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Phase Diagrams of Systems Containing Cholesterol, Cholesteryl Esters, and Triglycerides

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Binary and ternary systems of cholesterol, the three cholesteryl esters, linoleate, oleate, and stearate and the two triglycerides, triolein and tristearin were studied in order to determine the phase transitions and the conditions for the cholesteric and smectic mesophases. Phase transitions were determined using differential thermal analysis, melting point determination, and polarizing microscopy.

The cholesterol-cholesteryl ester systems studied are of the eutectic type with limited solid solubility. The mesophases, cholesteric and smectic, are monotropic as to the crystalline state and exist up to *ca.* 75 wt. % cholesterol. Ternary systems of cholesterol and two cholesteryl esters show the same general features as the binary systems.

The melting point of cholesterol is depressed by increasing amounts of triglycerides down to an eutectic point at high concentrations of the triglycerides. The solubility of anhydrous cholesterol and cholesterol monohydrate in triolein was found to be the same.

In mixed systems with cholesterol, triglycerides, and cholesteryl esters even low concentrations of the triglycerides removed the cholesteric mesophase typical for cholesteryl ester systems. At higher concentrations of triolein the smectic mesophase was also removed. In systems with cholesterol, a cholesteryl ester and tristearin an apparently smectic mesophase with mosaic texture was exhibited.

Cholesterol, cholesteryl esters, and triglycerides belong to the major components of atherosclerotic deposits and serum lipoproteins. From this standpoint, the phase conditions of these lipids help to understand the physicochemical mechanism of the lipid deposition in atherosclerotic arteries.

Cholesterol, cholesteryl esters and triglycerides are major lipids in atherosclerotic plaques.[1] Correlated morphological and chemical studies have shown that lipids, accumulating as liquid and liquid crystalline droplets in early stages of atherosclerosis, are mainly composed of cholesteryl esters.[2,3] In advanced plaques there is much solid material consisting of crystalline cholesterol and amorphous cholesteryl esters and triglycerides.[3]

Based upon their interactions with water cholesterol and triglycerides are classed as polar insoluble non-swelling amphiphiles and cholesteryl esters as non-polar lipids.[4]

On heating, cholesterol and triglycerides melt from a crystalline form directly to an isotropic liquid while long chain cholesteryl esters exhibit thermotropic mesomorphism.[5]

Regarding the interactions with polar insoluble swelling lipids (*e.g.* phospholipids) the difference between cholesterol, cholesteryl esters and triglycerides is marked. In bulk systems cholesterol can be solubilized in the molar ratio of 1:1 by phospholipids,[5,7] while triglycerides can be solubilized to a much smaller extent,[8] and cholesteryl esters to an almost negligible amount.[9]

This work is part of a program for studying the physical state of, and the interactions between, the different lipid classes which accumulate in the atherosclerotic lesions. Phase diagrams of systems containing cholesteryl esters and triglycerides have been presented in an earlier paper.[10]

MATERIALS AND METHODS

The cholesterol, cholesteryl oleate and cholesteryl stearate used were purchased from E. Merck AG. Cholesteryl linoleate was prepared by a modified acid chloride method.[11]

The cholesterol was recrystallized three times from 1,2-dichloroethane and the monohydrate of cholesterol was prepared by dissolving cholesterol in methanol and recrystallization by addition of water. The cholesteryl esters were recrystallized from pentyl alcohol with subsequent washing in an ethanol-water solution. Triolein and tristearin were purchased from Fluka AG. The triolein was purified by Florisil column chromatography and the tristearin by recrystallization from acetone.

Samples for analysis were prepared by dissolving the weighed components in chloroform which was then removed *in vacuo*. *Ca.* 30 mg of sample were weighed in an aluminium pan which was placed in a Fisher Model 370 Differential Thermal Analyzer (DTA). The heating curves were obtained with a scan speed of 10 °C/min and the cooling curves with a scan speed of 5 °C/min. When examining lipids with unsaturated fatty acid chains, an atmosphere of N_2 gas was used.

