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MOLECULAR PACKING OF CHOLESTEROL IN PHOSPHOLIPID VESICLES AS PROBED BY DEHYDROERGOSTEROL

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Ergosta-5,7,9,22-tetraen-3-β-ol (dehydroergosterol) was synthesized and employed as a probe of cholesterol behavior in phospholipid bilayers. Circular dichroism (CD) spectra were obtained. The CD of dehydroergosterol in sonicated egg phosphatidylcholine vesicles was dependent on cholesterol concentration, while in unsonicated egg phosphatidylcholine liposomes and in vesicles obtained by oxctylglucoside dialysis, the CD observed was independent of cholesterol content. The CD of dehydroergosterol in sonicated sphingomyelin vesicles exhibited a different dependence on cholesterol content than seen in sonicated egg phosphatidylcholine vesicles. These data are interpreted in terms of differences between the packing of cholesterol in systems of large and small radii of curvature and in different interactions between dehydroergosterol and phosphatidylcholine and sphingomyelin.

The question of the behaviour of cholesterol in phospholipid membranes has been the subject of intensive investigation for many years (for a recent review, see Ref. 1). Direct measurements of cholesterol behavior have been few because of the lack of suitable probing methods. The search for adequate probing methods has been hampered due to the sensitivity of sterol behavior to sterol structure [2]. A notable advance in the study of cholesterol behavior was the introduction of ^{13}C-enriched cholesterol as a non-perturbing probe of cholesterol behavior sensitive to ^{13}C nuclear magnetic resonance [3,4]. This approach proved useful in small, sonicated phosphatidylcholine vesicles but not in large unsonicated liposomes, where the resonance was not adequately distinguishable [3].

Recently, a sterol derivative with a conjugated triene carbon-carbon double bond system, ergosta-5,7,9,22-tetraen-3-β-ol (dehydroergosterol), was introduced as a cholesterol probe, exploiting its fluorescent properties [5]. This probe has been shown to be similar to cholesterol in water permeability studies [5], cell growth studies [5,6], glucose permeability [7] and in its affect on the motional order of the membrane [7]. The circular dichroism (CD) of this molecule has been used to study cholesterol behavior in human serum lipoproteins [7]. Here the circular dichroism of this probe is exploited to detect differences in cholesterol packing between small phospholipid vesicles and large unsonicated liposomes. Further, cholesterol-phospholipid interactions are found to be different between phosphatidylcholine and sphingomyelin.

Materials and Methods

Ergosterol was obtained from Sigma. Egg phosphatidylcholine and bovine brain sphingomyelin

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were purchased from Avanti Biochemicals, and each phospholipid showed a single spot on two-dimensional thin-layer chromatography: (a) chloroform/methanol/ammonia (65:25:5) and (b) chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1). Cholesterol and mercuric acetate were obtained from Fisher. Octylglucoside was purchased from Calbiochem.

Absorption spectra were recorded on a Cary 14 spectrophotometer. For each absorption spectrum obtained with probe, an equivalent sample without probe was used in the reference compartment. Spectra were recorded at 23°C.

Circular dichroism spectra were obtained on a Jasco J21 spectropolarimeter at 25°C in 1 cm pathlength cells. All samples for CD measurements were adjusted to exhibit an absorbance between 0.1 and 1 in the wavelength region scanned. For each preparation containing the dehydroergosterol from which a CD spectrum was obtained, a baseline was obtained from a sample containing no dehydroergosterol.

Uncorrected fluorescence spectra were obtained on a SLM fluorescence instrument.

Preparation of ergosta-5,7,9,22-tetraen-3-β-ol (dehydroergosterol)

Ergosterol was recrystallized from methanol before use. All reactions were peformed in the dark and in a glove box under N_2 to prevent decomposition. Ergosterol was dehydrated with mercuric acetate in chloroform and acetic acid for 18 h, following previously published procedures [9]. The acetate form of the product was recrystallized from diethyl ether and ethanol and exhibited a melting point of 168°C. The free alcohol had a melting point of 143°C. Both melting points are in agreement with previously published values [10].

