The phase behavior of hydrated cholesterol

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Abstract The thermotropic phase behavior of cholesterol monohydrate in water was investigated by differential scanning calorimetry, polarizing light microscopy, and x-ray diffraction. In contrast to anhydrous cholesterol which undergoes a polymorphic crystalline transition at 39°C and a crystalline to liquid transition at 151°C, the closed system of cholesterol monohydrate and water exhibited three reversible endothermic transitions at 86, 123, and 157°C. At 86°C, cholesterol monohydrate loses its water of hydration, forming the high temperature polymorph of anhydrous cholesterol. At least 24 hours were required for re-hydration of cholesterol and the rate of hydration was dependent on the polymorphic crystalline form of anhydrous cholesterol. At 123°C, anhydrous crystalline cholesterol in the presence of excess water undergoes a sharp transition to a birefringent liquid crystalline phase of smectic texture. The x-ray diffraction pattern obtained from this phase contained two sharp low-angle reflections at 37.4 and 18.7 Å and a diffuse wide-angle reflection centered at 5.7 Å, indicating a layered smectic type of liquid crystalline structure with each layer being two cholesterol molecules thick. The liquid crystalline phase is stable over the temperature range of 123 to 157°C before melting to a liquid dispersed in water. The observation of a smectic liquid crystalline phase for hydrated cholesterol correlates with its high surface activity and helps to explain its ability to exist in high concentrations in biological membranes.—**Loomis, C. R., G. G. Shipley, and D. M. Small.** The phase behavior of hydrated cholesterol. *J. Lipid Res.* 1979. **20:** 525–535.

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Cholesterol is universally distributed in all animal tissues where it fulfills a structural and functional role in cell membranes, serum lipoproteins, and bile. Practically insoluble in water, cholesterol is readily solubilized in solutions containing amphiphilic compounds (1–6). Cholesterol concentrations up to 50 mole% with respect to phospholipid are observed in biological membranes and lipid dispersions prepared from naturally occurring phospholipids (3, 7–9). Sonicated systems of synthetic homogeneous chain lecithins have been shown to contain up to 3 moles of cholesterol per 1 mole of phospholipid (10), and liposomes prepared from certain organic solvents contain nearly 2 moles of cholesterol to 1 mole of phospholipid in a metastable state (11). The high solubility of cholesterol in phospholipid bilayers is attributed partly to its intrinsic hydrophobicity and partly to specific head group interactions between the sterol OH group and the polar moiety of the lipid (5, 12). When the cholesterol concentration exceeds the solubility limit in lipid bilayers or micelles, deposition of crystalline cholesterol monohydrate is observed (1, 3, 7). This process of saturation and deposition of crystalline cholesterol is known to occur in certain pathological states in man, most notably, gallstone disease (4) and atherosclerosis (13, 14).

The effect of cholesterol incorporation on the structural and thermodynamic properties of model lipid systems has been intensively studied in recent years; however, only minor efforts have been made toward understanding the phase behavior of pure cholesterol in water (15). We believe that the cholesterol–water binary system needs to be described before the more complex multicomponent systems can be rigorously defined. In this report we describe the phase behavior of cholesterol monohydrate in water as a function of temperature. The physical states of hydrated and anhydrous cholesterol are compared, with particular reference to the behavior of cholesterol in aqueous biological systems.

MATERIALS AND METHODS

Cholesterol undergoes autoxidation under a variety of conditions (16, 17). Since degradative products affect the thermotropic and lyotropic phase behavior of a compound, great care was taken to ensure the purity of the samples. Cholesterol (Nu Chek Prep, Elysian, MN) was recrystallized three times from an ether–

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Abbreviations: DSC, differential scanning calorimetry.

methanol solution. The final recrystallized material was analyzed for impurities by infrared spectroscopy and by thin-layer chromatography using separate solvent systems of toluene–ethyl acetate 60:40 (v/v) and hexane–diethyl ether–acetic acid 70:30:1 (v/v/v). The recrystallized material was divided into 500-mg quantities and sealed in vials by flame under nitrogen before storing in the dark at 4°C. Each vial was analyzed on opening for autoxidative products as described above. No impurities were observed for the final recrystallized material nor for samples stored up to 9 months.

Anhydrous crystalline cholesterol was prepared by dissolving pure cholesterol in hot acetic acid and allowing the solution to slowly cool. The crystals were isolated on filter paper and placed in a heated desiccator (80°C) under vacuum for 72 hr to remove all remaining acetic acid. The anhydrous crystals were stored in a desiccator over phosphorous pentoxide at 4°C in the dark. Cholesterol monohydrate was prepared by recrystallization from 95% ethanol after the method of Stauffer and Bischoff (18). Monohydrate crystals, isolated by filtration, were washed repeatedly with triply distilled water and stored in the dark at 4°C in water. Cholesterol as a dry microcrystalline powder or dispersed in water did not show autoxidative degradation when stored up to 3 months.

Anhydrous cholesterol crystals (~5 mg) with an equal weight of distilled water were hermetically sealed in small stainless steel pans under nitrogen and heated or cooled at a programmed rate of 5°C/min in a Perkin-Elmer DSC-II differential scanning calorimeter. Samples for polarizing light microscopy were examined by direct light and between crossed nicols in order to document the number of phases and their textures. To insure that cholesterol monohydrate remained hydrated above 100°C, samples were placed in preflattened sealed glass capillary tubes (19). The sample tubes were immersed in a silicon oil bath fitted to a Leitz heating/CO_2 cooling microscope stage which allowed control of both the heating and cooling rates of the sample. The temperature apparatus was calibrated at heating rates of 1 and 2°C/min using known standards. The accuracy in measuring a sharp first-order transition on heating was determined to be ±1.0°C.

