of the former compound to 26-hydroxycholesterol was observed indicates that 26-hydroxycholesterol is probably not an intermediate in bile acid metabolism.

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Interaction of Egg Lecithin with Cholesterol in the Solid State*

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ABSTRACT: The attenuated total reflectance technique of infrared spectroscopy has been used to obtain spectra of solid films of egg lecithin–cholesterol mixtures. The presence of lecithin produces a low frequency shift of $\sim$150 cm^{-1} in the ^{-}OH stretching absorption of cholesterol. A maximum interaction was observed at a stoichiometry of 2 sterol:1 phospholipid molecules. The interaction is not produced by other lipids containing polar groups similar to those found in lecithin.

There is considerable evidence that cholesterol–lecithin interactions may be important in certain biological and biomedical systems. In particular, it has been recognized that these two compounds are major components of the lipid fraction in cell membranes (O'Brien and Sampson, 1965; Ways and Hanahan, 1964), and speculative models of cellular membranes often include a lecithin–cholesterol complex. For example, Finean (1966), Vandenheuvel (1963), and O'Brien (1965) have all discussed a model for the myelin sheath membrane which involves van der Waals interaction between hydrocarbon portions and coulombic forces between the positively charged choline nitrogen of lecithin and the ^{-}OH group of cholesterol in a bimolecular leaflet structure. The ability of lecithin to solubilize cholesterol in bile has also been recognized, and this interaction may be important in understanding the processes of gall stone formation (Niederhiser *et al.*, 1966).

By studying mixed monolayers of phospholipids and cholesterol, Leathes (1925) demonstrated a "condensing effect" which was thought to be due to interaction between the sterol and the phospholipids. Chapman and Penkett (1966) have published nuclear magnetic resonance data which show that the freedom of motion of lecithin side chains is greatly reduced in the presence of cholesterol, suggesting extensive interaction of the hydrocarbon moieties. On the other hand, Shah and Schulman (1967) have concluded that cholesterol actually reduces the hydrophobic interaction of the lecithin side chains in a monolayer, producing a liquid film. Furthermore, surface potential measurements led these authors to conclude that there was no significant interaction between the polar group of lecithin and cho-

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esterol in a monolayer, although such interactions are usually found in molecular models of cholesterol–lecithin complexes suggested in the literature (Vandenheuvel, 1963).

This paper presents spectral data which indicate that in films cast from organic solvents, cholesterol can form a 2:1 complex with lecithin which involves interaction of the cholesterol ⁻OH with lecithin polar groups. Association with the quaternary nitrogen of lecithin is ruled out, and a model which invokes hydrogen bonding of cholesterol with the esterified phosphate oxygens of lecithin is discussed.

Methods and Materials

Egg lecithin was prepared by a modification of the method of Singleton *et al.* (1965). The lecithin preparation gave a single spot on thin-layer chromatography, and upon chemical analysis, an ester phosphate ratio of 1.97, and a choline phosphate ratio of 1.05.

Cholesterol was purchased from Sigma Chemical Co.

Infrared spectroscopy was done on solid films of lipid utilizing the attenuated total reflectance technique (Harrick, 1963). This technique is useful for several reasons: (a) one can obtain intense spectra with small amounts of sample; (b) the crystals used are not hygroscopic and when studying ⁻OH vibrations, the danger of confusion due to water absorption on the crystals is avoided; (c) the fact that the radiation interacts with the entire surface of the attenuated total reflectance crystal makes it easy to obtain reproducible spectra; *i.e.*, one covers the entire crystal with the same amount of lipid each time, thus avoiding large variations in the amount of material the instrument "sees;" and (d) it is necessary to obtain addition spectra of varying amounts of cholesterol and lecithin separately, to compare with the interaction spectra. For the complex spectra of the lipids, this is most easily done experimentally. With the attenuated total reflectance technique, one need merely cover part of the crystal with cholesterol and part with lecithin, and it is not difficult to assure that the fraction of the surface covered by a single component is equal to the mole fraction of the component in the mixture. Thus, experimental addition spectra of, *e.g.*, cholesterol–lecithin (3:1), are obtained to compare with the similar mixture.