Preparation of liposomes and sonicated vesicles

The appropriate amounts of phospholipids, cholesterol and dehydroergosterol were dissolved in chloroform to ensure random mixing of the lipids. In each preparation, 2 mg total of phospholipid were used, along with 0.1 mg dehydroergosterol and the indicated amounts of cholesterol. The lipids were first dried under a stream of nitrogen gas and then under vacuum, forming a thin film on the bottom of the flask. Buffer (150 mM NaCl/10 mM histidine/1 mM EDTA (pH 7)) was added and the solution vortexed to disperse the lipid.

To form small unilamellar vesicles, the lipid dispersion was sonicated with a Branson 350 probe sonifier for three 5-min periods, with 2 min between each sonication. For vesicles of phosphatidylcholine, the sonication was performed in an ice bath. For the sphingomyelin vesicles, sonication was performed in a water bath at 25°C. In each case after sonication, the nearly clear solution was centrifuged at 45000 rpm using a 50 rotor in a Beckman L5-50 ultracentrifuge for 45 min. The clear supernatants were collected for spectral measurements. All samples were used within 24 h of formation. For short-term storage, the samples were kept in the dark, under argon at 4°C.

In the case of egg phosphatidylcholine a third system was created for study. Egg phosphatidylcholine (8 mg) and dehydroergosterol (0.5 mg) were solubilized with 30 mg octylglucoside in 0.5 ml 10 mm Tris/100 mM NaCl (pH 7.5). This material was then dialyzed for 16 h against two changes of 1 litre of the same buffer without octylglucoside. Vesicles were formed as shown by an increase in light scattering. This procedure is similar to one used previously for unilamellar vesicles of phosphatidylcholine [11].

The concentration of the probe in these samples was determined by ultraviolet difference spectroscopy, using samples without the probe as reference and a molar extinction coefficient of $\epsilon = 1 \cdot 10^4$ $cm^{-1} \cdot M^{-1}$. The samples were diluted before CD measurements so that the maximum absorbance in the region scanned was less than 1.0. Phospholipid concentrations were determined by the Bartlett assay [12], and cholesterol by enzymatic assay [18]. Samples were analyzed after preparation.

Freeze-fracture electron microscopy.

The methods for temperature controlled freeze-fracture experiments have been described elsewhere [20]. 0.1 μl of the sample was sandwiched between two 75 μm-thick copper foils, equilibrated at 23°C, and then rapidly quenched in liquid propane. Samples were fractured and replicated at −128°C in a Polaron E7500 unit at a vacuum of $5 \cdot 10^{-7}$ Torr. The replicas were viewed on a Siemens 101 electron microscope.

Results

The purity and integrity of the dehydroergosterol were verified several ways. As was already indicated above, the melting point of the pure compound agreed with literature values. The absorption spectrum of dehydroergosterol, in egg phosphatidylcholine vesicles is presented in Fig. 1. The band structure of this spectrum shows similar transitions to those seen in the spectrum of cholesta-5,7,9-trien-3β-ol, a related sterol also containing three conjugated double bonds [5]. Likewise the uncorrected fluorescence spectrum, also presented in Fig. 1, has a similar, though not identical structure to cholesta-5,7,9-trien-3-β-ol [5].

The usefulness of dehydroergosterol as a probe of cholesterol behavior has been examined previously [5–7]. The effects of dehydroergosterol on water permeability in membranes are similar to the effects of cholesterol [5]. *Tetrahymena* will use both dehydroergosterol and cholesterol as a sterol substitute for tetrahymenol [5]. *Mycoplasma mycoide* will also use dehydroergosterol to support growth [6]. Dehydroergosterol and cholesterol increase the motional order of phosphatidylcholine liposomes proportionally to sterol content in a similar way, as measured by an ESR spin label [7]. Permeability of phosphatidylcholine liposomes is inhibited by both sterols, though to a lesser extent by dehydroergosterol than by cholesterol. Thus, while the two sterols do not behave identically dehydroergosterol is similar enough to cholesterol to provide useful indications of cholesterol behavior in a variety of systems [7].