X-ray diffraction profiles were recorded using focussing cameras with either toroidal mirror (20) or double mirror (21) optics utilizing Ni-filtered Cu$K\alpha$ radiation from an Elliot GX-6 rotating anode generator. Anhydrous cholesterol or cholesterol monohydrate in an excess of water was placed in 1-mm quartz capillary tubes and sealed by flame. The sample tubes were located in a specially constructed brass sample holder capable of operating with either camera. The temperature of the sample was regulated by an electrically variable temperature controller over the temperature range of 20–300°C with an accuracy of ±0.5°C as determined using standards with known transition temperatures. X-ray films were analyzed with a Joyce-Loebl model III CS microdensitometer.

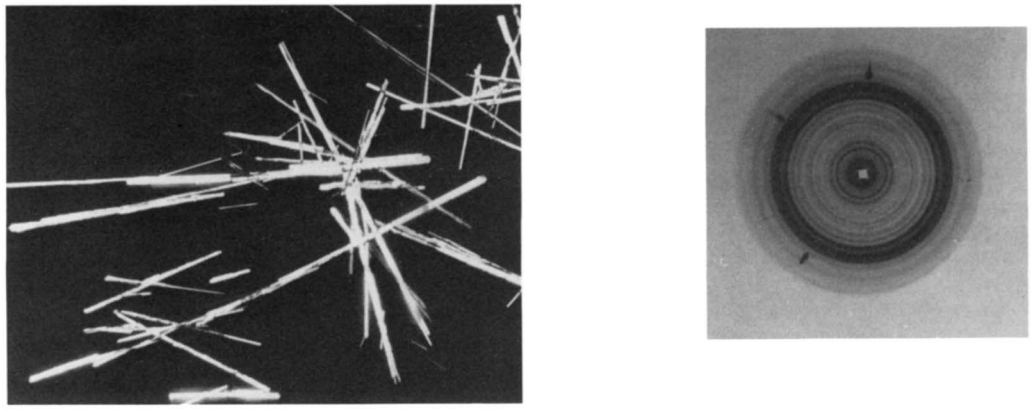
The rate of cholesterol hydration in different relative humidities was determined by measuring the weight change of a cholesterol sample suspended over different sulfuric acid solutions. A triturated 200-mg sample of anhydrous cholesterol was suspended from a Cahn microbalance inside a glass chamber. The microbalance was connected to a continuous recording device which allowed the weight change of the sample to be monitored throughout the entire experiment. The relative humidity inside the chamber was determined by the volume ratio of sulfuric acid to water and the atmospheric pressure. The apparatus was placed in a closed room and the room temperature was monitored by a constant recording temperature device. The temperature for all experiments was 22 ± 0.5°C.

RESULTS

Identification of cholesterol crystals

Gravimetric analysis, polarizing light microscopy, and x-ray diffraction were used to verify the crystal structures of anhydrous cholesterol and cholesterol monohydrate. Approximately 25 mg of wet cholesterol monohydrate crystals were placed on a microbalance pan and periodically weighed. When constant weight of the sample was observed over a period of 30 min, it was assumed that all excess water had evaporated from the crystal surfaces. The crystals were immediately heated until melted, allowed to cool, and reweighed for the weight of cholesterol alone. From the weight of cholesterol, the mole ratio of water to cholesterol was calculated for several samples. The mean of 0.98 ± 0.17 (SD) mol of H_2O/mol of cholesterol indicated that the samples were the monohydrate form of cholesterol. Anhydrous cholesterol crystals treated in a similar manner showed no change in weight after heating.

Identification of anhydrous cholesterol and cholesterol monohydrate was carried out using polarizing light microscopy and x-ray diffraction as shown in **Fig. 1.** At 20°C anhydrous cholesterol appears as needles and cholesterol monohydrate as plates when viewed under a polarizing light microscope. The angles formed by the edges of the monohydrate plates measured on a large number of crystals were 79.15



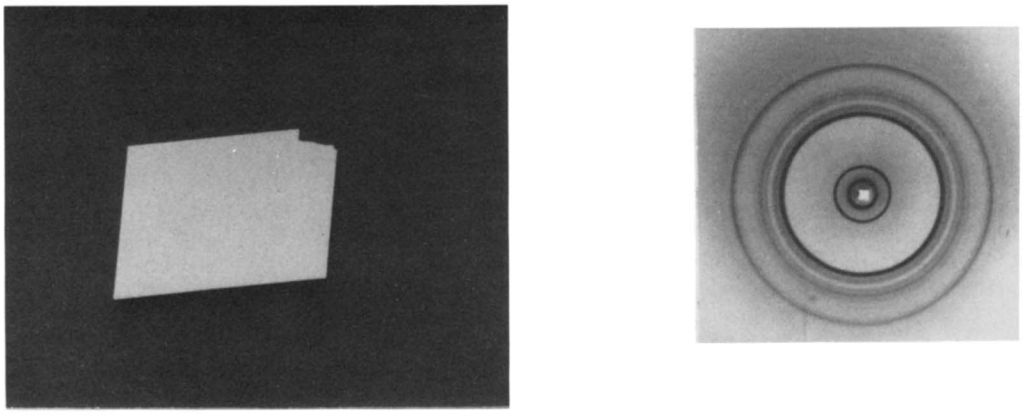


Fig. 1. Photomicrographs and x-ray diffraction patterns obtained from crystalline anhydrous cholesterol and cholesterol monohydrate. Photomicrographs were taken with the samples between crossed nicols. Magnification 70×.

and 100.85°. The larger angle agreed well with the angle γ (100.8°) of the unit cell of cholesterol monohydrate (22). The x-ray powder diffraction patterns obtained from the triturated samples at 20°C are shown on the right side of Fig. 1. The reflections in each powder pattern could be indexed according to the unit cell data in the crystal structure of anhydrous cholesterol (23) and cholesterol monohydrate (22).

