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Self-Association of Cholesterol and Its Interaction with Triglycerides. An Infrared Study*

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ABSTRACT: The self-association of cholesterol due to hydrogen bonding has been studied by infrared measurements of its OH-stretching band. At concentrations below 0.014 M in CCl_4, cholesterol exists only as a monomer.

As the concentration is increased it associates to form a dimer and at a concentration of ~0.06 M a higher aggregate begins to form which becomes the predominant species at a concentration of ~0.2 M. The dimerization constant has been determined ($K_{d,23°}$ = 4.5 l. mole^{-1}) at different temperatures from which the enthalpy of dimerization has been evaluated (ΔH = −1.8 kcal mole^{-1}). The ν_{max} of the OH-stretching bands of the dimer and trimer have also been reported. Infrared spectra of mixed solutions of cholesterol and the triglycerides gave evidence of formation of a 1:1 hydrogen-bonded complex. The equilibrium constants and enthalpies of formation of the complexes of cholesterol with triacetin, tributyrin, and trilaurin have been reported ($K_{23°}$ = 2.4–3.7 l. mole^{-1}; ΔH = −3.5 to −5.4 kcal mole^{-1}). The hydrogen-bonding properties of cholesterol are suggested as factors in the mechanism of plaque formation in atherosclerosis.

The mechanism of the deposition of cholesterol in atherosclerotic plaque is not well understood, and attention to some physicochemical factors may be of value in helping to understand this process. Recent studies attest to the need for further investigation of the physical chemistry of cholesterol, triglycerides, and other substances associated in the plasma with them (Sodhi and Gould, 1967; Chapman and Penkett, 1966; Paton, 1964). The proton-donating OH group of cholesterol immediately suggests hydrogen bonding as a possible mechanism of its interaction with triglycerides, which have proton-accepting carbonyl groups, and other acceptor molecules. Surprisingly enough there have been no reports of any such investigations on the hydrogen-bond interactions of cholesterol in the literature. There is only a passing reference to hydrogen-bonded aggregates of cholesterol as a probable cause for the line broadening of its nuclear magnetic resonance spectrum (Varian, 1957). We have carried out detailed studies of the hydrogen-bond interactions of cholesterol in terms of both its self-association and its association with triglycerides.

Experimental Section

Chromatographically pure (99+ %) cholesterol (General Biochemicals), reagent grade triacetin and tributyrin (Fisher), and trilaurin (Mann Research Labs) were used in the present studies. Spectrograde carbon tetrachloride (Merck) was dried over P_2O_5 in a desiccator

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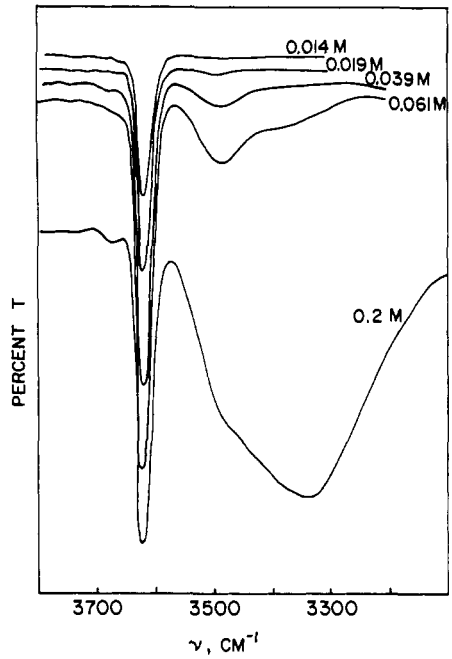

FIGURE 1: OH-stretching vibration bands of cholesterol at different concentrations. Solvent, CCl_4; temperature, 23°. The ordinate axis is not drawn to scale.

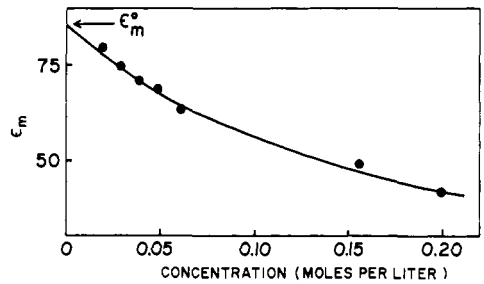

FIGURE 2: Apparent molar extinction coefficient *vs.* concentration of cholesterol in CCl_4 (Liddel and Becker plot).

prior to use. All the spectral measurements were made with freshly prepared solutions.