While several means exist to optically monitor the behavior of dehydroergosterol, circular dichroism (CD) was chosen in this study because it is a property greatly sensitive to the environment of the chromophore. Several asymmetric carbon atoms are found in the structure of dehydroergosterol, suggesting the possibility of intrinsic optical activity. However, no circular dichroism is detected from dehydroergosterol in chloroform [7]. In phospholipid bilayers, however, CD is detectable from this probe [7]. This phenomenon is explored further here.

Fig. 2 shows the typical band shape seen from dehydroergosterol in phospholipid vesicles. Apparently, four negative transitions are seen at 345 nm, 327 nm, 315 nm and 300 nm. The relative strengths of these transitions remain constant throughout all the systems studied here, although, as will be seen, the absolute magnitude of all the bands varies according to the conditions.

Light scattering is a problem in these CD spectra and it must be considered as a source of artifacts in these measurements. Light scattering is, however, less of a problem than normally encountered in CD studies of membranes because

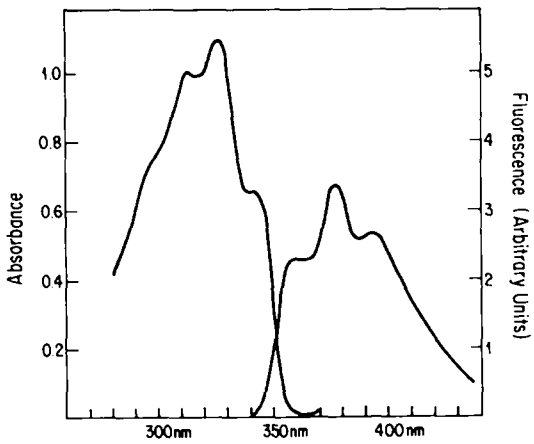

Fig. 1. Absorption (on the left) and fluorescence emission spectra (uncorrected) of dehydroergosterol 10 mol% in sonicated egg phosphatidylcholine vesicles containing no cholesterol. The fluorescence spectra were excited at 270 nm.

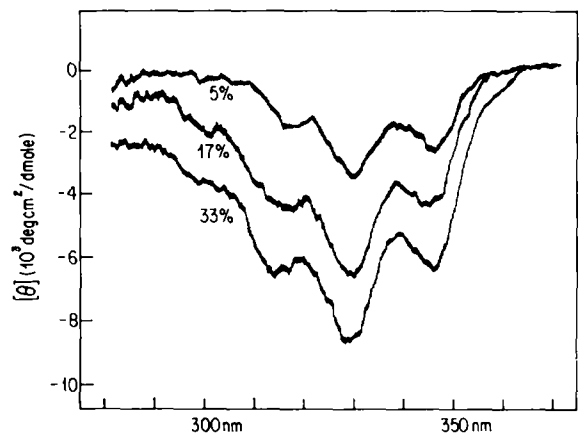

Fig. 2. CD spectra of dehydroergosterol in sonicated egg phosphatidylcholine vesicles containing the indicated mole percent of cholesterol. Spectra were obtained at 23°C in 1 cm pathlength cells.

these measurements are made in the near ultraviolet region rather than the far ultraviolet region. This expectation is supported by the observation that no noticeable shifts in the positions of the transitions occur between the CD of dehydroergosterol in small sonicated vesicles and in the large unsonicated liposomes, while large differences exist in the light scattering properties of these two systems. Shifts in the positions of transitions are normally seen when light scattering is causing artifacts.

As alluded to above, the CD arising from dehydroergosterol in phospholipid vesicles is sensitive to the environment in the membrane. Fig. 2 shows the effect on the CD spectra due to changing the cholesterol content of sonicated egg phosphatidylcholine vesicles containing the optically active sterol [7]. Little change is seen in the band structure of the CD spectra, while a monotonic increase in the intensity of the CD transitions is noted as the cholesterol content of the vesicles is increased. A more clear picture of this change in the magnitude of the CD transitions is presented in Fig. 3. In vesicles containing about 30 mol% cholesterol or more, no dependence of the CD on sterol content is observed. Also interesting is that at zero cholesterol, no CD is observed from dehydroergosterol, which is present at 10 mol%, in these sonicated egg phosphatidylcholine vesicles.