A film of lipid was deposited on the attenuated total reflectance crystal surface (usually KRS-5) by application of 0.25 ml of a lipid solution in CCl_4, or at times in a solution consisting of $CHCl_3$–CH_3OH (2:1). The solvent was evaporated by flushing the surface of the crystal with N_2 in a desiccator. When obtaining spectra, complete removal of H_2O from the atmosphere was necessary to prevent accumulation of water on the lecithin-covered crystals. This was accomplished by flushing the infrared spectrometer with dry air.

Spectra were run in a Model 521 Perkin-Elmer infrared spectrometer, with a Wilks, Model 12, double-beam attenuated total reflectance attachment. The reference beam was always directed through a clean reference crystal.

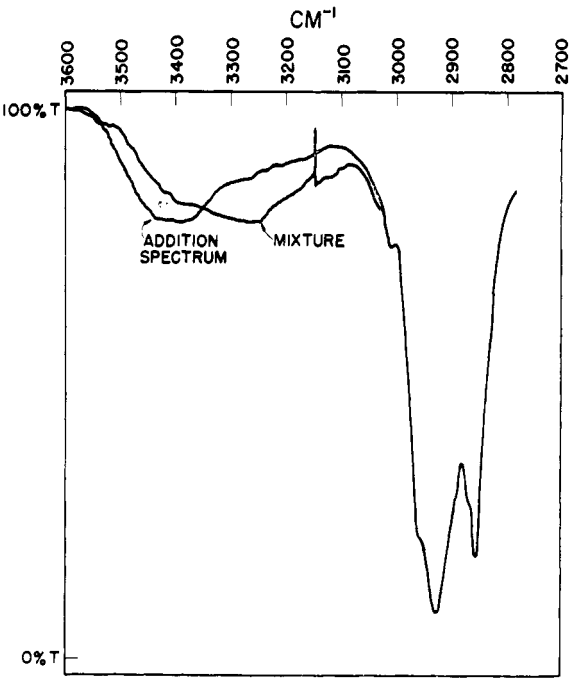

FIGURE 1: Attenuated total reflectance infrared spectra of a 1:1 molar mixture of cholesterol and lecithin; in the OH, CH stretching region; addition spectrum obtained by covering half the surface area of the KRS-5 crystal with lecithin and half with cholesterol.

Results

Attenuated total reflectance spectra of cholesterol and lecithin were obtained. The spectra obtained were directly comparable with transmission spectra (Chapman, 1965). A broad absorption peaking at 3380–3390 cm^{-1} was present in the lecithin spectrum. Since lecithin contains no NH or OH groups, and since chemical and thin-layer analysis indicated high purity of the preparation, it was suspected that this absorption was due to water. Upon overnight equilibration of the lecithin with 95% ethanol-1-*d*–5% D_2O (room temperature), this 3390-cm^{-1} absorption was greatly reduced, although not completely eliminated. It was then possible to look at the OH stretching frequency of cholesterol in cholesterol–lecithin mixtures.

Figure 1 gives the infrared spectrum of a 1:1 molar mixture of cholesterol and lecithin and a 1:1 addition spectrum in the ⁻OH and CH stretching regions. The addition spectrum clearly shows the strong ⁻OH absorption of cholesterol which peaks at 3420–3400 cm^{-1}. However, in the mixture this ⁻OH peak is shifted to about 3250 cm^{-1} and is considerably broadened, while a small shoulder remains at ~3380 cm^{-1}.

Use of the relatively new technique of attenuated total reflectance spectroscopy raised the possibility that the spectral shift was an artifact of this technique, or that special surface effects of the KRS-5 crystals were responsible for the observed ⁻OH shift. In particular, orientation of molecules at the crystal surface seemed a possible source of artifact, since the attenuated total reflectance spectra represent primarily the surface layers of lipid. This possibility appears to be negated

by the following information: (a) transmission spectra of cholesterol–lecithin mixtures, both on KRS-5 crystals and on NaCl, showed the same shift in ⁻OH stretching frequency; (b) the shift was independent of the type of attenuated total reflectance crystal used, germanium and silver chloride, both being employed on occasion. Thus, the ⁻OH shift appears to be real, and due to the presence of lecithin.

However, there were no significant differences in the areas of the spectrum other than the ⁻OH stretching region. We studied the 1250–1000-cm^{-1} region (P═O, C—O) (Bellamy, 1958; Chapman, 1965; Shimanouchi *et al.*, 1963; Tsuboi, 1964) and the 850–750-cm^{-1} region (P—O) extremely carefully in an attempt to locate the base involved in hydrogen bonding, but in no case were significant alterations observed.

