn*-1 phosphatidylcholine, cholesterol, and dicetylphosphate (molar ratios 0.48 : 0.48 : 0.04), collected them by centrifugation (48 000 × *g*, 60 min) and determined the phosphorus/cholesterol ratio in the pellet. It was found that the *sn*-1 lipid can accommodate an equimolar amount of cholesterol.

Fig. 1 shows the spectra for *sn*-3 and *sn*-1 lipids alone and with 17 and 33 mole % cholesterol. Table I gives band widths at half-height for the -CH_2- signals, and also the ratios of -CH_2- peak height to the, almost constant [6, 7], $N(CH_3)_3$ peak height. The data for *sn*-3-phosphatidylcholine plus cholesterol confirm published results [6, 7]; i.e., the band widths of -CH_2-, and, to a lesser degree, of -CH_3-, increase, while the peak heights decrease. The *sn*-1 phospholipid alone gives, as expected, a spectrum similar to that of the natural antipode, but responds very differently to the addition of cholesterol. At 17 mol % and 33 mol %, the band width has not increased but has actually slightly decreased. The -CH_2- peak height increases at 17 mol % cholesterol, suggestive of a loosening of fatty acid chain restraints. At 33 mol %, the peak

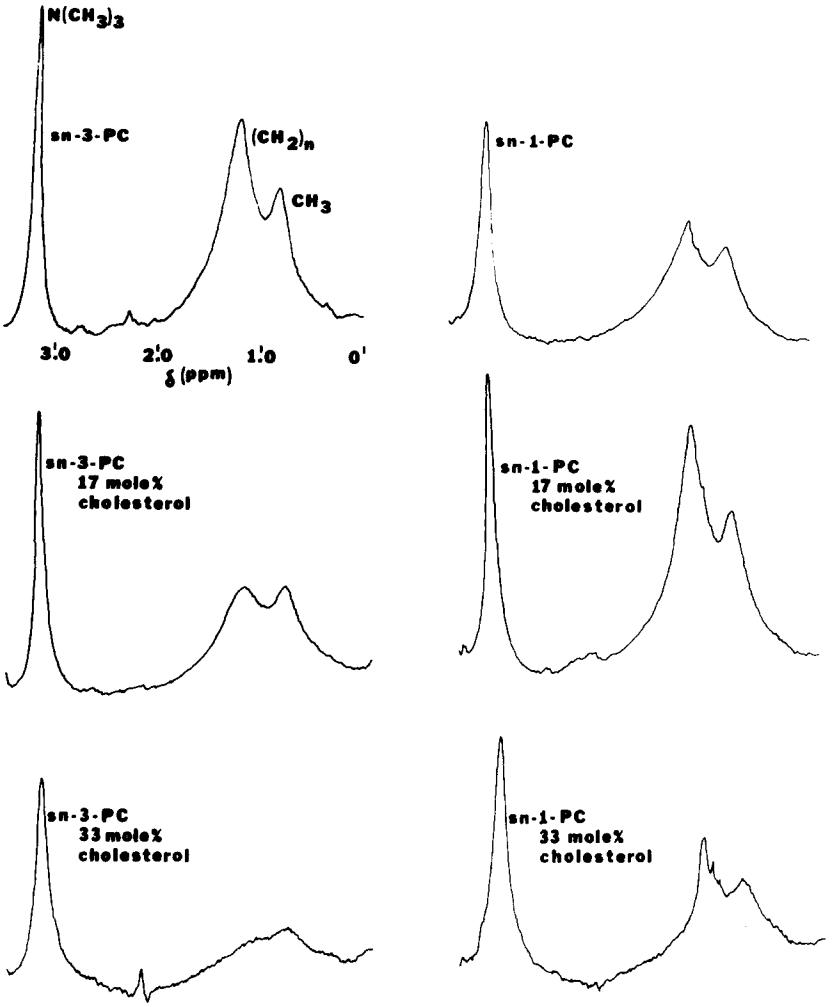

Fig. 1. Proton NMR spectra of *sn*-3 and *sn*-1 dipalmitoyl phosphatidylcholine/cholesterol vesicles (100 MHz, 45° C). PC, phosphatidylcholine.

TABLE I

CH_2 SIGNAL BAND WIDTHS AT HALF-HEIGHT AND $CH_2/N(CH_3)_3$ PEAK HEIGHT RATIOS OF PROTON NMR SIGNALS OF *sn*-3 AND *sn*-1-DIPALMITOYL PHOSPHATIDYLCHOLINE CHOLESTEROL VESICLES

Cholesterol (mol %)	CH_2 half-height width (Hz)		$CH_2/N(CH_3)_3$ intensity ratio	
	sn-3	*sn*-1	*sn*-3	*sn*-1
0	44	48	0.65	0.57
17	62	38	0.36	0.82
33	96	36	0.25	0.61

height is very slightly reduced, not nearly as much as in the *sn*-3 antipode. The spectrum gives the appearance of a sharp -CH_2- peak superimposed on a broader peak. This effect was also seen at 44 mol % cholesterol (not shown).

The striking difference between *sn*-3 and *sn*-1 phosphatidylcholine in re-

sponse to the addition of cholesterol clearly points to a cholesterol-phospholipid interaction in the vicinity of the anomeric C-2 of the glycerol. The CO of the secondary fatty acid ester, being nearest to the glycerol C-2, is the most likely candidate for such interaction; thus, the result is in harmony with our concepts of hydrogen belt structure and our suggestion that the secondary ester group may be hydrogen-bonded by cholesterol-OH [8]. This group is also more exposed to hydration than the primary ester [15].

The sharp -CH_2- peak upon a broader peak in the *sn*-1-phospholipid/33-mol %-cholesterol mixture may indicate that cholesterol is now, at higher concentration, exerting the familiar chain-freezing effect on the *sn*-1 isomer also (broad peak), but that many of the chains are still not affected (sharp peak). This might be due to a lack of cooperativity in the *sn*-1-phospholipid/cholesterol layer, or perhaps to phase separation [18—20]. Either explanation would underline the difference between the two isomers in interacting with cholesterol.

If membrane proteins compete with cholesterol for bonding to phospholipids in the hydrogen belt as we have suggested [8], a similar steric discrimination may be expected for lipid-protein bonding.

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