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Another crystalline form of cholesterol monohydrate and its connection with gallstones

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Abstract

The characteristics of anhydrous cholesterol (ACh) and cholesterol monohydrate (ChM) have been studied using measurements with a thermogravimeter, a differential thermoanalyser, an X-ray powder diffractometer and an infrared absorption spectrometer. ACh is observed to display polymorphism. ChM, obtained from 95% ethanol by a common procedure, is unstable above 72°C. A new form of ChM has been detected after exposing ACh to heat and moisture under high pressure. The newly found ChM is unstable in air and changes easily and irreversibly to ACh like the known form of ChM. The DTA curve of the new ChM shows endothermic peaks at 76, 120 and 138°C when measured using a closed pan. Its X-ray pattern coincides with that of human mixed gallstone. The results suggest that the newly found form of ChM plays an important role in the human body.

INTRODUCTION

Cholesterol is widely distributed in animal tissues and plays important roles in the biomembrane, lipoprotein and mixed micelle in bile. In bile, if cholesterol levels are very high in comparison with other biliary lipids, cholesterol monohydrate crystals can often be observed microscopically [1–3]. Anhydrous cholesterol (ACh), cholesterol monohydrate (ChM) and cholesterol derivatives have been observed in crystalline forms [4–8], but their polymorphism has only been reported by Loomis et al. [9]. Cholesterol makes up 95% of human mixed gallstone, probably occurring as a ChM. Interestingly, differential thermal analysis (DTA) has shown that gallstones examined immediately after surgical removal differ from those that had been stored in water at 4°C [10]. We therefore studied the crystalline form and crystalline transition of ACh and ChM by DTA, X-ray powder diffractometry and other methods to determine the crystalline form of cholesterol in human mixed gallstones.

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EXPERIMENTAL

Materials

ACh purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan) was recrystallized from chloroform, milled in a mortar and kept in a reducing desiccator over phosphorous pentoxide.

ChM was recrystallized from 95% ethanol by the method of Stauffer et al. [11] and washed twice with water. This ChM was named ChM/Et. Another ChM obtained from ACh was exposed to heat and moisture under high pressure in an autoclave at 120°C for 2 h. This compound was named ChM/auc. The two forms of ChM were milled in a mortar and kept in a reducing desiccator with a saturated solution of potassium nitrate which corresponded to 92% relative humidity.

Human mixed gallstones were obtained at surgery and kept in bile. They were used for the experiments within 2 h, being wiped with tissue and milled before the experiments.

Determination of thermal properties

The thermal properties of the sample were determined by thermogravimetry (TG) and DTA using the TG–DTA CN8089 model of Rigaku Corp. (Tokyo, Japan).

Most of the measurements were performed under the following conditions: sample mass, 2 mg; sample pan, open aluminium pan or pressureproof closed stainless steel/aluminium pan, $2.5 \times 5\ mm^2$ internal dimensions; temperature range, 4–170°C; heating and cooling rate, 10°C min^{-1}; flow rate of nitrogen gas, 150 ml min^{-1}.

In another TG examination, the samples were kept at a certain temperature under the following conditions: sample mass, 20 mg; sample pan, open aluminium pan, $5 \times 5\ mm^2$ internal dimensions; temperature range, 15–30°C; holding time, within 3 h; flow rate of nitrogen gas, 50 ml min^{-1}.

X-ray powder diffractometry

A Rigaku X-ray powder diffraction analyser RAD-2B with a monochromator and a controlled heat attachment was used to obtain X-ray diffractograms. The examination conditions were as follows: Cu Kα radiation, $\lambda = 1.54178$ Å, 40 kV, 40 mA; slits, DS = 0.5 mm, RS = 0.3 mm, SS = 1.0 mm; scanning speed, 3 deg min^{-1}; angle of data sampling $2\theta = 2$–30°; interval of sampling, 0.02°; measured temperature point, room temperature (before and after the DTA peak).

