Correlation between crystal habit and the composition of solvated and nonsolvated cholesterol crystals

N. Garti, L. Karpuj, and S. Sarig

Casali Institute of Applied Chemistry, School of Applied Science and Technology, Hebrew University of Jerusalem, Jerusalem, 91904 Israel

Abstract The correlation between the crystal habit and the composition of cholesterol crystals formed in four organic solvents (methanol, acetonitrile, ethanol, and acetone) was studied. Anhydrous and monohydrate cholesterol were precipitated in anhydrous and aqueous organic solvent mixtures, respectively. The main conclusions derived from the study were that *1*) the appearance of plates does not automatically guarantee the presence of hydrated cholesterol, and *2*) the presence of 5% or more of water in the crystallization solvent may not result in the formation of monohydrate cholesterol.—**Garti, N., L. Karpuj, and S. Sarig.** Correlation between crystal habit and the composition of solvated and nonsolvated cholesterol crystals. *J. Lipid Res.* 1981. **22:** 785–791.

Supplementary key words cholesterol hydrates · crystal structure · differential scanning calorimetry · differential thermal analysis · x-ray diffraction · atherosclerosis

The process of saturation and deposition of crystalline cholesterol is known to occur in certain pathological states in man causing gallstone disease (1) and atherosclerosis (2, 3). Several investigators have studied the effect of cholesterol deposition on the structural and thermodynamic properties of model lipid systems (4–6). In numerous reports it has been shown that cholesterol monohydrate is the crystal modification deposited from biological membranes and lipid dispersions prepared from naturally occurring phospholipids (5, 7, 8).

It has been also shown that hydrated cholesterol can be obtained by a recrystallization procedure from 95% ethanol or acetone–water solutions (7, 9, 10). Cholesterol monohydrate was well-defined by gravimetric analysis, polarizing light microscopy and x-ray diffraction (9, 11). Since most procedures describing identification of cholesterol crystals (both anhydrous and monohydrate) were carried out in the same way, one can get the impression that anhydrous cholesterol appears always as needles and cholesterol monohydrate as plates when viewed under a light microscope or even by the naked eye.

The identification of hydrated cholesterol seems to be rather tedious because of spontaneous loss of water from the crystal at room temperature (9). Therefore the easiest way of identifying the harmful hydrated modification is frequently done by checking the crystal habit of the deposit cholesterol (10).

In the course of our studies of the effects of solvents on crystal habit, composition, and structure of precipitated cholesterol it was noticed that the relation between the observed habit and crystal structure is neither obvious nor self-evident.

We wish to report several experiments from which the relation between crystal habit and structure of cholesterol, formed in various organic solvents and in water, has been obtained. Four organic solvents were chosen: methanol, ethanol, acetonitrile and acetone, since they are the solvents from which cholesterol monohydrate has been obtained and studied.

EXPERIMENTAL

Cholesterol was purchased from Aldrich (99% pure by GLC) and was further purified by repeated crystallization from methanol. The final recrystallized material was analyzed by GLC and elemental analysis and stored in the dark and under a dry nitrogen atmosphere.

Two types of solvent were used: extra pure dry solvent (>99.8%) and spectroscopic grade with up to 5% water (obtained from Baker and Mallinckrodt). The amount of water in each solvent was checked by the

Abbreviations: DSC, differential scanning calorimetry; DTA, differential thermal analysis; TG, thermogravimetric analysis; DTG, differential thermogravimetric analysis; GLC, gas–liquid chromatography.

Karl-Fisher technique and by GLC (using a Porapak column).

Cholesterol solutions were prepared in 100-ml Erlenmeyer flasks fitted with elongated necks. Solubility curves were determined using standard procedures (12). A special crystallization cell was built and placed in a water bath equipped with a controlled cooling rate unit. The crystallization cell was heated above the equilibrium temperature and cooled at 4°C per hr. Crystals appeared slowly and were allowed to deposit for approximately 2 hr. Two sets of experiments were carried out. In one series the cholesterol was crystallized from >99.8% absolute solvent and kept in the solvent solution. The crystals were removed and analyzed by DSC and DTA. In the second series of experiments cholesterol was crystallized from 95% organic solvent in aqueous solution. The product was filtered and divided into two parts. One portion was kept under the crystallizing solution and then removed prior to each analysis (DSC and DTA) while the other portion was equilibrated with distilled water for several hours (up to 48 hr). The crystals from this last experimental series were analyzed by DTA only.