The infrared spectra were recorded with a Perkin-Elmer Model 521 spectrophotometer (fitted with a Model 621 glower). Matched pairs of Irtran-2 demountable cells (Limit Corp.) were used. The cells were provided with metallic jackets (Limit Corp.) through which water at the desired temperature was circulated. The cell thickness varied between 0.5 and 2.0 mm for the self-association studies and was 1.5 mm for the donor–acceptor studies.

For the self-association studies, the concentration of cholesterol ranged from 0.02 to 0.2 M. In the donor–acceptor studies, the concentration of cholesterol used was in the neighborhood of 0.013 M, while that of the triglycerides was about 0.25 M.

Results

Self-Association of Cholesterol. The OH-stretching absorption bands of carbon tetrachloride solutions of cholesterol at different concentrations are shown in Figure 1. At low concentrations (0.014 M), cholesterol shows a single sharp peak at 3620 cm^{-1} due to the OH-stretching vibration of the free molecule. As the concentration is increased, a new broad band appears on the low-frequency side at 3470 cm^{-1} in addition to the monomer peak at 3620 cm^{-1}. The 3470-cm^{-1} band is due to the bonded OH group of the dimer. With further increase in the concentration (~0.06 M), a third band is observed at 3330 cm^{-1} in addition to the monomer and dimer bands and is probably due to a trimer (or other higher aggregate). As the concentration of cholesterol is increased, the intensity of the band at 3330 cm^{-1} increases more than does that of the dimer band, and at a concentration of ~0.2 M the dimer band is seen only as a shoulder on the band at 3330 cm^{-1}.

The association constant for the dimer was calculated from measurements of the intensity of the monomer band at different concentrations by use of the limiting slope method of Liddel and Becker (1957). At low concentrations the dimer is the predominant species and hence the limiting slope method would still be applicable. In this method, the apparent molar extinction coefficient of the monomer band is plotted against the concentration of the associating solute, and the dimerization constant (K_d) is obtained from the limiting slope of the resulting curve by use of eq 1, where ϵ_M^0 is the

$$\left[\frac{d\epsilon}{dc}\right]_{c\to 0} = -2K_d\epsilon_M^0 \qquad (1)$$

molar extinction coefficient of the monomer extrapolated to infinite dilution ($c = 0$). A typical curve obtained in the case of cholesterol is shown in Figure 2. The equation above is derived for the case of a cyclic dimer where the OH groups of the dimer would make no contribution to the absorption coefficient of the monomer peak. In the case of an open-chain dimer, the free OH group of the dimer will also absorb in the region of the monomer peak. If it is assumed that the absorption coefficient of the free OH group of the dimer is the same as that of the monomer OH, the dimerization constant (K_d) will be given by (Liddel and Becker, 1957) $[d\epsilon/dc]_{c\to 0} = -K_d\epsilon_M^0$, *i.e.*, it will be twice that for the case of the cyclic dimer. Since the two equilibrium constants are proportional to each other, the enthalpy value obtained from the slope of the plot of log K_d *vs.* $1/T$ would remain the same regardless of the choice of the structure (cyclic or open).

The dimerization constants for cholesterol have been calculated on the basis of an open-chain dimer. The enthalpy of dimerization was determined from the dimerization constants measured at different temperatures between 5 and 50°. The spectral and thermodynamic data of the self-association of cholesterol are summarized in Table I.

Figure 3 shows the effect of temperature on the association of a 0.06 M solution of cholesterol. At 5°, the OH band due to the trimer (or higher aggregate) at 3330 cm^{-1} is quite intense and much more so than the OH band due to the dimer at 3470 cm^{-1}. This trend is reversed as the temperature is raised and at 50°

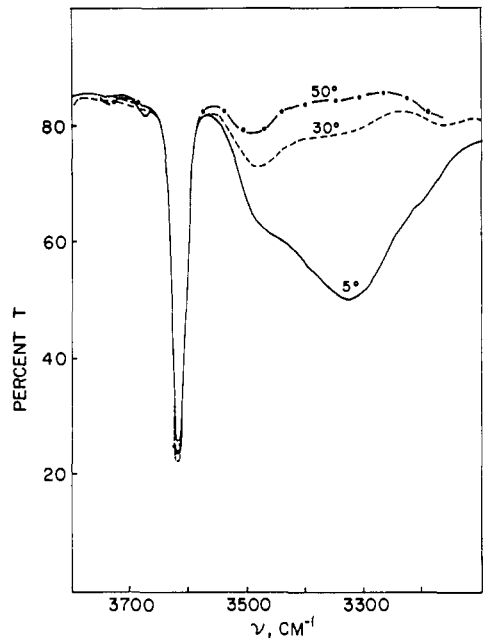

FIGURE 3: Effect of temperature on the bonded OH peaks of cholesterol (0.06 M). Solvent, CCl_4.