Infrared (IR) absorption spectroscopy

IR spectra of the compounds mulled in Nujol were measured with a Jasco IR spectrometer A-702 of Japan Spectroscopic Co. Ltd.

RESULTS AND DISCUSSION

Microscopic observations of the samples with a Nikon Stereo Photo 102 revealed that before milling ACh, ChM/Et and ChM/auc were in the forms of a needle shape, a plate and coagulation, respectively. Slight milling of ChM/auc made it assume a plate shape like that of ChM/Et.

Moisture contents of ChM/Et and ChM/auc, measured by the Karl Fisher method, were 4.58% and 4.36%, respectively. Therefore, both compounds had one mole of water per mole.

Crystalline transition of ACh

DTA curves of ACh obtained using an open pan are given in Fig. 1. Increasing temperature (first run in Fig. 1), as shown by the DTA curve of ACh which had been kept in a Dry Ice box, resulted in several endothermic peaks at 24°C and 148°C. The cooling DTA curve (second run) of the same sample gave several exothermic peaks at 135 and 17°C. The reheating DTA curve (third run) gave a similar initial heating curve. TG curves remained at the baseline for the entire range (not shown). The higher peak probably occurred due to melting. The lower one was considered to be a crystalline transition peak.

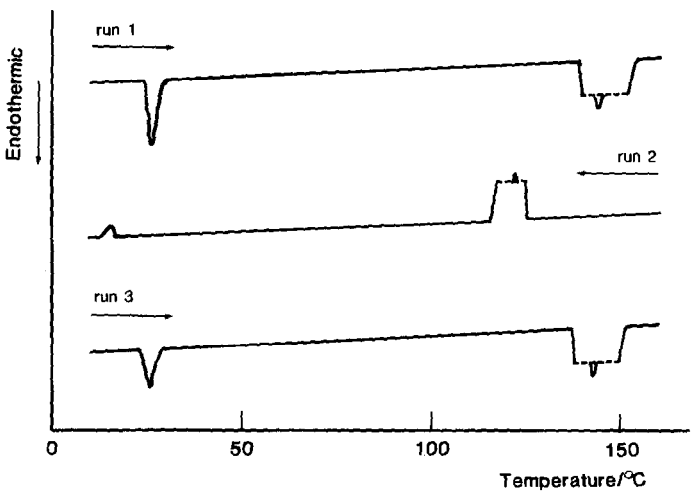

Fig. 1. DTA curves of anhydrous cholesterol. Temperature scanning rate, 10°C min^{-1}; sample mass, 3 mg; sample pan, aluminium open pan; standard, alpha-alumina; atmosphere, nitrogen gas.

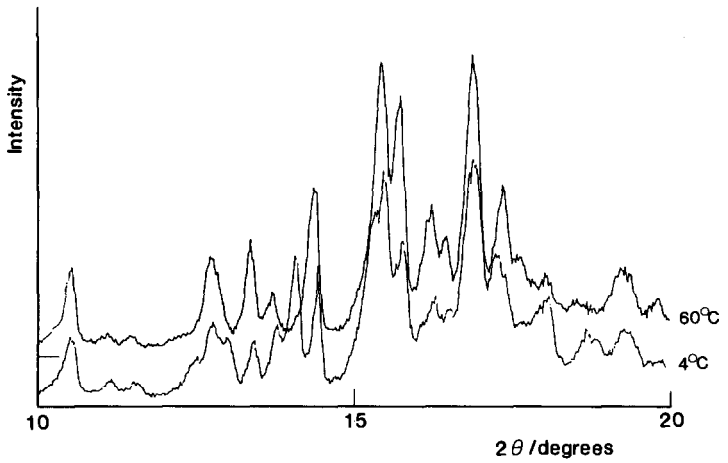

Fig. 2. X-ray powder diffractograms of anhydrous cholesterol immediately after 4°C and 60°C.