The melting point values obtained by DTA measurements were complemented by examinations with a Gallenkamp melting point apparatus. In order to identify the phase changes recorded by the DTA measurements a Wild polarizing microscope equipped with a thermostated stage was used.

RESULTS

Individual lipids. The thermal properties of the three C_{18} cholesteryl esters (stearate, oleate, and linoleate) and the two C_{18} triglycerides (triolein and tristearin) used in this study have been presented in a previous report.[12] Also in this study precautions were taken to obtain the stable higher melting modification of the unsaturated cholesteryl esters and to bring the tristearin into the triclinic β_L form.

On heating, the anhydrous cholesterol showed a reversible endotherm at 38 °C and melted at 148 °C. The 38 °C transition of cholesterol was exhibited also in mixtures with other lipids but is for simplicity omitted from the diagrams. The monohydrate of cholesterol showed no transition at 38 °C, lost its hydration water between 100 and 120 °C and then melted at the same temperature as the anhydrous cholesterol.

Mixtures of cholesterol and cholesteryl esters. The condensed binary phase diagrams of cholesterol and the cholesteryl esters are presented in Fig. 1. The melting point of cholesterol is depressed continuously by increasing amounts of esters down to an eutectic point at high concentrations of the esters.

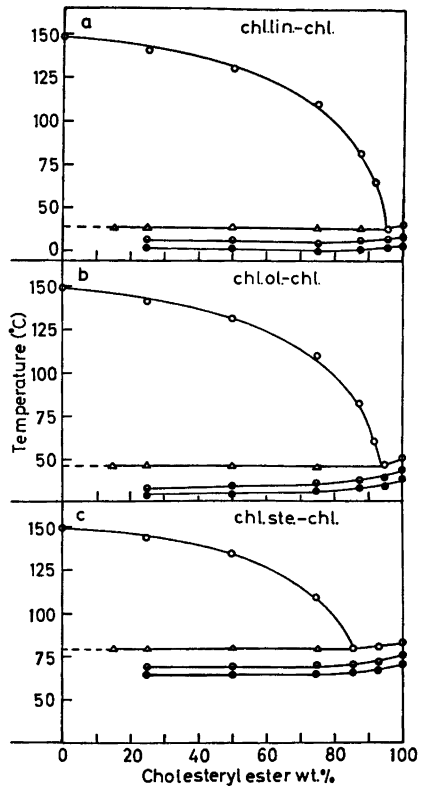

Fig. 1. Condensed binary phase diagrams for the systems (a) cholesterol – cholesteryl linoleate, (b) cholesterol – cholesteryl oleate, and (c) cholesterol – cholesteryl stearate. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, solid-(solid + liquid) △.

On heating, the DTA curves for blends with composition within the miscibility gap have two peaks; the first corresponding to the melting of the eutectic composition and the second one to the melting of the solid portion of the blend. At compositions approaching the eutectic point the second peak diminishes. (Fig. 2a)

On cooling from isotropic melt cholesteric and smectic mesophases were found to exist up to about 75 % cholesterol.

Ternary systems of cholesterol and two cholesteryl esters showed the same general features as the binary systems. This is illustrated in Fig. 3 where the proportion of cholesterol is held constant and the mutual concentrations of the esters are changed. The linear curve for the final melting points of cholesterol shows that