All the DTA analyses were performed in the dry form, i.e., the samples were kept under nitrogen flow in the analyzer crucible, at 25°C or 35°C, until constant weight was obtained.

In an additional set of experiments, anhydrous cholesterol was precipitated according to the procedure of Loomis, Shipley, and Small (11), filtered, dried and equilibrated with triple-distilled water for 48 hr, and analyzed by DTA.

The differential thermal analysis (DTA) of each crystal batch was carried out on a Mettler Thermoanalyzer under a controlled dry nitrogen flow at a rate of 5 l per hour. The weights of the samples were of the order of 100–150 mg and the heating rate was $4°C \cdot min^{-1}$. The DTA sensitivity was 50 μV for the recorder span.

The DSC experiments were carried out in a Perkin Elmer DSC in small stainless steel pans under nitrogen (in the closed system) and heated at a programmed rate of $5°C \cdot min^{-1}$.

Scanning electron microscopy (Cambridge 10/4) and x-ray diffraction (Phillips, utilizing Ni-filtered CuKα radiation) were used to examine each crystal batch.

RESULTS

Fig. 1 presents microphotographs of cholesterol crystals obtained several hours before the examination from absolute (>99.8%) and aqueous methanol

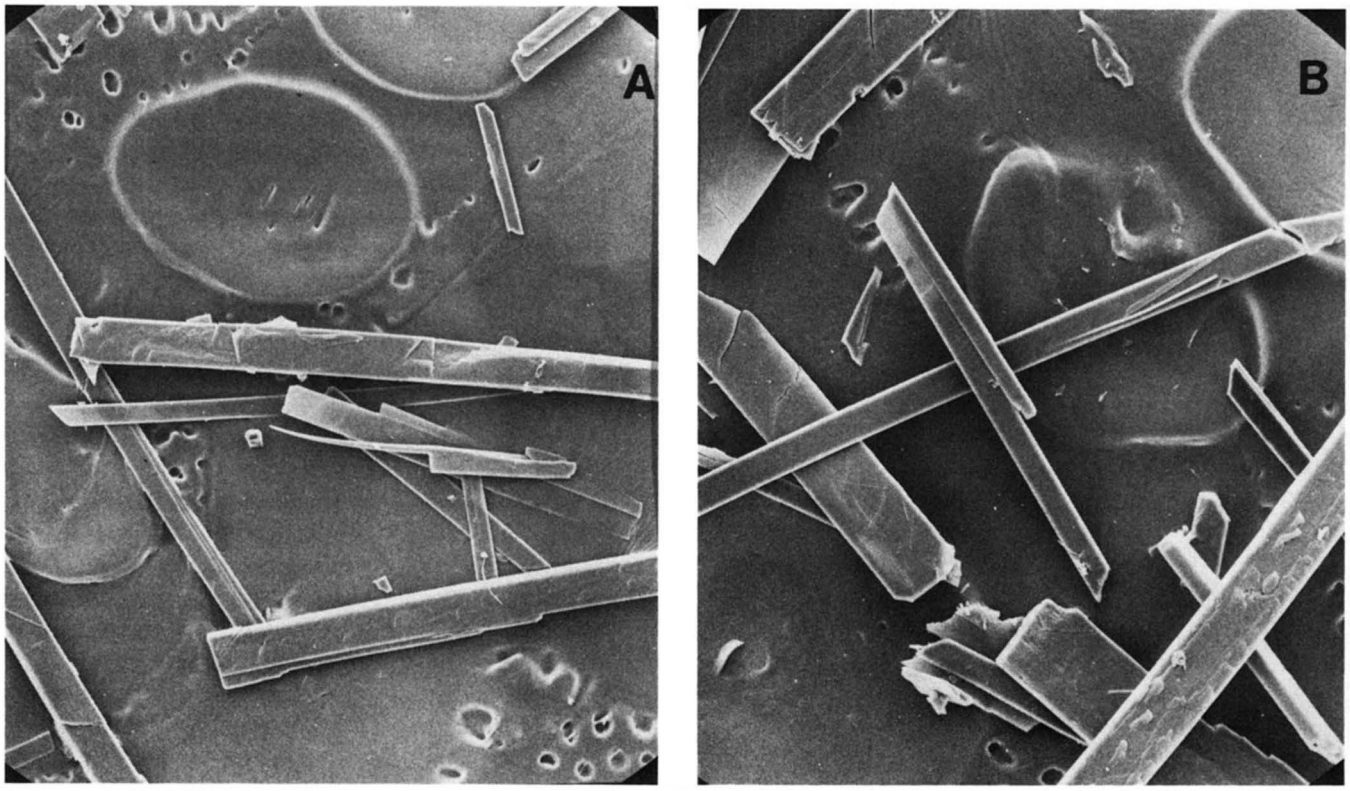

Fig. 1. Crystal habit of cholesterol obtained from A) dry methanol (×60), and B) methanol–water 95:5 (×40).

(>95%) solutions. No significant change was noticed due to the drying procedure. The habit of those crystals was needle-like regardless of the amount of water present in the crystallizing solution. X-ray diffraction patterns (presented in **Fig. 2**) for the same crystals (either wet or dry) indicated that they were pure anhydrous cholesterol crystals in spite of the presence of water. Differential thermal analysis (DTA), coupled with TG and DTG measurements showed no loss of any amount of water in samples obtained from methanol at room temperature