Thermotropic phase behavior of the two crystalline forms of cholesterol

Fig. 2 shows a typical heating scan of anhydrous cholesterol on first heating from 0 to 170°C. A small, relatively broad endotherm was observed centered at 38.9 ± 1.4°C and having a calculated heat change of 0.91 ± 0.5 Kcal/mol cholesterol. No further changes were observed on heating until 150.7 ± 0.8°C when a large, sharp endotherm occurred with a calculated heat change of 6.59 ± 0.25 Kcal/mol. Both transitions were reversible on cooling, with the large transition at approximately 115°C and the small transition undercooling to approximately 20°C. On the second heating of the sample, the small transition sharpened and moved down to 36°C. The larger transition remained at 150°C. At the bottom of Fig. 2, x-ray diffraction patterns show that there are structural changes associated with the two transitions of cholesterol. The x-ray powder diffraction pattern obtained at 20°C is typical of anhydrous cholesterol as shown previously in Fig. 1. On heating the sample to 45°C, the dif-

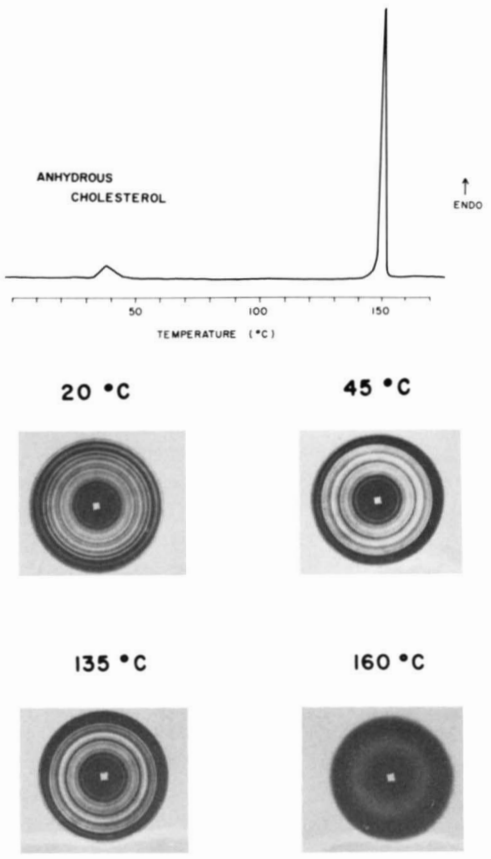


Fig. 2. Differential scanning calorimetry and x-ray diffraction results obtained from an anhydrous cholesterol sample as a function of temperature.

fraction pattern has changed from that observed at 20°C in accordance with the small transition observed by DSC at 39°C. Heating the sample to 135°C did not change the powder diffraction pattern from that observed at 45°C. At 160°C, above the large 150°C DSC endotherm, the diffraction pattern dramatically changed. Two diffuse scattering maxima centered at equivalent Bragg spacings of 6 and 21 Å indicated that the sample had melted to a liquid.

X-ray diffraction patterns obtained above and below the 39°C transition and the microdensitometer traces from these patterns are shown in **Fig. 3.** The traces clearly show that several crystal reflections have changed intensity during the transition (dashed lines indicate reflections that have changed intensity). Except for the appearance of one new reflection at a Bragg spacing of 5.4 Å (arrow), no change in the diffraction spacing was observed. These results are consistent with the existence of a crystalline polymorphic transition of cholesterol first described by Spier and van Senden (24) and later confirmed by van Putte et al. (25), and suggest that the crystal structures of the two polymorphic forms are similar.

In contrast to anhydrous cholesterol, cholesterol monohydrate in excess water produced a different DSC profile on first heating the sample from 10°C as shown in **Fig. 4.** Three endotherms were observed at 86.4 ± 0.5, 123.4 ± 0.8, and 156.8 ± 0.5°C. The cal-

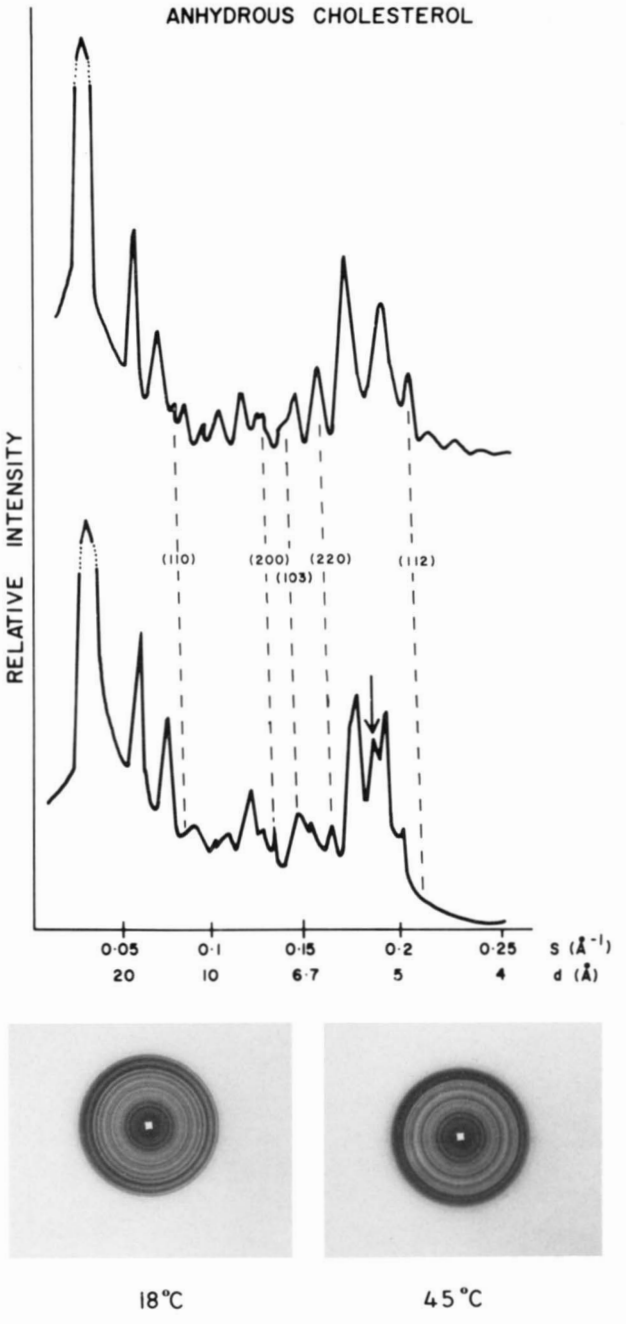


Fig. 3. X-ray diffraction patterns of anhydrous cholesterol above and below the 39°C polymorphic transition. The microdensitometer traces of the two x-ray diffraction patterns are shown at the top of the figure. The dashed lines indicate the reflections that undergo intensity changes during the transition. These reflections have been indexed according to the unit cell parameters of anhydrous cholesterol (23). The arrow designates a new reflection not seen in the low temperature polymorph.