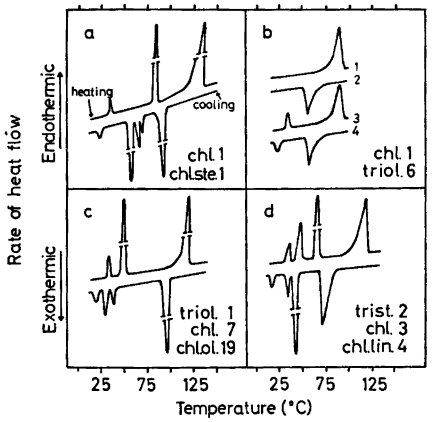

Fig. 2. DTA curves of mixtures of cholesterol, the three cholesteryl esters, linoleate, oleate, and stearate, and the two triglycerides, triolein, and tristearin. In the figures the heating curves are above and the cooling curves below. (a) The heating curve of a 1:1 mixture of cholesterol and cholesteryl stearate showing the 38 °C endotherm of cholesterol and the melting peaks of cholesteryl stearate and cholesterol. The cooling curve shows the crystallization of cholesterol, two mesomorphic transitions (liquid-cholesteric and cholesteric-smectic), the crystallization exotherm of cholesteryl stearate and the reversible crystal transformation of cholesterol. (b) (1) The melting endotherm of cholesterol monohydrate in a 1:6 mixture with triolein. (2) Cooling curve of the same mixture showing the crystallization exotherm of cholesterol monohydrate. (3) Reheating of the same mixture after removing the crystal water of the cholesterol monohydrate by heating to 150 °C. The curve shows the 38 °C and the melting endotherms of anhydrous cholesterol. (4) Cooling curve of the same mixture showing the crystallization and crystal transformation of the anhydrous cholesterol. (c) The heating curve of a 1:7:19 mixture of triolein, cholesterol, and cholesteryl oleate showing the 38 °C endotherm of the cholesterol and the melting peaks of cholesteryl oleate and cholesterol. The cooling curve shows the crystallization peak of cholesterol, two mesomorphic transitions (liquid-cholesteric and cholesteric-smectic) and the crystal transformation of cholesterol. (d) The heating curve shows 38 °C endotherm of cholesterol and the melting peaks of cholesteryl linoleate, tristearin, and cholesterol of a 2:3:4 mixture of tristearin, cholesterol, and cholesteryl linoleate. The cooling curve shows the crystallization exotherms of cholesterol and tristearin, the mosaic phase transition and the cholesterol crystal transformation.

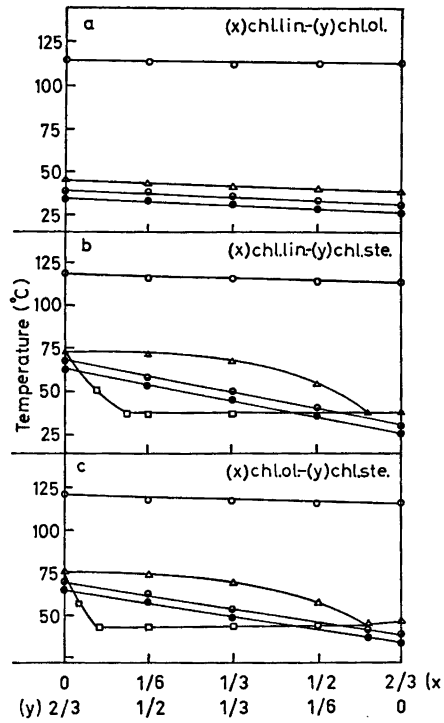

Fig. 3. Phase transitions in the ternary systems with cholesterol (chl) and two of the cholesteryl esters, cholesteryl linoleate (chl. lin.), cholesteryl oleate (chl. ol.), and cholesteryl stearate (chl. ste.). (a) Chl. – chl. lin. – chl. ol., (b) chl. – chl. lin. – chl. ste., (c) chl. – chl. ol. – chl. ste. Proportion of cholesterol is constant at the weight fraction of 1/3 and the proportions of the cholesteryl esters vary between 0 and 2/3. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, solid-(solid + liquid) △ and □.

the melting point depressing properties are additive in an ideal manner. Also the melting point of cholesteryl stearate is depressed by the unsaturated esters. The unsaturated esters show complete miscibility in both the liquid and solid phases.

On cooling from melt, cholesteric and smectic mesophases were found to exist for all mutual concentrations of esters and the fixed weight fraction of 1/3 cholesterol.