The X-ray powder diffractograms of ACh at room temperature (20°C), immediately after 4 and 60°C are shown in Fig. 2. The X-ray pattern of ACh immediately after 4°C differed from that after 60°C. For example, the pattern after 4°C had two peaks of 14.060–14.400° in the range 14–15° of 2θ as compared with one peak of 14.360° in the pattern after 60°C. Therefore, ACh displays polymorphism, with a transition temperature of 24°C.

Crystalline transition of ChM/Et

TG–DTA curves of ChM/Et showed equilibrium moisture contents with 92% relative humidity in an open or pressureproof closed pan as shown in Fig. 3. With an open pan, the DTA curve showed large duplex endothermic peak at lower temperature and a melting peak of 146°C. The decrease in the TG curve was considered to occur due to water desorption at the temperature range of the DTA lower peak. The TG curve did not show any large change above the point of the transition temperature.

With decreasing temperature, the DTA curve was the same as the second run curve in Fig. 1 and the reheating DTA curve. ChM/Et seemed to have changed to ACh.

Using a pressureproof closed pan, the DTA curve showed a broad endothermic peak of lower temperature and two peaks of 70 and 134°C. After heating to 160°C and cooling to 1°C in turn several times, the heating curve was the same as the third run in Fig. 1. The results showed that ChM/Et in the open pan changed to ACh and had a melting peak of 146°C. ChM/Et with a high moisture content gave a drastic change of DTA curve. An exothermic peak of 23°C appeared in the endothermic

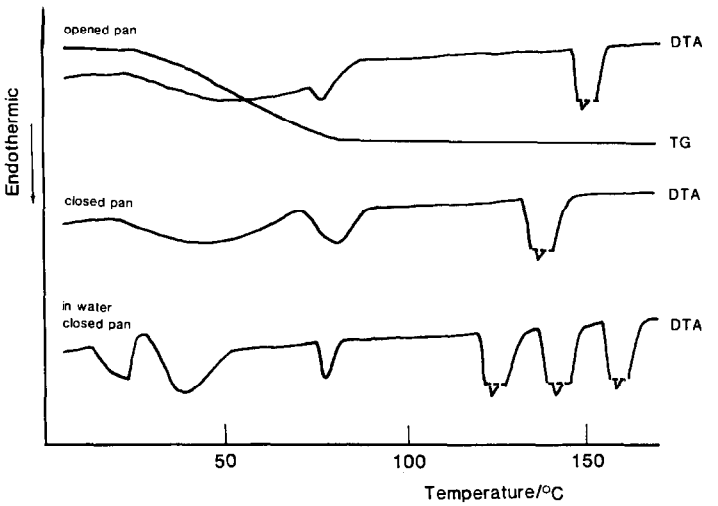

Fig. 3. TG–DTA curves of cholesterol monohydrate from 95% ethanol. Upper two samples have equilibrium moisture contents with 92% relative humidity and the bottom sample has about triple the amount of water.

peak and four endothermic peaks of 75, 121, 135 and 152°C were found. The reheating curve had the 121 and 152°C peaks not the 75 and 135°C ones. The 135°C peak in the DSC curve of ChM in water was not observed by Loomis et al. [9], but was distinctly detected in our system. However, our reheating curve coincided with theirs.

ChM/Et on the DTA settings at various temperatures gave TG curves that showed decreases in mass and stability at the ACh level. It took 150, 70 and 46 min to reach stability when ChM/Et had been held at 23, 29 and