Although it was obvious that unequivocal identification of the base involved in the interaction could only be obtained from data on the lecithin–cholesterol mixtures directly, the failure to observe any consistent spectral differences other than the ⁻OH stretching frequency prompted us to look at mixtures of cholesterol with other lipids containing polar groups similar to those found in lecithin. Table I shows the result of these studies. Of the lipids studied, a cholesterol ⁻OH stretching frequency shift is observed in the presence of the quaternary ammonium salts, and in mixtures with lysolecithin. The spectrum obtained from lysolecithin–cholesterol mixtures appeared identical with lecithin–cholesterol mixtures in the ⁻OH stretching region. However, that obtained with cetyltrimethylammonium salts was quite different. Figure 2 shows a typical spectrum obtained from such a mixture. Although superficially the observation of this shift may appear to be evidence in favor of the choline-$N^+\cdots$O(H)-cholesterol hypothesis of Finean (1966) and others (Vanderheuvel, 1963; O'Brien, 1965), the data in fact indicate that this shift is due to hydrogen bonding of the cholesterol hydroxyl to the anion of the quaternary ammonium salt. As Table I shows, the cetyltrimethylammonium salt–cholesterol interaction depends upon the anion in the salt, the chloride enhancing the ⁻OH shift by some 30 cm^{-1}. Therefore, one cannot invoke the choline nitrogen on the basis of these data, but rather the interaction is due to ⁻OH hydrogen bonding to the anion. Furthermore, the ⁻OH peak is shifted by $\sim$100 cm^{-1}, and is highly symmetrical. This is in contrast to the lecithin–cholesterol interaction where the ⁻OH absorption is broad, unsymmetrical, and shifted by at least 150 cm^{-1}.

TABLE I: Cholesterol Hydroxyl Infrared Frequency in Lipid Mixtures.[a]

Lipid	⁻OH Max (cm^{-1})
Lecithin[b]	3280–3230
Lysolecithin[c]	3280–3200
Cetyltrimethylammonium bromide[c]	3310
Cetyltrimethylammonium chloride	3280
Tristearin[b]	3420–3400
Dicetyl phosphate[c]	3420–3400

[a] Cholesterol concentration was 4.0 mg/ml in all mixtures. [b] Applied to attenuated total reflectance crystal as solution of 8.0 mg/ml in CCl_4. [c] Applied to crystal as solution of 4.0 mg/ml in CH_3OH–$CHCl_3$ (1:2).

We studied the lecithin–cholesterol interaction further by attempting to define its stoichiometry. In the mixtures with an excess of cholesterol, there exist two absorptions, one for the lecithin-complexed cholesterol (3250 cm^{-1}) and one for the noninteracting cholesterol (3400 cm^{-1}). A plot of the ratio of the intensities of these two absorptions *vs.* the molar ratio of cholesterol to lecithin is presented in Figure 3. These data are compared with those obtained from addition spectra. It can be seen from this plot that the ratio A_{3400}/A_{3250} reaches its minimum at a ratio of two cholesterols to one lecithin.

These data were obtained from studies in the solid state. Spectra obtained in CCl_4 solution showed no hydrogen bonding. These results were obtained with cholesterol concentrations just below those necessary to observe cholesterol–cholesterol hydrogen bonding. In these solutions the molecules must be at least loosely ordered in a manner similar to that obtained in the solid films. Thus, the absence of an ⁻OH shift in solution suggests that hydrogen bonding is not the primary driving force in the interaction observed in the solid state, since this polar association should predominate in nonpolar solvents.

During this study we did observe that concentrated solutions of cholesterol show intermolecular association with an ⁻OH stretching frequency which maximizes near 3400 cm^{-1}. Thus, in the solid state, it appears that intermolecular hydrogen bonding in cholesterol is no stronger than that seen in concentrated solutions.