there is very little absorption below 3470 cm^{-1}. This indicates that the variation in the concentration of the aggregate (and hence its association constant, K) with temperature is much more marked in the case of the trimer (or higher aggregate) than that of the dimer. The enthalpies of formation, which are proportional to the slopes of the plots of log K *vs.* $1/T$, should therefore follow the same trend, *i.e.*, the enthalpy of formation of the trimer should be considerably higher than that of the dimer. It is interesting to note that this trend is also indicated by the position of the absorption bands of the associated species. From the time Badger and Bauer (1937) proposed a relationship between $\Delta\nu$ (the shift in frequency of the OH band of the associated molecule from that of the free molecule) and ΔH of association (hydrogen bonding), this relation has been tested in several cases and it is generally accepted that $\Delta\nu$ is an index of ΔH of hydrogen-bond formation (Pimentel and McClellan, 1960). Referring now to Table I, it can be seen that the frequency shift ($\Delta\nu$) of the trimer is almost twice that of the dimer.

TABLE I: Spectral and Thermodynamic Data of the Self-Association of Cholesterol in CCl_4.

Associated Species	ν_{OH} (cm^{-1})	$\Delta\nu_{OH}$ (cm^{-1})[a]	K (l. $mole^{-1}$)[b]	$-\Delta H$ (kcal $mole^{-1}$)
Dimer	3470	150	4.5	1.8
Trimer	3330	290		

[a] Shift from the ν_{OH} of the monomer at 3620 cm^{-1}. [b] At 23°.

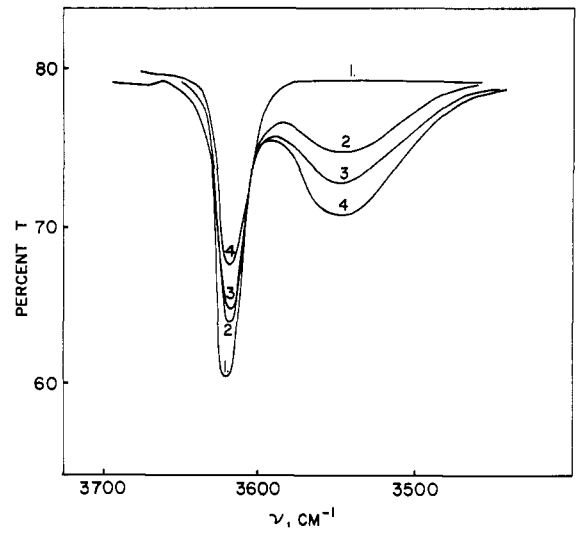

FIGURE 4: Effect of varying the concentration of tributyrin on the associated OH band of cholesterol–tributyrin complex. Solvent, CCl_4; temperature, 23°. Curve 1: cholesterol (0.01 M); curves 2–4: cholesterol (0.01 M) plus tributyrin (0.10, 0.15, and 0.25 M).

Based on this we can estimate the ΔH for the trimer to be approximately -3.6 kcal $mole^{-1}$.

Interaction of Cholesterol with Triglycerides. The infrared spectrum of a carbon tetrachloride solution of cholesterol (0.01 M) and tributyrin (0.25 M) (Figure 4, curve 4) shows, in addition to the OH band at 3620 cm^{-1}, a new band at a lower frequency (3540 cm^{-1}). At this concentration cholesterol exists only as a monomer (curve 1) and since the spectrum is recorded against a blank solution containing the triglyceride at the same concentration as in the mixed solution, it is obvious that the new peak is not due to either component alone. It can also be seen from the figure that the intensity of the OH peak of cholesterol at 3620 cm^{-1} in the mixture is less than that in the pure