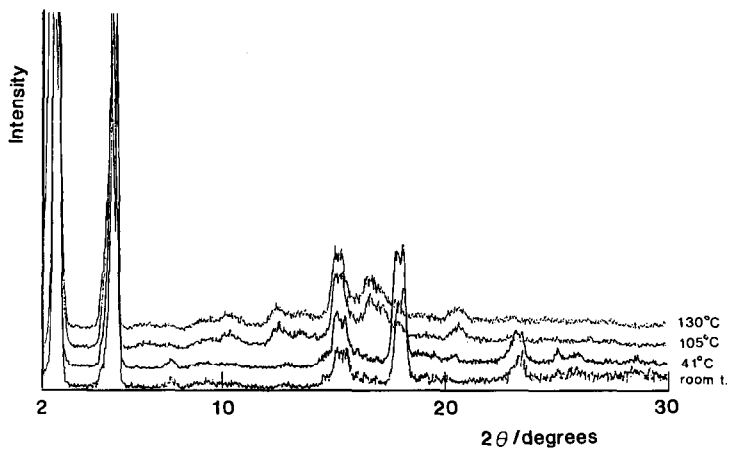

Fig. 4. X-ray powder diffractograms of cholesterol monohydrate from 95% ethanol at various temperatures.

34°C respectively. TG curves of ChM/Et at the heating rate of 0.33–1°C min^{-1} gave similar results to the curve obtained upon holding at 50°C, i.e. the TG curve decreased simply and substantially and was stable. ChM/Et changes easily to ACh, and the water of crystallization of ChM/Et is readily disengaged like adsorbed water.

Figure 4 gives the X-ray powder diffractograms of ChM/Et at room temperature, 41, 105 and 130°C before and after DTA peaks. The diffractogram at room temperature seemed to be about the same as that at 41°C. Therefore, although the peak in the DTA curve at the same range of room temperature to 41°C with the decreasing TG curve was recognized, a crystalline transition did not occur in the range studied. With increasing temperature, the diffractogram pattern at 41°C changed markedly to that at 105°C but not to that at 130°C. Thus, the peak of 80°C in the DTA curve was clearly at the crystalline transition point.

Crystalline transition of ChM/auc

IR spectra of various samples are shown in Fig. 5. The IR pattern of ChM/auc coincided with that of ChM/Et and could be distinguished from ACh in the ranges of 1680–1620 cm^{-1} and 3500–3200 cm^{-1} which correspond to water and the hydroxy radical, respectively.

The DTA curve of ChM/auc differed not only from that of ACh but also that of ChM/Et as shown in Fig. 6. DTA measurement of ChM/auc using an open pan did not reveal a higher peak. The reheating DTA curve of ChM/auc was the same as that of the third run in Fig. 1. According to the reheating DTA curve and the IR pattern of Fig. 5, cholesterol of ChM/auc seemed to be decomposed on autoclave treatment.

ChM/auc in a pressureproof closed pan showed the three peaks of 76, 120 and 138°C, which differed from those of ChM/Et. However, the DTA

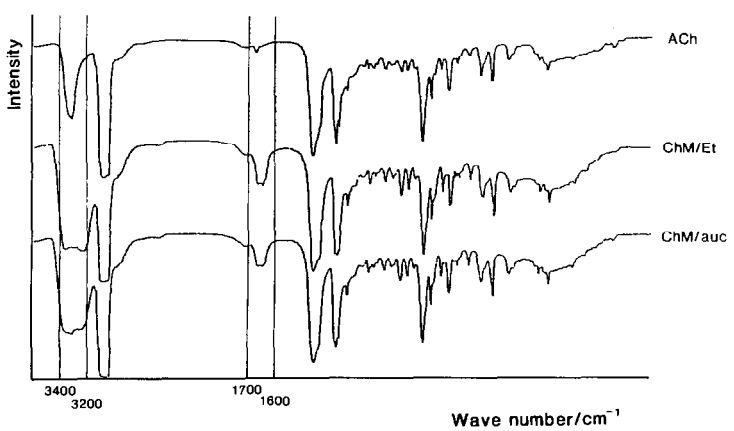

Fig. 5. IR spectra of the various forms of cholesterol.