As a comparison it was of interest to see the behavior of dehydroergosterol in phospholipid bilayer systems with less curvature on their surfaces than is found in the small sonicated vesicles. Two systems were used. In the first, unsonicated liposomes of egg phosphatidylcholine were explored at several mole percentages of cholesterol. Surprisingly, in these large systems, no dependence of CD intensity is seen with changes in cholesterol content, as shown in Figs. 3 and 4. Further, at zero cholesterol, CD transitions are seen in these unsonicated systems, in contrast to the results in the sonicated vesicles. Note also that the CD intensity in the unsonicated system never reaches the magnitude seen in sonicated egg phosphatidylcholine vesicles with high cholesterol content.

The second system that was studied has a much greater radius of curvature than sonicated vesicles and thus has some common properties with the unsonicated liposomes. This recently described system [11] is made by solubilization of egg phosphatidylcholine with octylglucoside followed by dialysis to form vesicles that were closer in size to the multilamellar unsonicated liposomes but were unilamellar like the small sonicated vesicles. Freeze-fracture electron micrographs show predominantly large unilamellar vesicles in the preparations used here (see Fig. 5). Again the CD of dehydroergosterol was examined as a function of cholesterol content. The results obtained were virtually identical with the CD spectra from the unsonicated liposome system described above. The CD spectra were weak in intensity and independent of cholesterol content. Fig. 6 shows the CD spectra for one of these vesicle systems. The plot

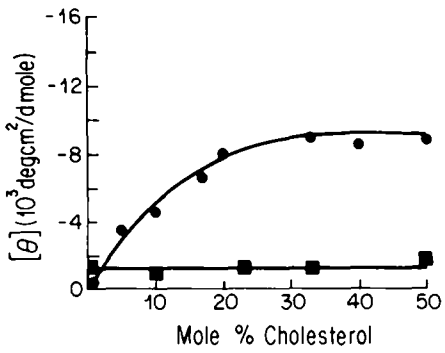

Fig. 3. The magnitude of the CD spectra at 327 nm of dehydroergosterol in egg phosphatidylcholine bilayers containing the indicated mole percentage of cholesterol. ●, sonicated vesicles; ■, unsonicated liposomes.

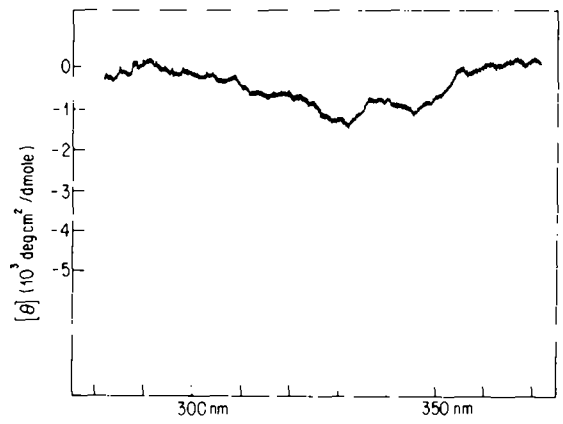

Fig. 4. CD spectra of dehydroergosterol in unsonicated egg phosphatidylcholine liposomes.