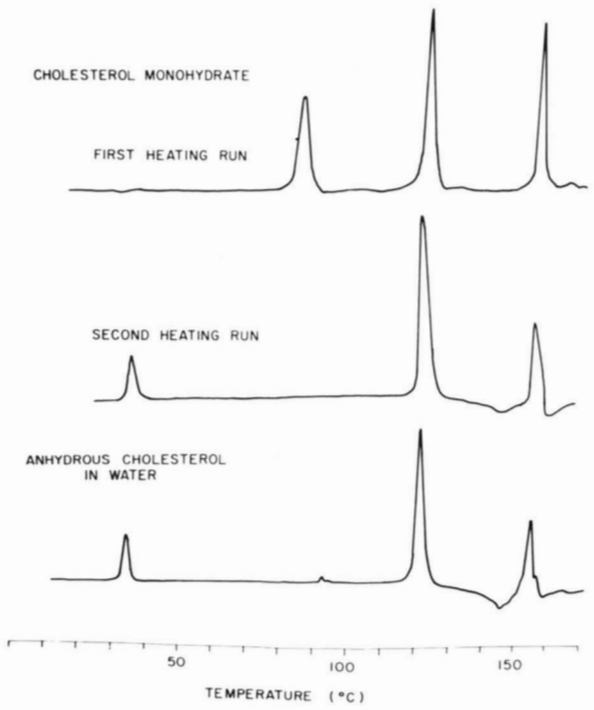


Fig. 4. Comparison of differential scanning calorimetry heating scans of cholesterol monohydrate and crystalline anhydrous cholesterol in water.

culated heat change for each transition was 2.35 ± 0.22, 3.42 ± 0.20, and 2.29 ± 0.14 Kcal/mol cholesterol, respectively. If the sample was cooled at 5°C/min., three exotherms were observed at 149, 85, and 10°C. The latter two transitions showed marked undercooling. The calculated heat change of the 149 and 85°C exotherms compared well with those of the 156 and 123°C transitions observed on heating. The 10°C exotherm gave an average heat change of only 0.8 Kcal/mol cholesterol, well below that calculated for the 86°C endotherm of the heating run. The second heating run, shown in Fig. 4, no longer exhibited an 86°C transition but instead showed a small endotherm at 36.4 ± 0.5°C. The two high-temperature endotherms remained unchanged. The calculated heat change for the 36°C transition was 0.81 ± 0.21 Kcal/mol cholesterol, which is similar in temperature and enthalpy to the low-temperature polymorphic transition of anhydrous cholesterol described in Fig. 2. To verify that the cholesterol monohydrate sample exhibited a polymorphic crystalline transition on the second heating run, anhydrous cholesterol crystals were placed in a DSC pan, an equal weight of water was added, and the pan was immediately sealed and heated from 10°C at 5°C/min in the differential

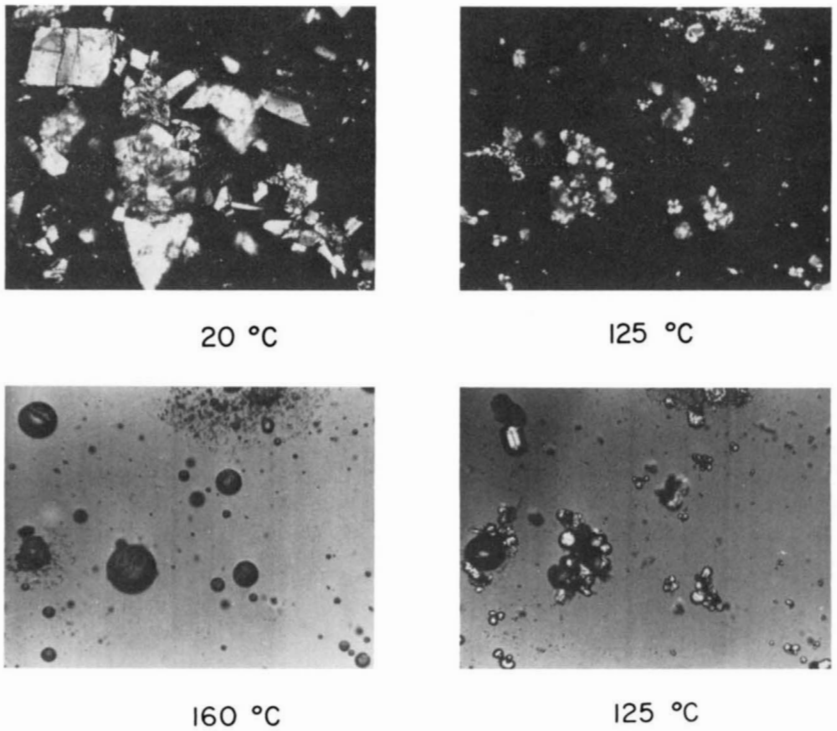


Fig. 5. Photomicrographs illustrating the changes in cholesterol monohydrate in water as a function of temperature. All pictures are of same field, magnification 100×. Top left, cholesterol monohydrate crystals, crossed nicols; bottom left, melted cholesterol droplets, direct light; top right, cholesterol smectic liquid crystals, crossed nicols; bottom right, same field, crossed nicols with full wave compensators inserted. Sign of birefringence +.