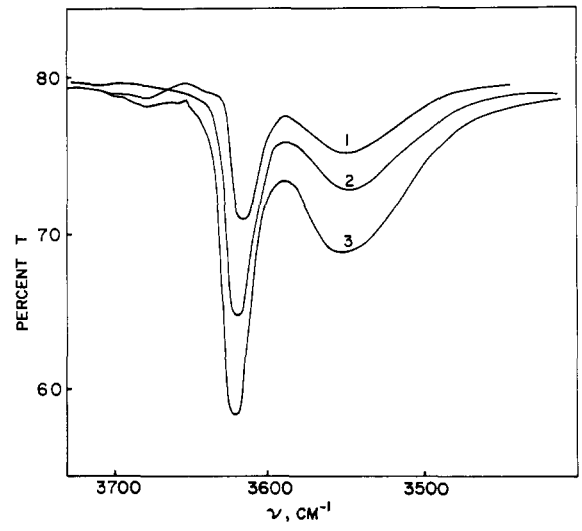

FIGURE 5: Effect of varying the concentration of cholesterol on the associated OH band of cholesterol–tributyrin complex. Solvent, CCl_4; temperature, 23°. Curves 1–3: cholesterol (0.006, 0.01, and 0.015 M) plus tributyrin (0.15 M).

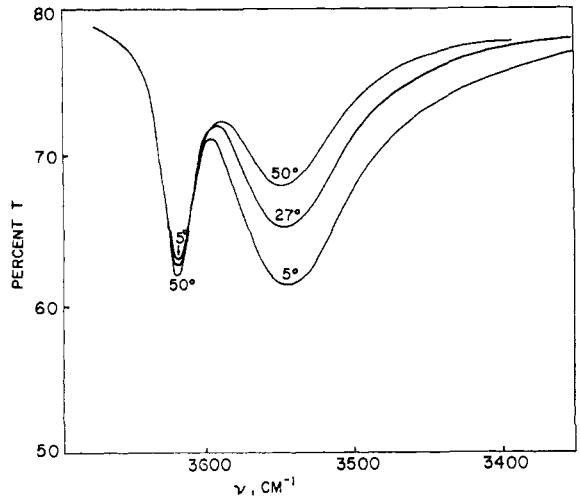

FIGURE 6: Effect of temperature on the intensities of the free and associated OH bands of cholesterol (0.012 M)–tributyrin (0.25 M) complex. Solvent, CCl_4.

solution. Thus, the new peak is due to a hydrogen-bonded complex between cholesterol and the triglyceride. The triglyceride has three potential acceptor groups (C=O), and it was therefore necessary to establish the stoichiometry of the complex before equilibrium and thermodynamic data could be evaluated. Within the range of concentrations that could be employed (cholesterol, 0.006–0.015 M; triglyceride, 0.1–0.3 M) only one band was observed on the low-frequency side of the OH band, indicating formation of only one type of complex. Steric considerations would seem to favor a 1:1 complex. Further confirmation that the complex has 1:1 stoichiometry was obtained by examining the intensity of the complex band with varying concentration of either component (Figures 4 and 5). It was found that the intensity of the complex band was directly proportional to the concentration of both components, thus proving the complex to be of 1:1 stoichiometry.

The equilibrium constant for a 1:1 complex is given by eq 2, where [DA] = equilibrium concentration

$$K = \frac{[DA]}{[D][A]} \tag{2}$$

of the complex, [D] = equilibrium concentration of the free proton donor, and [A] = equilibrium concentration of the free proton acceptor.

Since the acceptor concentration (~0.25 M) is considerably higher than that of the donor (~0.013 M), the equilibrium concentration of the free acceptor is not significantly different from its initial (total) concentration. With this assumption the only unknowns in the above equation are the equilibrium concentrations of the complex and the free donor. Both are easily determined from measurements of the intensities of OH absorption bands of cholesterol (3620 cm^{-1}) in the pure solvent (CCl_4) and in the presence of the acceptor. If a_1 is the absorbance at the ν_{max} of the free OH band (3620 cm^{-1}) of a dilute cholesterol solution

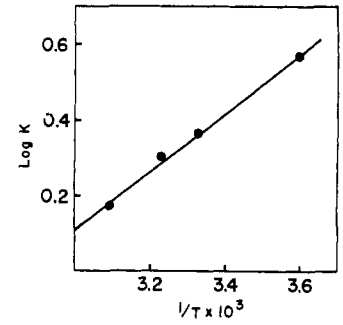

FIGURE 7: Log K *vs.* $1/T$ plot for the cholesterol–tributyrin complex.