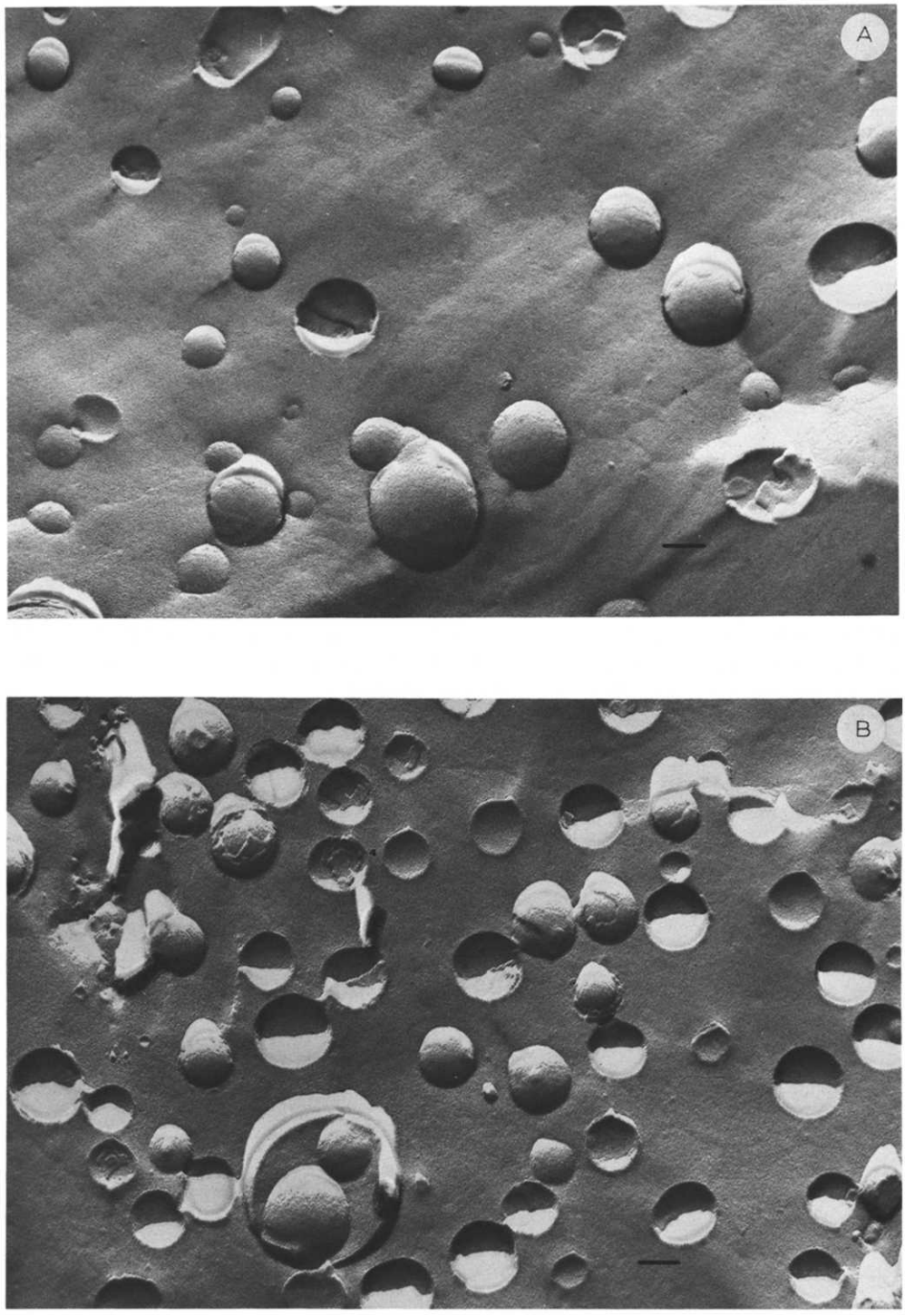

Fig. 5. Freeze-fracture electron micrographs of vesicles formed by octylglucoside dialysis. Bar represents 1000 Å. (A) Egg phosphatidylcholine/cholesterol/dehydroergosterol (5 : 5 : 1); (B) Egg phosphatidylcholine/dehydroergosterol (9 : 1).

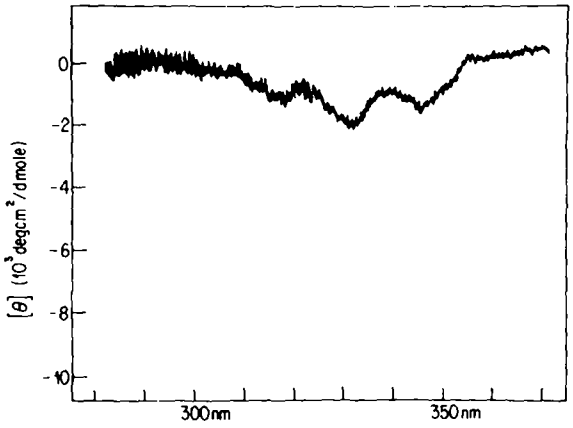

Fig. 6. CD spectra of dehydroergosterol in vesicles formed by octylglucoside dialysis, containing egg phosphatidylcholine and dehydroergosterol (9 : 1).