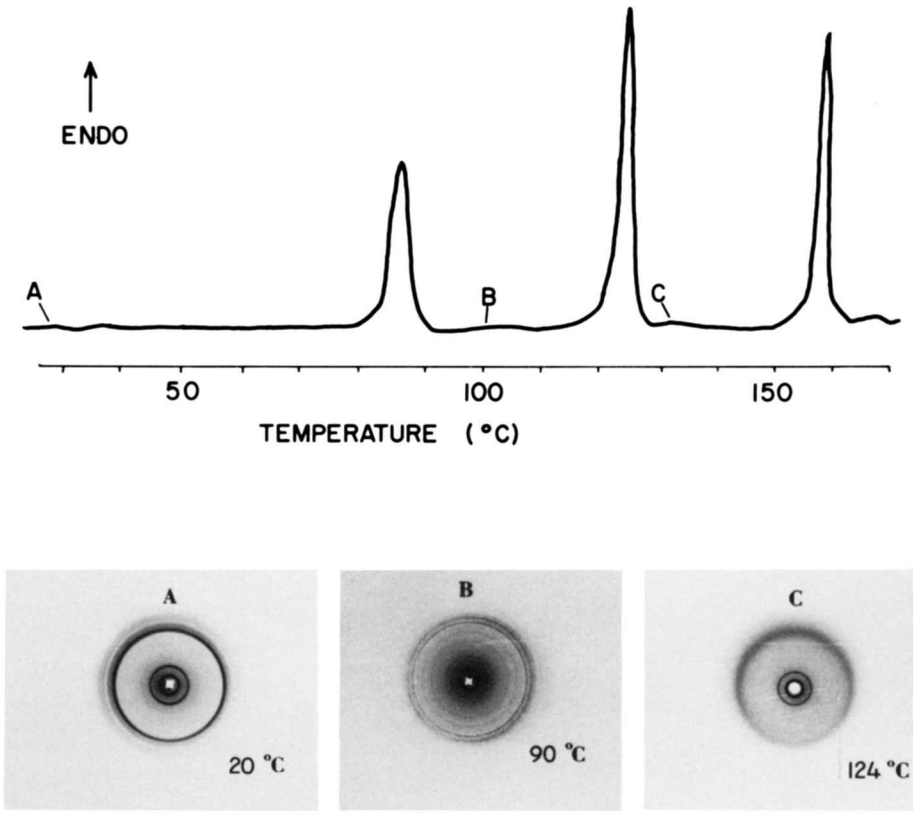


Fig. 6. Differential scanning calorimetry and x-ray diffraction results obtained from a cholesterol monohydrate sample in water as a function of temperature.

scanning calorimeter. At the bottom of Fig. 4 is shown the resulting heating scan which remained unchanged on consecutive heating runs. The scan was identical to the second heating scan of cholesterol monohydrate. This identity indicated that after cholesterol monohydrate was heated to high temperatures and cooled in water, the cholesterol crystallized to the anhydrous form.

Cholesterol monohydrate samples sealed in capillary tubes with water were studied by polarizing light microscopy and x-ray diffraction in an attempt to identify the phases undergoing the transitions observed by DSC. The types of phases observed by polarizing light microscopy during heating are shown in the photomicrographs of **Fig. 5.** At 20°C, cholesterol monohydrate displays birefringent plate crystals. No consistent changes could be discerned on heating the sample from 20° to 123°C. At 124°, there was a sudden change from crystalline cholesterol to a birefringent phase exhibiting flow characteristics. The two photomicrographs on the right of Fig. 5 show the same field with crossed nicols (top) and with crossed nicols plus the full wave compensator inserted (bottom). Notice the myelin figure (upper right at 125°C) and the large number of focal conics dispersed in a continuous water phase. The focal conics gave a positive sign of birefringence, indicating a smectic type of liquid crystalline phase. This is the first observation that pure cholesterol will form a liquid crystalline phase in water. On further heating, the liquid crystalline phase melted at 157°C to an isotropic oil dispersed in water (Fig. 5, bottom, left).

Fig. 6 shows the results from the x-ray diffraction experiment. The powder diffraction pattern of cholesterol monohydrate, identical with that shown in Fig. 1, was observed at 20°C. Between 20 and 85°C, the diffraction pattern remained unchanged, but at 90°C a different powder pattern was recorded. The new diffraction pattern was identical to one obtained from the anhydrous cholesterol sample at 90°C. Thus the transition observed by DSC at 86°C represents the transformation of cholesterol monohydrate to the high-temperature anhydrous cholesterol polymorph with the loss of the water of hydration. At 124°C, a new diffraction pattern was obtained which contained two sharp low-angle diffraction maxima at the equivalent Bragg spacings of 37.4 and 18.7 Å and a diffuse wide angle maximum at 5.7 Å. Although only two low-angle diffraction maxima were observed, their ratio of 1:½ was compatible with a smectic liquid crystalline

phase. The diffuse wide-angle maximum indicated a semi-melted or fluid state in the short-range packing of the molecules.

The effect of temperature on the liquid crystal structure was studied by obtaining x-ray diffraction patterns at 2°C intervals between 124 and 156°C. The experimental results are shown in **Fig. 7.** Over the total temperature range of the liquid crystal, only two sharp low-angle diffraction maxima could be obtained. This graph shows that the three liquid crystal reflections remain invariant with temperature. When the sample was heated above 156°C, two diffuse maxima were observed at approximately 25 and 5.9 Å, indicating that the cholesterol liquid crystal had melted to a liquid with no long-range order.

Hydration rate of cholesterol

Anhydrous cholesterol suspended in water will form cholesterol monohydrate with time (26). Since anhydrous cholesterol undergoes a polymorphic transition near physiologic temperature (36–39°C), the dependence of the hydration rate on the crystal form of cholesterol was investigated. These studies were carried out in bulk water at 20 and 45°C and in different relative humidities at 22°C.