and no loss of weight during the heating process. Only one phase transition was observed at 37°C characteristic to the anhydrous cholesterol. There was no additional endothermic peak at 80°C as might have been expected for monohydrate modification (**Fig. 3**). Similar results were obtained by DSC when wet samples obtained from absolute methanol and methanol–water solution withdrawn directly from the reaction cell were analyzed. The phase transition at 37°C was less than 0.7 kcal/mol in agreement with previously reported data (13).

The use of an ethanol–water mixture resulted in the formation of plate-like crystals. The first crystals to appear in the supersaturated solution were in a star-shape, subsequently growing into laths. The final plates were clearly identified by both light microscope and SEM (see **Fig. 4**). Significantly, the same habit was obtained when cholesterol was crystallized from 95% and 80% ethanol–water solutions (Fig. 4B).

The DTA curves for crystals obtained from absolute ethanol were interesting since they were not characteristic of either the anhydrous or monohydrate forms. There was no 37°C phase-transition peak and instead

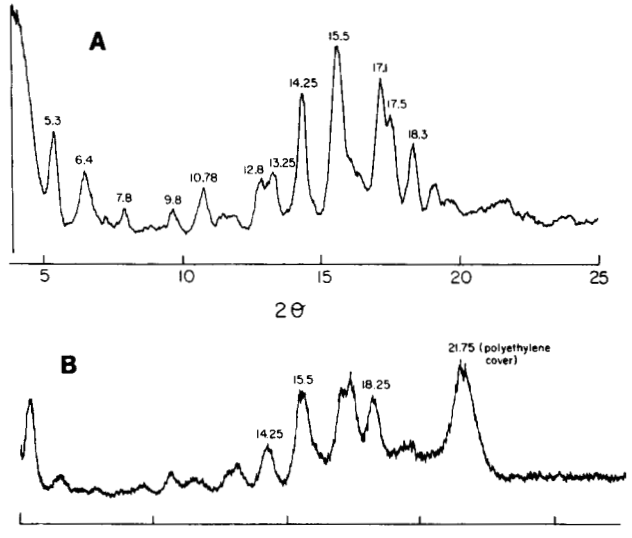

Fig. 2. X-ray diffractions of cholesterol obtained from A) dry methanol, and B) methanol–water 95:5.

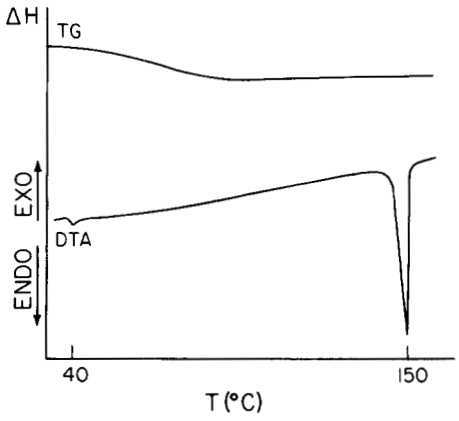

Fig. 3. DTA thermogram of cholesterol crystals precipitated from methanol–water 95:5 solution.

a large endothermic peak appeared at 100–106°C. Although the samples were brought to constant weight at room temperature, there was a loss of half mole of solvent (calculated for ethanol) during the heating for each mole of cholesterol (**Fig. 5**). The crystals did not contain any traces of water since no water was detected when they were dissolved in another dry solvent and analyzed by GLC. Only the 0.2% of water present in the ethanol originally could be detected in the mother liquor.