(~0.013 M) and a_2 that of an equal concentration of cholesterol in the presence of a large excess of the acceptor, then

$$\frac{a_1 - a_2}{a_2} = \frac{[DA]}{[D]} \tag{3}$$

and

$$K = \frac{a_1 - a_2}{a_2}\frac{1}{A}$$

where A is the initial concentration of the acceptor.

The enthalpy of complex formation was determined by measuring the equilibrium constants at different temperatures. Since the complex band (3540 cm^{-1}) showed more marked intensity changes with temperature than did the free OH band (3620 cm^{-1}) (Figure 6), the former band was made use of in the determination of ΔH. To do this it is necessary to determine the molar extinction coefficient of the complex band by the following procedure. The concentration of the complex is given (in terms of the absorbance measurements referred to in the preceding paragraph) as [DA] = $((a_1 - a_2)/a_1)D$, where D is the initial concentration of cholesterol and the other terms have the same meaning as before. By use of this value of the concentration and the absorbance of the complex band its molar extinction coefficient (ϵ) can be calculated by the familiar Beer's law equation, absorbance = $\epsilon[DA]l$, where l is the optical path length. The extinction coefficient thus calculated can in turn be used to determine the concentration of the complex at different temperatures. A typical plot of log K *vs.* $1/T$ obtained for the cholesterol–tributyrin complex is shown in Figure 7. The spectral and thermodynamic data of the cholesterol–triglyceride complexes are summarized in Table II.

Discussion

The results show that hydrogen bonding could play an important role in interactions involving cholesterol. Although the enthalpy of dimerization of cholesterol is below the range found for OH····O systems (3–10 kcal $mole^{-1}$) (Singh and Rao, 1967), the effect of temperature on the intensity of the trimer (or higher aggregate) band indicates a higher enthalpy

TABLE II: Spectral and Thermodynamic Data of the Cholesterol–Triglyceride Complexes.[a]

Triglyceride	ν_{OH} assocd (cm^{-1})	$\Delta\nu_{OH}$ (cm^{-1})[b]	K (l. $mole^{-1}$)[c]	$-\Delta H$ (kcal $mole^{-1}$)
Triacetin	3540	80	2.9	4.3
Tributyrin	3545	75	2.4	3.5
Trilaurin	3545	75	3.7	5.4

[a] The solvent is CCl_4. [b] Shift from the free cholesterol peak at 3620 cm^{-1}. [c] At 23°.

of aggregation for this species, probably in the range quoted above. The enthalpy of association of cholesterol with triglycerides is considerably higher than that of dimerization and probably would be the more favored of the two types of interactions.

Since the interactions have been studied in a nonaqueous solvent it is pertinent to question the relevance of the results obtained here to biological systems. It will be obvious that solubility considerations dictated the choice of solvent, but the same consideration might result in the lipids existing in a more or less nonaqueous environment in the human system. It is known that in blood plasma most of the lipid is associated with protein in the familiar lipoprotein. While the exact nature of the latter is still not clearly known, there is evidence that "the hydrophilic portions of the molecule such as the protein and phospholipids are on the outside in contact with water, while the hydrophobic portions such as the triglycerides, cholesterol and cholesterol esters are in the interior, sheltered from contact with water molecules" (Hoffman, 1964). The interactions described in the present paper could therefore be of considerable importance. In a recent paper on the hydrogen-bonding interactions of adenine and uracil derivatives in a nonaqueous solvent ($CHCl_3$), Kyogoku *et al.* (1967) have proposed similar arguments to emphasize the relevance of their studies to biological systems. Franzen and Stephens (1963), following up the investigations on the instability of interamide hydrogen bonds in polar media (Klotz and Franzen, 1962), have proposed that in proteins such bonds are stable only in the well-shielded, nonpolar, hydrocarbon-rich regions (Klotz, 1960) of the protein molecules. The long hydrocarbon chain of cholesterol is likely to result in nonaqueous regions as suggested for proteins.

The clinical aspects of the hydrogen-bonding interactions reported here are, at present, a matter of speculation. Hydrogen bonding may contribute to the formation of atherosclerotic plaques. It would appear quite possible for plasma cholesterol to hydrogen bond to cholesterol previously deposited in arterial tissue in a locality that would be essentially nonaqueous at certain stages of plaque development. The suggestion by Samuels *et al.* (1965) that spontaneous aggregation of gangliosides, cholesterol, and phospholipids to form a molecular complex results in the Tay-Sachs membranous cytoplasmic bodies would be an interesting example in this connection.

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