For the bulk water experiments at 20 and 45°C, the degree of hydration in a sample was measured by differential scanning calorimetry using the areas of the 36 and 86°C endotherms of anhydrous cholesterol and cholesterol monohydrate, respectively. The ratio

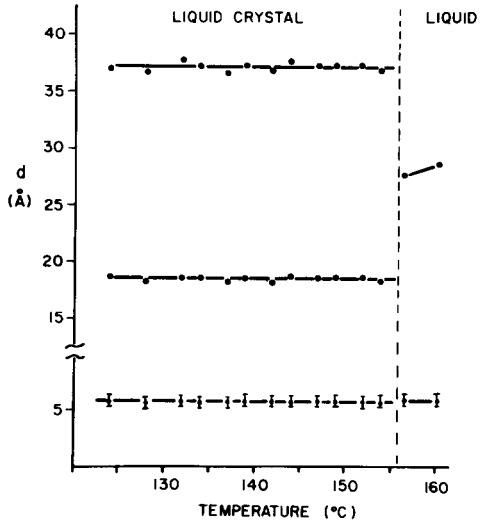


Fig. 7. Variation in d spacing as a function of temperature for the liquid crystalline phase of cholesterol and water. The symbol ⧳ designates the width of the diffuse maxima at wide angle, where the solid circle represents the d spacing at maximum intensity determined from the microdensitometer analysis. The figure shows that the diffuse maxima are generally symmetric over the total temperature range.

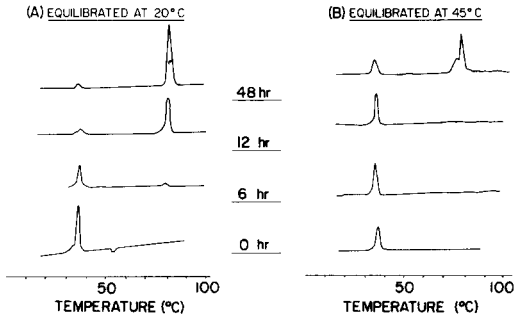


Fig. 8. Differential scanning calorimetry curves for anhydrous cholesterol equilibrated in water at different temperatures for different periods of time. *A*, 20°C; *B*, 45°C. Samples were quickly cooled to 10°C and then immediately heated to 100°C at 5°C/min.

of the areas under the peaks, standardized to the mean area per mole of cholesterol, gave the ratio of anhydrous cholesterol to cholesterol monohydrate in the sample. A series of DSC pans were prepared, each containing 7 mg of anhydrous cholesterol. Since the crystal surface area will greatly affect the hydration rate, the cholesterol in each pan was heated above the melt and cooled to form a thin crystalline film on the bottom of the pans of approximately equal surface area from sample to sample. Half of the pans were placed in a 20°C constant temperature room and half were placed in an oven at 45°C. At time = 0, each pan received 7 μl of triply distilled water and was sealed under nitrogen. Equilibration was carried out at the appropriate temperature and each pan was examined by DSC after a specified period of time ranging up to 48 hr.

Several of the DSC heating scans from samples equilibrated at 20 and 45°C are shown in **Fig. 8.** On the left, the samples equilibrated at 20°C have formed the monohydrate in the first 24 hr. In contrast, the samples equilibrated at 45°C showed no evidence of monohydrate formation until after 36 hours. The rate of cholesterol monohydrate formation may be determined from a plot of percent cholesterol monohydrate vs. time. This is shown in **Fig. 9,** where the rate of formation is given by the slope of the line. Assuming all the samples equilibrated at 20°C have reached maximum hydration in 24 hr, the data points were fitted to a straight line by least-squares linear regression analysis ($m = 4.17, b = -6.5$). A regression coefficient of $r = \pm 0.927$ indicates the assumption is valid. The rate, determined from the slope and standardized to 1 g of cholesterol, was found to be 1.94×10^{-3} g H_2O/g cholesterol per hr. If the sample of anhydrous cholesterol was introduced to water as a powder, the rate was increased.

In contrast, samples equilibrated at 45°C did not readily convert to cholesterol monohydrate in 48 hr.

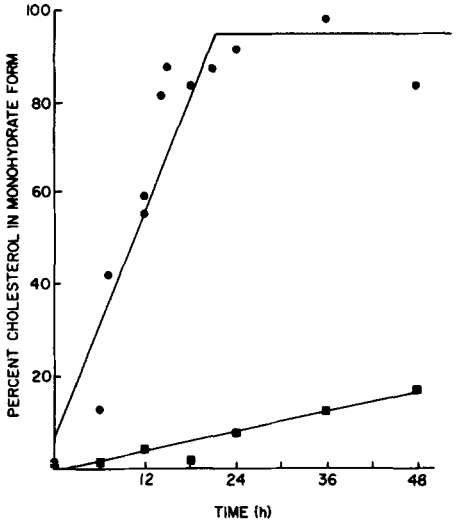


Fig. 9. The rate of hydration of cholesterol in bulk water. (●) Anhydrous cholesterol and water equilibrated at 20°C; (■) anhydrous cholesterol and water equilibrated at 45°C.

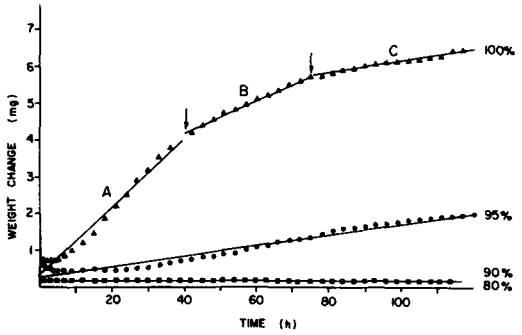


Fig. 10. Absorption isotherms of anhydrous cholesterol equilibrated in different relative humidities at 22°C. (■), Pan with no sample, samples equilibrated in 80 and 90% relative humidity; (●) 95% relative humidity experiment; (▲) 100% relative humidity experiment.

Least-squares linear regression analysis gave a line ($m = 0.3341, b = -0.359$) with a regression coefficient of 0.94. The rate of monohydrate formation at 45°C was calculated as 1.56×10^{-4} g H_2O/g cholesterol per hr. Thus, there is a large difference in the rate of cholesterol monohydrate formation between the two polymorphic forms of anhydrous cholesterol.