The DSC measurements on samples withdrawn from the reaction vessel and analyzed immediately without any drying procedure showed a large peak at 114°C followed by a melting point peak at 154°C (**Fig. 6**).

Crystals removed from ethanol–water solution, which were examined in their wet form by the DSC technique, showed a large 112°C endothermic peak followed by the melting point (at 145°C) peak. The shape of the DTA thermogram was surprising when compared to that obtained from crystals formed in dry alcohol. The main endothermic peak was at 80–86°C with a loss of weight corresponding to 1 mole of water (see Fig. 5B). When samples were dried prior to the DTA analysis, smaller amounts of water weight were lost at that temperature.

In another experiment the crystals were removed from their mother liquor and equilibrated for 48 hr in distilled water (as proposed by Loomis et al. (11). The crystals were filtered and kept under air (in the apparatus) for various periods of time to reach constant weight and then analyzed. Upon heating, an endothermic peak at 80°C was observed. All the samples showed different amounts of released water varying from 1:2 ratio to 2:1 ratio on a molar basis of cholesterol, depending on the time of drying in air.

There are some apparent discrepancies between the

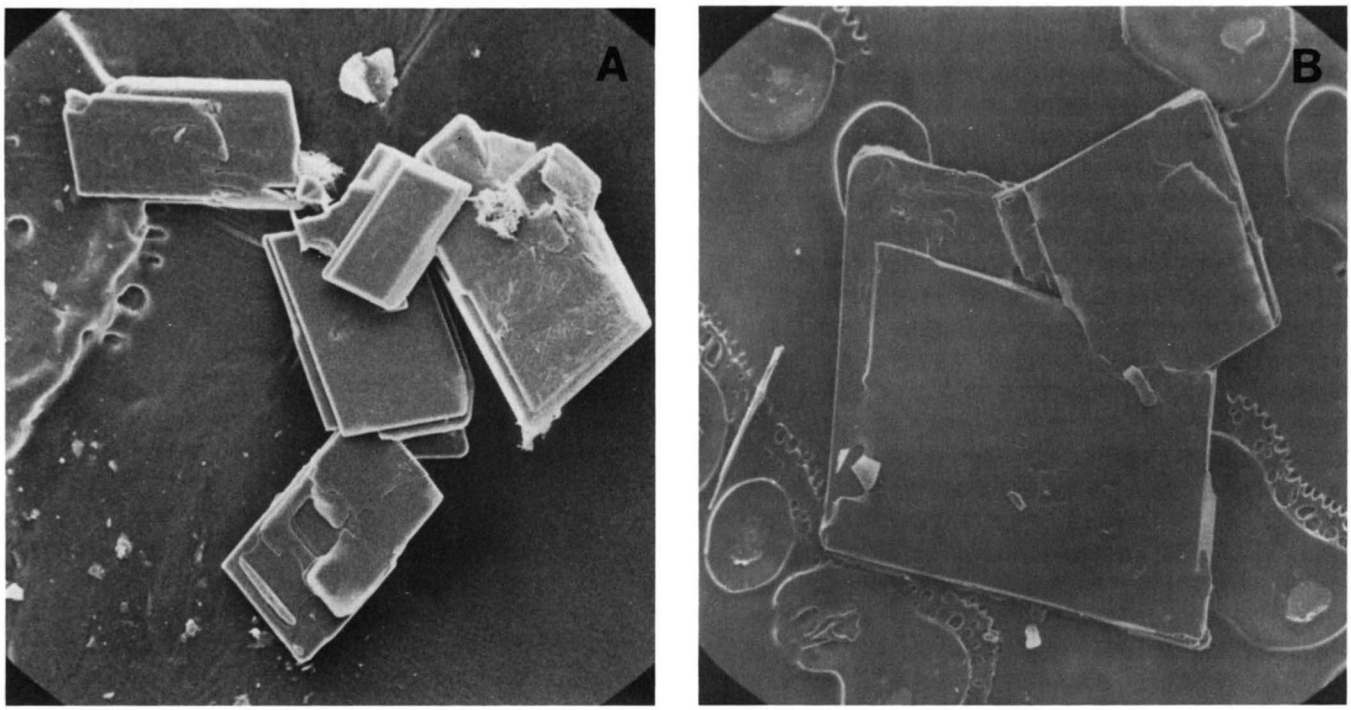

Fig. 4. Photographs of cholesterol crystals isolated from A) absolute ethanol (99.8%) (×78), and B) ethanol–water 95:5 (×78).