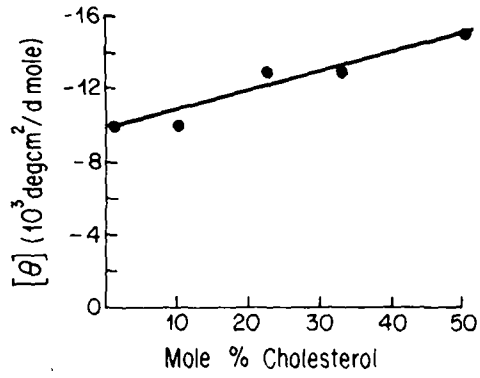

Fig. 8. The magnitude of the CD spectra at 327 nm of dehydroergosterol in sphingomyelin containing the indicated mole percentage of cholesterol. ●, sonicated sphingomyelin vesicles.

of intensity versus mole percent cholesterol is superimposable on the graph for the unsonicated phosphatidylcholine liposomes (see Fig. 3).

Having studied the CD of dehydroergosterol in phosphatidylcholine membranes, it became important to compare this behavior reported above with the behavior of dehydroergosterol in other phospholipid systems. Sphingomyelin was one of the other phospholipids studied. Sonicated vesicles of sphingomyelin containing a constant mole percent dehydroergosterol and varying mole percentage of cholesterol were formed as described above. CD spectra were obtained of dehydroergosterol in these vesicles. While sphingomyelin itself exhibits CD, it is found in the far ultraviolet region which does not overlap the near ultraviolet region measured here. The results are presented in Figs. 7 and 8. These results are clearly quite different from results for the CD of dehydroergosterol in sonicated egg phosphatidylcholine vesicles. Only a modest change in CD intensity is observed as a function of cholesterol content. Instead of the curve observed in Fig. 3, these data are better approximated by straight line. Finally, instead of the CD intensity reducing to zero at zero cholesterol, a relatively intense CD spectrum is observed in sonicated sphingomyelin vesicles without cholesterol.

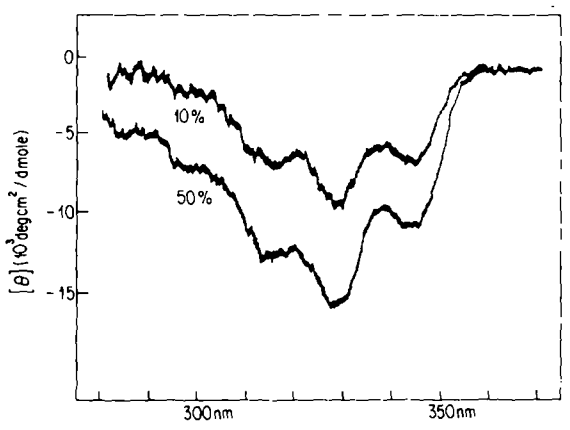

Fig. 7. CD spectra of dehydroergosterol in sonicated sphingomyelin vesicles, at the indicated mole percentages of cholesterol.

Comparison experiments with unsonicated sphingomyelin were not useful, because sphingomyelin is not stable in large liposomes and can spontaneously form small vesicles which complicate the CD spectra observed [13].

Discussion

These results demonstrate that the overlapping absorption bands of dehydroergosterol in the 300 nm to 350 nm region are optically active. This observation is not surprising, since seven asymmetric carbons are found in the dehydroergosterol structure. However, environmental interactions are required to produce detectable circular dichroism, since no spectra can be obtained from dehydroergosterol in $CHCl_3$ [7] or in sonicated egg phos-

phatidylcholine vesicles without cholesterol.

It would appear that at least four factors present in phospholipid bilayers could perturb the strength of the CD bands, and the possible influence of these factors will be used to discuss the results of the present study. One of these factors is the partial ordering of the molecular motion of the sterol by the phospholipid bilayer. In the membrane, cholesterol rotates predominantly about its long axis, although some wobble of the director is expected [4]. In solution the motion is not nearly as restricted and this is reflected in changes in rotational rates of cholesterol when moving from solution to the membrane. Long axis rotation does not change much but other motions possible in solution are restricted in the membrane [4]. Presumably, dehydroergosterol also experiences similar restrictions on its motion in the membrane. The presence of either cholesterol or dehydroergosterol also increases the motional order of the other lipid components in the membrane as measured by ESR spin labels [7,14] and by ^2H nuclear magnetic resonance [14].