A second set of experiments was designed to measure the rate of monohydrate formation at different relative humidities. These studies were carried out on the low-temperature cholesterol polymorph at 22°C using the microbalance apparatus described in the Methods section. To reduce to a minimum the differences in surface area between samples, all samples were ground to a fine powder immediately before beginning the experiment. The increase in sample weight at different relative humidities is shown in **Fig. 10.** The bottom experimental line (squares) represents the weight gained by the sample pan without sample at 100% relative humidity (blank) and the weight increase for samples equilibrated at 80 and 90% relative humidity. All three preparations fall on approximately the same line. Samples equilibrated in relative humidities up to 90% therefore do not absorb water. Samples equilibrated at 95 (circles) and 100% relative humidity (triangle) showed significant increases in weight with time. At 95% relative humidity, the samples were assumed to hydrate linearly and all data points were subjected to a least-squares linear regression analysis ($r = 0.97$). The slope of this line gave a hydration rate of 7.27×10^{-5} g H_2O/g cholesterol per hr.

The sample equilibrated at 100% showed an asymptotic curve and was analyzed differently. The curve was taken to represent at least two different hydration rates and therefore was broken arbitrarily into three zones of 0 to 40, 40 to 75, and 75 to 120 hr. It was assumed that the data points within each zone could be approximated by a straight line and a least-squares linear regression analysis was carried out. This was a valid assumption based on the high regression coefficients obtained ($r > 0.99$). The rates calculated from the slopes of the lines are presented in **Table 1.** It is apparent that cholesterol initially hydrates more rapidly at 100% than at 95% relative humidity (zone A). However, the final hydration rate is nearly the

TABLE 1. Hydration of cholesterol at different relative humidities

Relative Humidity		mg Weight Change over 40 hr	80 hr	120 hr	Slope	Regression Coefficient	Rate[a]
Blank		0.15	0.25	0.15			
80%		0.19	0.19	0.19			
90%		0.15	0.15	0.15			
95%		0.65	1.52	1.95	0.0145	0.979	7.27×10^{-5}
	Zone A	3.95			0.0925	0.992	4.62×10^{-4}
100%	Zone B		5.80		0.0446	0.998	2.23×10^{-4}
	Zone C			6.44	0.0154	0.991	7.68×10^{-5}

[a] Rate expressed as g H_2O/g cholesterol per hr.
Each sample contained 200 ± 2 mg of cholesterol.

same as the rate calculated from 95% relative humidity. Based on this final hydration rate, it would take about 28 days to transform one gram of anhydrous cholesterol to cholesterol monohydrate in a 100% relative humidity atmosphere.

DISCUSSION

The thermotropic phase behavior of cholesterol monohydrate in water is distinctly different from anhydrous cholesterol. Anhydrous cholesterol undergoes two enantiotropic transitions: a polymorphic crystalline transition at 39°C and a crystalline to liquid transition at 151°C. In contrast, cholesterol monohydrate undergoes three transitions at 86, 123, and 157°C. The 86°C transition, as determined by x-ray diffraction, represents the conversion of cholesterol monohydrate to anhydrous cholesterol.[3] This transition is not readily reversible and only the polymorphic crystalline transition is observed at 36°C on reheating the sample to 100°C. However, experiments designed to measure the rate of cholesterol hydration indicate cholesterol monohydrate will reform at room temperature (22°C) over a period of 24 hr. Thus, the true equilibrium state of cholesterol in water below 86°C is cholesterol monohydrate.

The experimental conditions required to study the phase behavior of cholesterol monohydrate above 100°C, e.g., a closed system at high temperature, invariably caused the system to experience increasingly high pressures with increasing temperature. The calculated pressure increase of 1 atm at 100°C would not be expected to significantly affect the results. However, the pressure increases rapidly above 100°C, reaching ~8 atm at 160°C, and would be expected to affect the transition temperatures and measured thermodynamic quantities. The influence of pressure on these quantities cannot be determined without knowing the change in molar volume associated with each transition. Therefore, the values given for the hydrated system above 100°C must be considered approximate.

The high temperature behavior of hydrated cholesterol was also different from the anhydrous state. A sharp transition at 123°C was shown by polarizing light microscopy and x-ray diffraction to represent a crystalline to liquid crystalline transition for cholesterol. This is the first observation that cholesterol is capable of forming a liquid crystalline phase by itself in water. Previously Mlodziejowski (27) had shown that mixtures of cholesterol and glycerol yield a mesomorphic transition at high temperature. The two low-angle x-ray diffraction maxima obtained from the mesophase indicate the structure is made up of a repeating lamellar unit of length 37.4 Å. The 37.4 Å spacing is similar to that of the crystallographic long axis of both cholesterol and cholesterol monohydrate and suggests that the bilayer organization of the cholesterol molecules is maintained in the smectic–liquid crystal phase. The sharpness of the two low-angle maxima suggests that the structure is periodic over quite large dimensions. The low-angle maxima plus the diffuse wide-angle maximum at 5.7 Å indicates the structure contains only one-dimensional order. Thus, the phase is probably a smectic–liquid crystalline phase. A series of x-ray diffraction patterns obtained between 124 and 156°C shows no variation in the structural parameters up to the liquid crystalline to liquid transition at 157°C, indicating the structure shows no expansion or contraction in the direction corresponding to the layers.

The finding that the x-ray diffraction crystalline pattern from cholesterol in water at 90°C was identical to the crystalline pattern obtained from anhydrous cholesterol at the same temperature allows us to speculate in a general way about the behavior of cholesterol in water. Since the formation of cholesterol monohydrate from anhydrous cholesterol occurs spontaneously in the presence of water, the amount of energy absorbed by the monohydrate over the temperature range of 20–90°C minus the amount of energy absorbed by anhydrous cholesterol over the same range is an approximation of the energy of formation of cholesterol monohydrate from cholesterol and water. We may neglect the capacity of heat required to raise the temperature of both systems to 90°C because the differences are very small in relation to the latent heats and because no large changes were observed in the heat capacities over the temperature range of interest. The latent heat of the 86°C monohydrate transition of 2.35 Kcal/mol cholesterol minus the latent heat of the polymorphic transition of 0.91 Kcal/mol cholesterol gives us a heat of formation of 1.44 Kcal/mol cholesterol. The mole ratio of water to cholesterol in the monohydrate is 1 and therefore the energy contribution of water to the hydrated system is ~1.44 Kcal/mol water. This value is almost identical to the heat of fusion for water of 1.43 Kcal/mol.