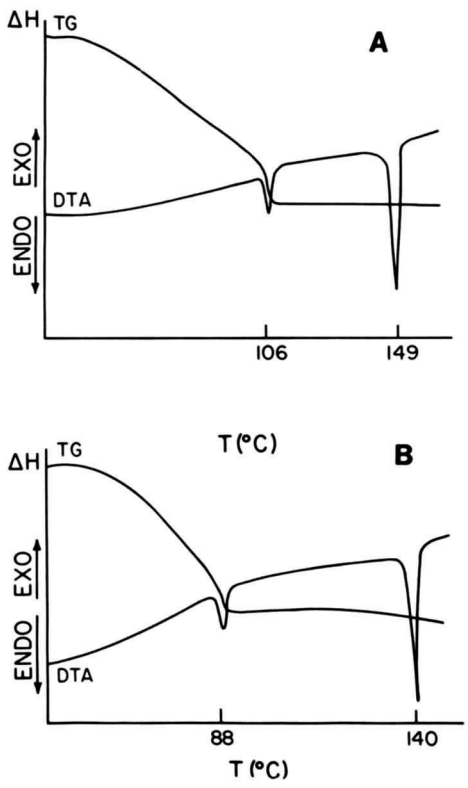

Fig. 5. DTA curves of cholesterol crystals obtained from A) absolute ethanol, and B) ethanol–water 95:5.

DSC and DTA thermograms. First, the low temperature transitions of solvated cholesterol occur in DSC 10–20°C above the temperatures in DTA, while the melting points are nearly the same in both systems. This can be explained by the different experimental conditions that affect the low temperature transition, namely the decomposition, much more strongly than the high temperature transition, the melting of cholesterol.

The decomposition in DTA is performed under dry gas flow. The evolved solvent is immediately carried away and no gas–solid equilibrium can be established. Therefore, the decomposition takes place at a temperature lower than in a static DTA system and much lower than in the closed DSC.

Another point of apparent disagreement is the anomaly of peaks of the ethanolate cholesterol in DSC (Fig. 6A). The ethanol evolved at 114°C is not drawn away from the DSC. Remaining there at high temperature, it may dissolve some of the cholesterol thus changing the net heat effect (14). It cannot happen in other systems: cholesterol is insoluble in water (Fig. 6B) and both solvents are expelled from the DTA system (Fig. 5).

The x-ray diffractograms of crystals grown from absolute alcohol solutions (hemiethanolate) in **Fig. 7A** and from organic-aqueous solutions (monohydrate)

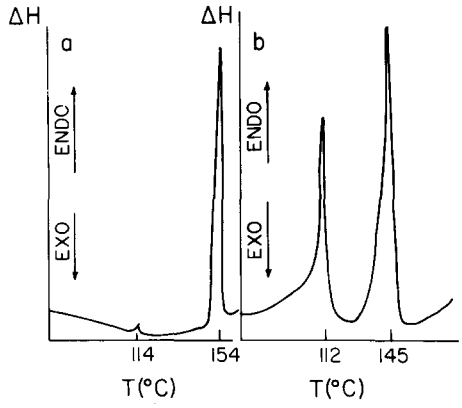

Fig. 6. DSC curves of cholesterol obtained from A) absolute ethanol, and B) ethanol–water 95:5.

in Fig. 7B show distinct differences. The prominent characteristic of cholesterol monohydrate diffraction is the systematic absence of hkl reflections at low angles when h and l are odd (9).

It is worth noticing that crystals obtained from any of the crystallization solutions will turn into anhydrous cholesterol upon prolonged drying even at room temperature and will yield their characteristic thermograms (by both DSC and DTA techniques) and x-ray diffraction without any habit change. The transition to the anhydrous form can be speeded up by increasing the drying temperature.

When absolute acetone was used, needle-like crystals were obtained (**Fig. 8A**) with anhydrous characteristic x-ray pattern and typical anhydrous DTA and DSC curves with large phase transition at 37°C (for DTA) or 45°C (for DSC). Crystals obtained from aqueous acetone were plates (Fig. 8B). The DTA curves showed an endothermic peak at 80–85°C with loss of weight followed by a regular melting point.