Mixtures of cholesterol and triglycerides. In Fig. 4 the phase diagrams of cholesterol-triolein and cholesterol-tristearin are presented. As for the cholesteryl esters increasing amounts of triglycerides continuously depress the melting

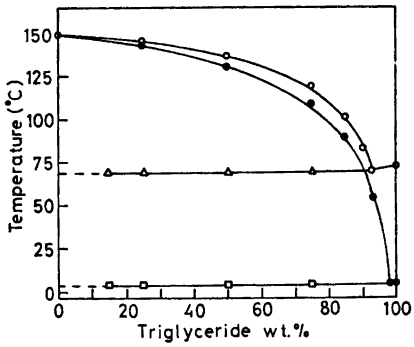

Fig. 4. Condensed binary phase diagrams for the systems (a) cholesterol-triolein, solid-liquid ●, solid-(solid + liquid) □, and (b) cholesterol-tristearin, solid-liquid ○, solid-(solid + liquid) △.

point of cholesterol down to an eutectic point at high concentrations of the triglycerides. The melting point depressing effect of triolein is somewhat greater than that of tristearin.

On heating, the cholesterol monohydrate showed no transition at 38 °C in mixtures with triolein, but if the mixtures were heated above 100 °C and the monohydrate lost its water a transition occurred (Fig. 2 b). No significant differences in solubility of anhydrous cholesterol and cholesterol monohydrate in triolein were found.

Mixtures of cholesterol, triglycerides, and cholesteryl esters. The phase diagrams of cholesterol, triolein, and the three cholesteryl esters respectively are presented in Fig. 5. The concentration of cholesterol is held constant at the weight fraction of 1/3 and the mutual concentrations of triolein and the cholesteryl esters are varied between the weight fractions of 0 and 2/3. The linear curves for the solid-liquid transition show that the melting point depressing effects are additive in an ideal manner. Also the melting points of the cholesteryl esters are depressed by triolein.

On cooling from isotropic melt both cholesteric and smectic mesophases were recorded at low triolein concentrations (Fig. 2 c). The smectic mesophases was recorded to somewhat higher triolein concentrations than the cholesteric one.

In Fig. 6 the phase diagrams of cholesterol, tristearin and the three cholesteryl esters are presented. From the linear liquidus line it can

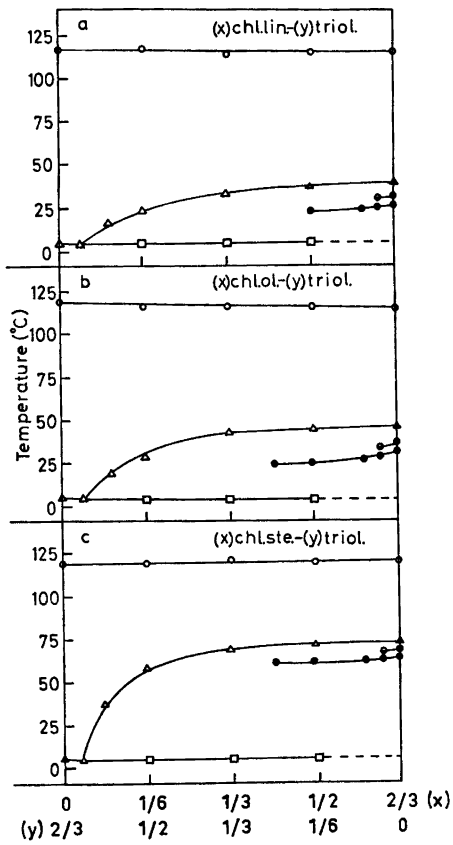

Fig. 5. Phase transitions in ternary systems of cholesterol (chl.), triolein (triol.) and the three cholesteryl esters, cholesteryl linoleate (chl. lin.), cholesteryl oleate (chl. ol.) and cholesteryl stearate (chl. ste.). (a) Chl. – triol. – chl. lin., (b) chl. – triol. – chl. ol. (c) chl. – triol. – chl. ste. Proportion of cholesterol is constant at the weight fraction of 1/3 and the proportions of triolein and the cholesteryl esters vary between 0 and 2/3. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, solid-(solid + liquid) △ and □.