Comparison of the cholesterol monohydrate and anhydrous cholesterol crystal structures suggests that the latent heat associated with the 86°C transition is not due to a large disruption of the monohydrate crystal structure but results from the release of the

[3] Earlier work had suggested that cholesterol monohydrate reverted to the anhydrous form at about 90°C (15).

water of hydration into the surrounding water. The water molecules in the crystal lattice of cholesterol monohydrate form three hydrogen bonds per oxygen atom (22). In addition, every other cholesterol hydroxyl/group is hydrogen bonded to its neighbor. Therefore, three hydrogen bonds must be broken to release one water molecule. In anhydrous cholesterol at 22°C and presumably in its higher melting polymorph, every hydroxyl group contains two hydrogen bonds (23). Thus, when cholesterol monohydrate loses its water of hydration, one new hydrogen bond must form for every cholesterol hydroxyl group. The net energy absorbed during the release of water from the cholesterol monohydrate structure is equivalent to the breaking of two hydrogen bonds or approximately the absorption of 9 Kcal/mole of cholesterol.

In ice, a water molecule tends to hydrogen bond with four neighboring water molecules in a tetrahedral arrangement (28). When ice melts, the energy required to break all the hydrogen bonds would be 9 Kcal/mol of water (one hydrogen bond is formed between two water molecules; therefore, two moles of hydrogen bonds are broken per mole of water). However the heat of fusion of water is much smaller (1.43 Kcal/mol) and indicates that only 16% of the hydrogen bonds are actually broken in the ice–liquid transition. The water released from the monohydrate structure will presumably form the same number of hydrogen bonds as the surrounding water. Thus, approximately 16% fewer hydrogen bonds are formed per mole of released water than are broken in the transition to anhydrous cholesterol. In other words, a small amount of energy, equivalent to the heat of fusion of water, must be added to the system to bring about the transition to anhydrous cholesterol.

A similar approach may be applied to the behavior of hydrated cholesterol at high temperatures (>100°C). At 90°C, cholesterol is in the same crystalline state regardless of the presence of water and the final state of cholesterol at high temperatures is a liquid for both systems. If we assume that the change in enthalpy is not influenced by the path taken to get from initial to final state and that variables which specify the state of the system, such as temperature and pressure, have a small effect on the enthalpy, then the total enthalpy change should be the same for the anhydrous and hydrated systems. A total enthalpy change of 6.59 Kcal/mol cholesterol was obtained from the anhydrous system and 5.71 Kcal/mol for the hydrated system. The difference between the two values of 0.88 Kcal/mol cholesterol was highly significant. A possible explanation for this difference may be that the liquid phases in the dry and hydrated systems are not exactly the same. X-ray diffraction patterns obtained on the liquids at 160°C show differences in the low-angle maxima of 25 and 21 Å for hydrated cholesterol and anhydrous cholesterol, respectively, which could indicate that water of hydration is present in the melted hydrated system.

Two separate experiments were designed to measure the hydration rate of crystalline anhydrous cholesterol. The first experiment determined the rate of hydration in bulk water above and below the 39°C polymorphic transition. The results conclusively showed that the low-temperature polymorph hydrated faster than the polymorph present at 45°C, the difference in the hydration rates being 1.9×10^{-3} g H_2O/g cholesterol per hr and 1.56×10^{-4} g H_2O/g cholesterol per hr for 20 and 45°C, respectively.

The second experiment was designed to measure the rate of hydration of anhydrous cholesterol at 22°C in different relative humidities. Relative humidities less than 95% failed to hydrate cholesterol. Stauffer and Bischoff (18) reported that cholesterol monohydrate remained stable at 80% relative humidity and at 38°C. It may be that after formation of the monohydrate, it will remain stable at lower relative humidities. At 95% relative humidity, the hydration rate of cholesterol held a constant value of 7.3×10^{-5} g H_2O/g cholesterol per hr over a period of 200 hr. Crystalline cholesterol at 100% relative humidity showed a sharp increase in the amount of hydration for the first 40 hr before the rate slowed and reached a constant value of 7.7×10^{-5} g H_2O/g cholesterol per hr. This value is very close to the rate obtained at 95% relative humidity. However, the maximum rate in 100% relative humidity of approximately 4.6×10^{-4} g H_2O/g cholesterol per hr is an order of magnitude below the rate obtained in bulk water at 20°C by DSC. Although the discrepancy cannot be adequately explained at this time, the hydration experiments have shown that formation of cholesterol monohydrate in water is a slow process and is dependent on the polymorphic crystalline form of anhydrous cholesterol.

In biological systems, cholesterol is usually found in cellular membranes. Such membranes have a general structure of a bilamellar leaflet of phospholipid into which proteins and other molecules are imbedded (29). Cholesterol is readily incorporated into pure phospholipid lamellar liquid crystalline phase (2, 3). In fact, cholesterol can form lamellar liquid phases with a large number of aliphatic amphiphiles (1, 30). Therefore, it would appear that high temperatures *or* the presence of fatty acyl chains is required to disrupt the steroid ring packing. In systems containing only cholesterol and water the interaction between the sterol hydroxyl group and water is apparently not strong enough to overcome the packing forces at low temperatures. The maximum amount of cholesterol that can be incorporated into membranes might also

depend on the sterol hydroxyl–phospholipid head group interactions (5, 12). Cholesterol in excess of a 1:1 mole ratio with phospholipid would experience both side-to-side packing and competition for phospholipid head groups with other cholesterol molecules. The excess cholesterol would eventually precipitate as crystalline cholesterol monohydrate (3), a process which also occurs in atherosclerosis (13, 14).

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