When acetonitrile was used as crystallizing solvent, a needle-like anhydrous crystal was obtained both with aqueous and dry solvent (**Fig. 9**).

Table 1 summarizes the results obtained for the four solvents.

DISCUSSION

The results presented in this study clearly indicate that the solvent from which the cholesterol is crystallized can affect both the habit and the crystal structure. The fact that plates were observed is not indicative enough for the unequivocal identification of the monohydrate modification.

When absolutely dry solvents (or with up to 0.2% moisture) were used as crystallizing solvents, anhydrous cholesterol was precipitated out having needle-like habit (methanol, acetone, acetonitrile) but it could also have plate-laths-like habit (ethanol), with characteristic x-ray diffractions and phase transitions (37–45°C and 149–154°C). Ethanol was the only solvent in which ethanolic-solvate was detected with phase transition at 100–106°C. The weight loss corresponded to the ratio of one ethanol molecule to two cholesterol molecules. The existence of both triclinic and monoclinic cholesterol hemiethanolates has been reported by Sheih and Nordman (15), although these structures were previously ascribed erroneously to the hydrate.

Cholesterol solvates of methanol, acetone, and acetonitrile have not been detected by us in crystals grown at the employed experimental conditions.

The presence of 5% or more water in the system caused a formation of hydrated-cholesterol when acetone and ethanol were used. In both cases x-ray diffractions and phase transitions were similar to those detected by Loomis et al. (11) and evidently are characteristic of the hydrated crystals. The hydrated

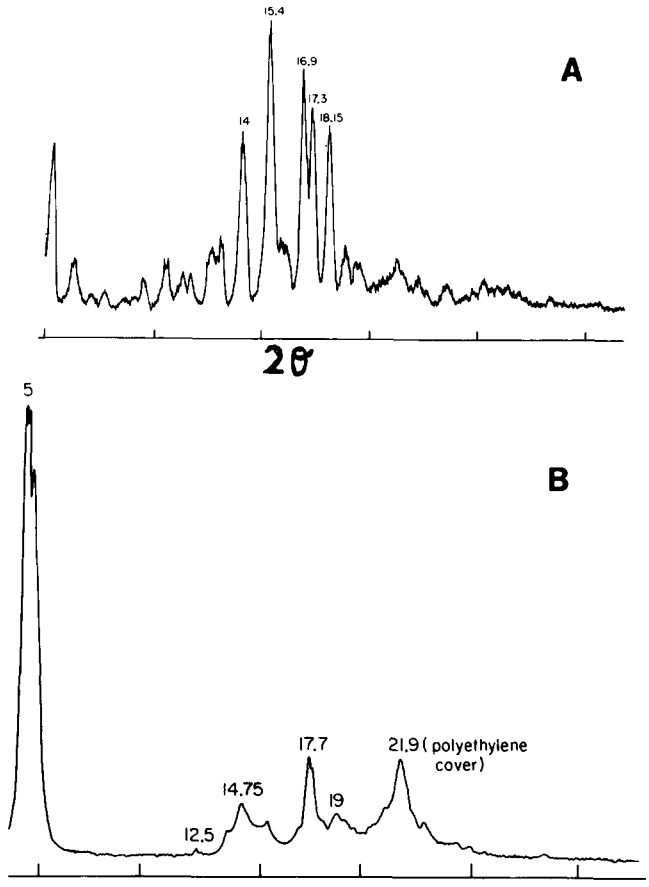

Fig. 7. X-ray diffraction of cholesterol obtained from A) absolute ethanol, and B) ethanol–water 95:5.