Discussion

From the present data, the origin of the ⁻OH shift cannot be stated. The most obvious interpretation is hydrogen bonding of cholesterol to some polar site in the lecithin molecule. However, cholesterol–cholesterol polymeric association induced by lecithin is a possible alternative interpretation, although it seems unlikely. This concept involves the end-to-end interaction of cholesterol hydroxyls to form a bridging polymeric system. Such systems have been reported to have ⁻OH stretching frequencies as low as those observed here (Chapman, 1965). However, this can involve lecithin only in utilizing the hydrocarbon chains as space fillers in the hydrocarbon matrix. It is difficult to envision such a situation when the observed effect depends upon the insertion of lecithin hydrocarbon moieties between some cholesterol molecules. This would tend to decrease rather than increase hydrogen bonding.

In addition, studies with space-filling molecular

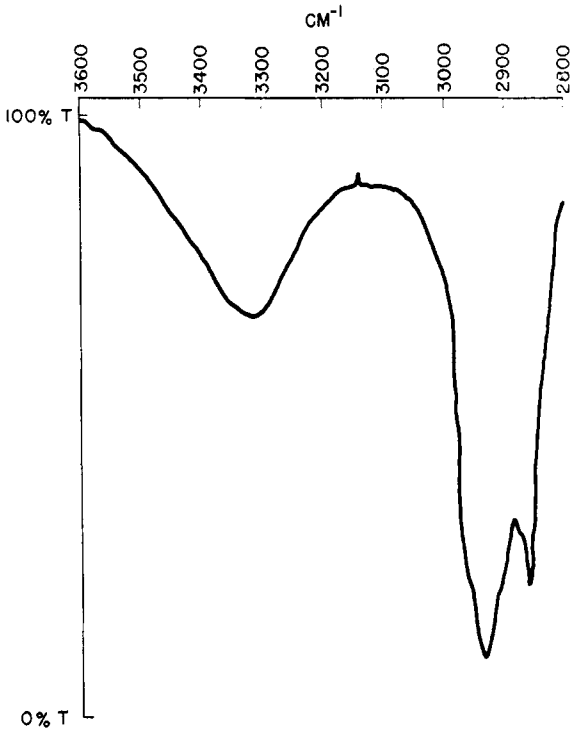

FIGURE 2: Attenuated total reflectance spectrum of cholesterol–cetyltrimethylammonium bromide (1:1 molar mixture). Each material was dissolved in $CHCl_3$–CH_3OH (2:1), and equal volumes were mixed. This solution (0.25 ml) was applied to the KRS-5 attenuated total reflectance crystal.

models show that it is impossible to form an extensive polymeric system with cholesterol because of the bulk of the hydrocarbon moiety. It seems likely, in fact, that solid cholesterol alone is as extensively associated as it can be, probably by end-to-end dimer formation, as suggested by the infrared adsorption of the ^-OH group at 3420 cm^{-1}.

The interpretation that the cholesterol hydroxyl forms a hydrogen bond with some polarizing group in lecithin therefore seems most likely. Although unequivocal identification of the functional group(s) in lecithin which cause the OH shift is not presently feasible, one can eliminate certain possibilities. It seems clear that the carbonyls of the ester moieties of lecithin are not involved because of the lack of an effect on the C=O stretching band in the spectra. In addition, involvement of the choline nitrogen as speculated by Finean and others seems highly unlikely. The data in Figure 2 and Table I show that the ^-OH of cholesterol does approach the polar end of cetyltrimethylammonium bromide closely enough to produce a Br^-–HO^- interaction, but that even in this case, the ^-OH shift is not as great as that with lecithin.

Intuitively, one would expect that the nonesterified phosphate oxygens might be implicated because of their high net negative charge, and thus favorable hydrogen-bonding properties. However, shifts of 50–80 cm^{-1} in the P=O stretching frequency are reported to be seen with hydrogen bonding of these oxygens (Bellamy, 1958). Furthermore, we recently observed shifts in this peak upon mixing cholesterol with the sterol, vitamin D (J. E. Zull and C. G. Sciotto, unpublished data). These vitamin D induced P=O stretching shifts are accompanied by sterol ^-OH shifts of the order of magnitude reported in this paper. It therefore appears that hydrogen bonding of sterols to the P=O group can be detected by our technique, and thus the cholesterol–lecithin interaction probably does not involve these oxygen atoms.