A second factor which could perturb the strength of the CD transitions is a change in the hydrogen bonding experienced by dehydroergosterol. Hydrogen bonding effects have been noted previously in the CD of sterols [15]. A third factor which could affect the strength of the CD bands of dehydroergosterol is an interaction between the chromophore of the sterol and either the ester bonds in phosphatidylcholine and phosphatidylethanolamine or the amide bond in sphingomyelin.

A fourth factor which might perturb the CD would be direct interactions between sterols, perhaps even dimer formation [16]. This could involve either dehydroergosterol-cholesterol interactions or perhaps dehydroergosterol-dehydroergosterol interactions.

The first results to be considered are those with egg phosphatidylcholine. In sonicated vesicles, the CD intensity increases with increasing cholesterol content, but insufficient interactions are present in the absence of cholesterol to produce detectable CD. Of particular note is that the effect becomes independent of cholesterol at concentrations higher than about 30 mol%.

One interpretation of these results is sterol dimer formation at high sterol concentrations, and that it is the dimer which leads to the CD spectrum observed. However, this interpretation does not then explain the results in the large vesicle systems, unless the monomer-dimer equilibrium in those systems is independent of sterol concentrations, which is unlikely. This, however, does not rule out dimer formation, but may suggest that the CD is not sensitive to that structure.

A second interpretation is that the CD changes reflect packing changes of cholesterol in the sonicated vesicles as a function of cholesterol content. If the CD arises from interactions between the ester bond of the phospholipid and the chromophore of dehydroergosterol, perhaps from some π oribital perturbations, then properties which effect the packing of cholesterol with phospholipid might also affect the CD observed. Because the radius of curvature is much smaller on the inside of the vesicle than on the outside, the glycerophosphocholine portion of the phospholipids are more tightly packed on the inside of the vesicles than on the outside [17]. Previous studies demonstrated that up to 30 mol% cholesterol, cholesterol is packed symmetrically in these egg phosphatidylcholine vesicles [19]. At higher concentrations, the cholesterol is preferentially distributed to the inside of the vesicle [19]. Therefore, the changes in CD intensity must reflect packing of cholesterol in the outer monolayer of the vesicle, since no changes are observed above 30 mol% cholesterol for the CD spectra. The packing in the two monolayers is different as described above, so the effects on the CD due to the ester bonds could be expected to be different.

From this model one can predict the results from bilayer structures with large radii of curvature. The packing of cholesterol with phospholipids in these structures will not be very asymmetric across the bilayer. Further, the packing will be intermediate between the outer and inner monolayers of the phospholipid vesicle. Therefore, one would expect a CD intensity intermediate between that expected for the outer and inner monolayers of the small vesicle, or a relatively low value. Further, it should be independent of cholesterol content, because of the lack of asymmetric packing of the sterol across the membrane. These are in fact the results observed for all the systems with

large radii of curvature studied here.

The results for the sphingomylein sonicated vesicles do not fit this model, and it may be that cholesterol behavior in sphingomyelin is substantially different from that in phosphatidylcholine. In sphingomyelin vesicles, the CD may only reflect the ordering of the membrane. The steady increase in CD intensity parallels the increase in membrane motional order due to addition of sterol [7,14]. If the CD effects from dehydroergosterol are due to interactions with the ester bonds of phosphatidylcholine, then in sphingomyelin the interactions would be with the amide bond. This difference could of itself lead to a difference in results between these two systems, without necessarily invoking large differences in cholesterol behavior in sphingomyelin.

A contribution to the CD from changes in hydrogen bonding of the probe hydroxyl may also be possible. One would expect a greater exposure of the probe hydroxyl to water in the outside monolayer than in the inside monolayer, due to the short radius of curvature in the small, sonicated vesicles. Again, the CD spectra would be representative of differences in sterol packing between the systems of small and large radii of curvature.

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