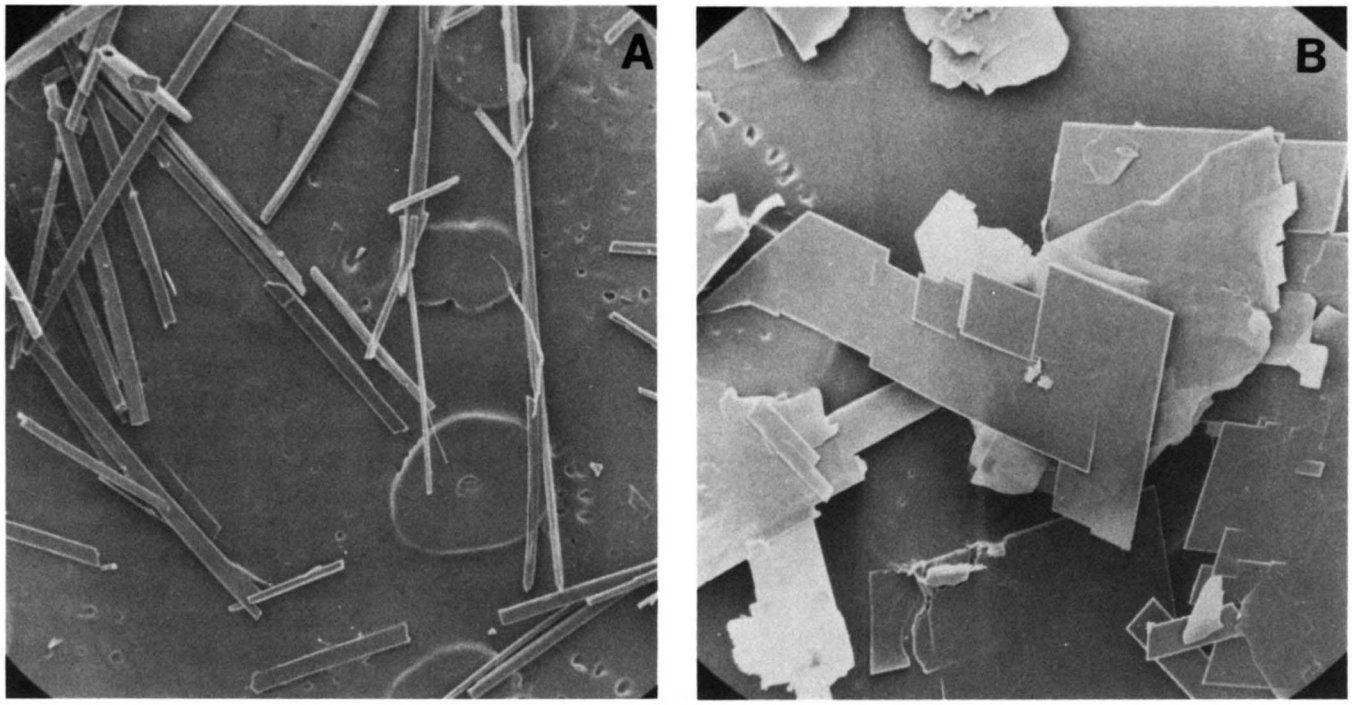

Fig. 8. Photographs of cholesterol crystals precipitated from A) dry acetone (×32), and B) acetone-water solution (×36).

crystals are thermodynamically unstable and tend to lose their crystallization water upon storage at room temperature. A characteristic phase transition was detected at 80–85°C that corresponds to a loss of water.

No solvents or hydrates were observed with aqueous acetonitrile or methanol. To the best of our understanding, this is due to the affinity of those solvents' molecules to cholesterol and their small dimensions. They can compete with water molecules for the