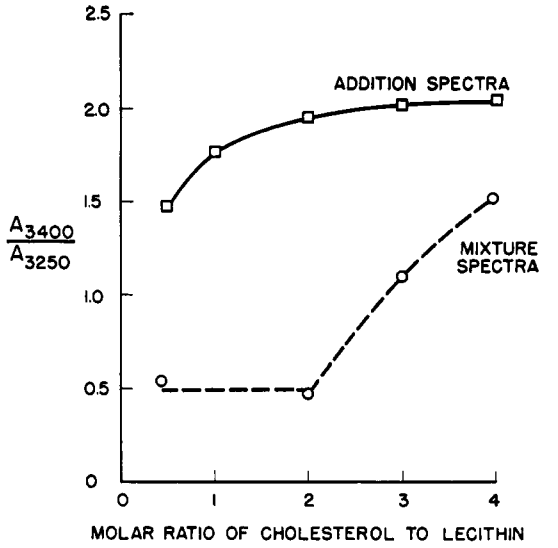

FIGURE 3: Relative absorbancy of "free" (3400 cm^{-1}) and associated (3250 cm^{-1}) form of cholesterol at different cholesterol–lecithin proportions.

The remaining possible electronegative sites for hydrogen bonding in lecithin are the ester oxygens of the phosphate and the carboxylic acid ester oxygens. On the basis of electronegativities the phosphate ester oxygens would be expected to be the more likely of these two sites for possible hydrogen bonding with cholesterol.

The stoichiometry of the interaction suggests, but does not prove, that two electronegative sites in lecithin are involved, one each with a cholesterol molecule. Also, the broadening of the ^-OH band implies that the electronegative groups are not equivalent. These properties are fulfilled if one assumes that each lecithin molecule is associated with two cholesterols through its two phosphate-ester oxygens. Whether this tentative conclusion is valid must await more definitive work.

The question of the orientation of the molecules relative to one another in the film is clearly of interest. There appear to be two reasonable possibilities. (a) A side-to-side association of cholesterol and lecithin in the manner suggested for the lipid bilayer in cell membranes; (b) an end-to-end association, of individual molecules or of micellar units. However, it is known that lecithin in organic solvents exists in a micellar state with the polar group directed inward away from the bulk solvent (Dervichian, 1965). In order for cholesterol to associate in an end-to-end fashion, the sterol molecule must penetrate and essentially disperse the lecithin micelles. This seems unlikely. On the other hand, a side-to-side association of the lipid molecules is easily envisioned if one assumes that cholesterol essentially fills in gaps in the lecithin micelle, the polar hydroxyl

group being inserted into the polar region of the lecithin micelle, to form a mixed micelle.

However, the results imply that the polar association is not the cause of the interaction, but rather the effect. Polar interactions should persist in nonpolar solvents and some hydrogen bonding of cholesterol–lecithin mixtures should be evident in the solution spectra. This is not the case. The observation that a nonpolar solvent disrupts a polar interaction leads to the idea that the observed shift of the hydroxyl stretching frequency is probably due to packing of cholesterol and lecithin in such a manner as to insert the hydroxyl group into a polar region of the lecithin molecule. Clearly the molecules must be loosely ordered in a similar fashion in solution, but only when the solvent is removed and the molecules are closely packed does the hydroxyl group come under the influence of the polar moieties in lecithin.

It should be mentioned that while we can observe a two cholesterol to one lecithin stoichiometry, we do not intend to suggest that a complex of this stoichiometry must exist as such *in vivo*, or that only lecithin can participate in such an interaction. Data on the ratio of cholesterol to phospholipid in various membrane preparations range from 0.1 to 1.32 (cholesterol:phospholipid) (Ashworth and Green, 1966). Thus, in cell membranes, phospholipids undoubtedly attain less than the theoretically possible 2:1 (cholesterol:phospholipid) ratio.

Finally, the observations of Shah and Schulman (1967) are clearly relevant to our work. They reported that in a monolayer, there was no observable deviation from additivity in the surface potential of cholesterol–lecithin monolayers. This was interpreted as an indication that cholesterol and lecithin do not interact through their polar groups when present in a monolayer, which may well be correct. However, it should be pointed out that lack of an effect on the surface potential of these complex molecules does not prove there is no interaction, but merely that there is not net change in the vertical component of the sum of all the dipoles in the molecules. At least, it seems important to reconsider the conclusions of Shah and Schulman in view of our data. In fact, it is difficult to imagine how the cholesterol hydroxyl can exist in close proximity with lecithin without some dipole–dipole or hydrogen bonding occurring.

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