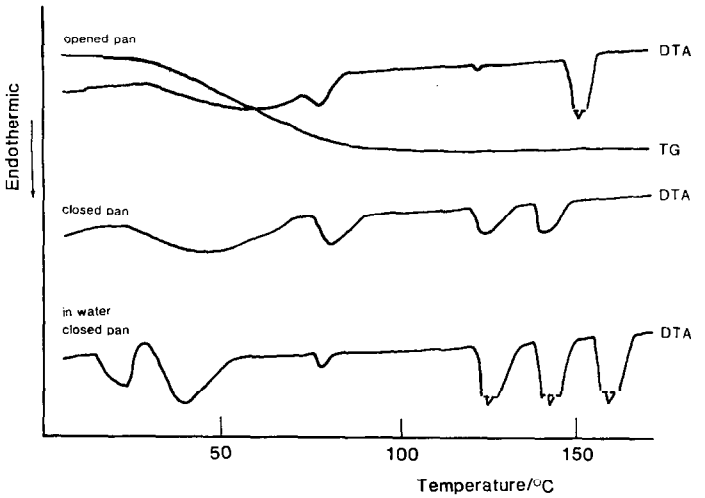

Fig. 6. TG–DTA curves of cholesterol monohydrate after autoclaving.

curve of ChM/auc having a high moisture content was the same as that of ChM/Et. An exothermic peak may indicate recrystallization.

When ChM/auc was held on the DTA setting at a definite lower temperature, the time required to stabilize TG was longer than for ChM/Et. ChM/auc seemed to be unstable and changed easily to ACh, but was slightly more stable than ChM/Et according to comparisons of the time requirement and the melting temperature.

X-ray diffractograms of various cholesterol samples and human mixed gallstone are shown in Fig. 7. Repeated testing of various batches of ChM/auc at room temperature gave diffractograms similar to that shown

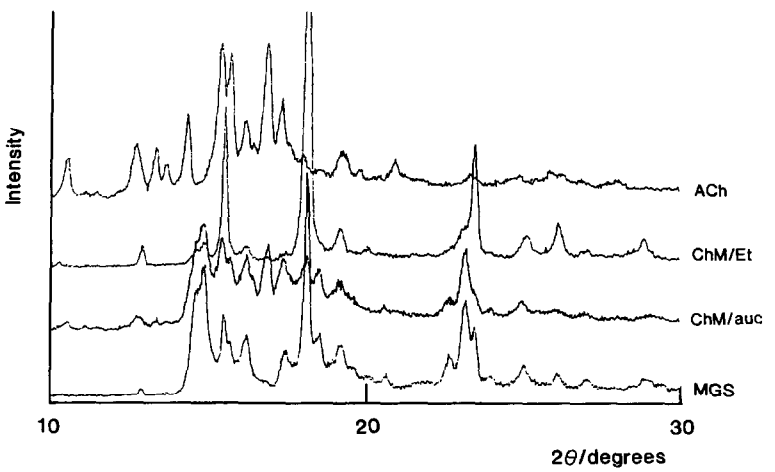

Fig. 7. X-ray powder diffractograms of various cholesterol samples and human mixed gallstones (MGS).

in Fig. 7. A few batches gave a mixture of the patterns of ChM/Et and ChM/auc presented in Fig. 7. Loomis et al. [9] reported ACh in water at 100°C changed to ChM/Et, according to the DSC pattern. We found another form of ChM after treatment at a high temperature and high pressure. Therefore, ChM/auc was recognized to be a different crystalline form from ChM/Et.

Comparison of the X-ray powder diffractograms of human mixed gallstone showed coincidence with that of ChM/auc, as can be seen in Fig. 7. These findings may suggest how the cholesterol of gallstone is formed in bile. ChM has been known to exist in one crystalline form with crystalline transition. This study revealed the existence of another crystalline form in the ChM system.

CONCLUSION

ChM/auc was clearly shown to be a cholesterol monohydrate from the Karl Fisher moisture contents, the DTA reheating curve and the IR pattern. The crystalline form of ChM/auc was considered to be different from ChM/Et which had been studied at the crystalline transition. As a single crystal of ChM/auc was not available, the space group and cell parameter of the crystalline form could not be determined. CHM/auc is closely related to the cholesterol of gallstones and may play an important role in the living body system.

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