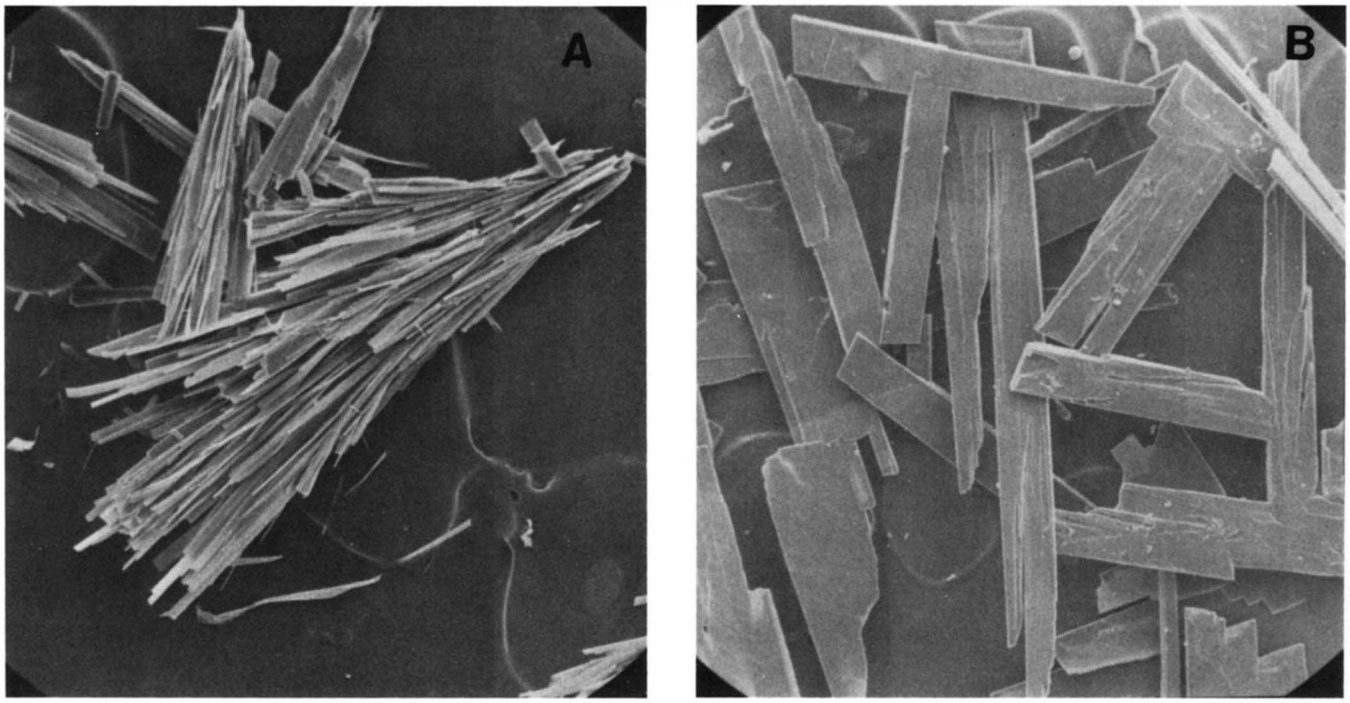

Fig. 9. Photographs of cholesterol crystals precipitated from A) acetonitrile (×54), and B) acetonitrile–water 95:5 (×30).

TABLE 1. Phase transition temperatures and crystal habit of cholesterol crystals

Solvent	DSC[a] Wet Crystals $T_{1(°C)}$	$T_{2(°C)}$	$T_{3(°C)}$	DTA[a] Crystals Dried to Constant Weight $T_{1(°C)}$	$T_{2(°C)}$	$T_{3(°C)}$	Crystal Habit	Figure
Methanol (<0.2% H_2O)	45		155	40		151	needles	Fig. 1A
Methanol (5% H_2O)	45		155	40		150	needles	Fig. 1B
Acetonitrile (<0.2% H_2O)				39		150	needles	Fig. 9A
Acetonitrile (95% H_2O)				40		151	needles	Fig. 9B
Ethanol (<0.2% H_2O)		114	154		106	149	plates[b]	Fig. 4A
Ethanol (5% H_2O)		112	145		88	150	laths[c]	Fig. 4B
Acetone (<0.2% H_2O)	50		156	40		151	needles	Fig. 8A
Acetone (5% H_2O)		98, 127	155		80–85	150	plates	Fig. 8B

[a] Temperature measured at the peaks-maxima.
[b] Angles of 71° and 110°, typical for cholesterol ethanolic solvate.
[c] Angles of 79° and 100.8°, typical for cholesterol monohydrate.

entrance to cholesterol crystals during the process of nucleation and growth and are small enough to leave the structure without staying incorporated in the crystal. The acetone and ethanol molecules, being less polar and larger in size, will not compete with water when the latter is present in excess and thus allow the formation of cholesterol-hydrate. When anhydrous ethanol is used, the solvent may enter the crystal and remain there forming cholesterol hemiethanolate (see Table 1).

Some conclusions can be drawn from this study. The identity between needles-anhydrous structure and plates-cholesterol monohydrate was observed and proved in specified studied systems (11). It seems that this identity does not exist without exception in new unexplored systems. The appearance of plates does not automatically guarantee the hydrated cholesterol composition and the needle habit does not guarantee the anhydrous form.

The interplay of solvent-solute interactions and crystallization conditions will govern the formation of both crystal structure and habit of cholesterol.

The fascinating problem is whether cholesterol crystallization in biological liquids always preserves the identity between plates and monohydrate structure.

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