be seen that the melting point depressing effects of tristearin and the cholesteryl esters are additive in an ideal manner. The melting point of tristearin is depressed by the unsaturated cholesteryl esters and the melting point of cholesteryl stearate is depressed by tristearin. On cooling from melt ternary systems with cholesterol, tristearin and either of the cholesteryl esters exhibited both cholesteric and smectic mesophases at low tristearin con-

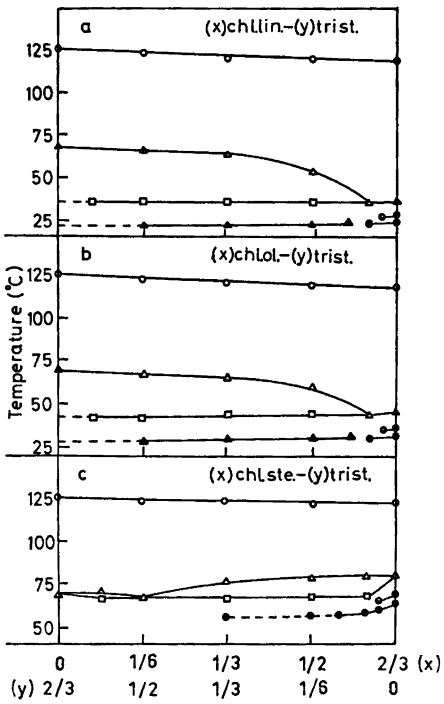

Fig. 6. Phase transitions in ternary systems of cholesterol (chl.), tristearin (trist.), and the three cholesteryl esters, cholesteryl linoleate (chl. lin.), cholesteryl oleate (chl. ol.), and cholesteryl stearate (chl. ste.). (a) Chl. – trist. – chl. lin., (b) chl. – trist. – chl. ol., (c) chl. – trist. – chl. ste. Proportion of cholesterol is constant at the weight fraction of 1/3 and the proportions of tristearin and the cholesteryl esters vary between 0 and 2/3. Solid-liquid ○, liquid-cholesteric ◒, cholesteric-smectic ●, liquid-mosaic smectic ▲, solid-(solid + liquid) △ and □.

centrations. For the unsaturated esters the smectic phase remains at high tristearin concentrations, but the microscopic texture changes considerably; a mosaic texture phase is formed (Fig. 2 d). At low cholesteryl ester concentrations the smectic transition cannot be recorded but the curve obtained by plotting composition *versus* the transition heat for the smectic mesophase indicated that this phase exists down to very low cholesteryl ester concentrations.

The interaction of cholesteryl stearate with tristearin and cholesterol is somewhat different from that of the unsaturated esters. On cooling from melt, cholesteric and smectic mesophases are exhibited at low tristearin concentrations but no mosaic type mesophase is formed at higher tristearin concentrations.

BIOLOGICAL CONCLUSIONS

This work is part of a project to elucidate the factors governing the deposition of lipids in atherosclerotic plaques through studies of the physical state of and interaction between lipids in adequate model systems. This study deals with the phase behaviour of three major atheroma lipids; cholesteryl esters, triglycerides and cholesterol.

All binary systems studied with cholesterol and one cholesteryl ester or triglyceride are of the eutectic type with limited solid solubility. The melting point of cholesterol is depressed to the eutectic point at a high percentage of the cholesteryl ester or triglyceride. The ternary systems show that the melting point depressing capacity is additive in an ideal manner. These facts have to be kept in mind when considering the physical state of lipids in atherosclerotic plaques.

No solid-phase solubility of the cholesteryl esters and triglycerides in cholesterol was detected. This must be due to limited calorimeter sensitivity because complete immiscibility of solid phases in eutectic systems never occurs. The low mutual solid solubility of cholesterol, cholesteryl esters and triglycerides accounts for the fact that crystals of cholesterol, with no chromatographically detectable impurities were found together with amorphous cholesteryl esters in atherosclerotic plaques.[3]

Typical for the early stages of atherosclerosis is a large accumulation of lipids especially cholesteryl esters, in isotropic and birefringent droplets. The polarizing microscopic pattern and freeze-etching electron microscopic pictures indicate a similarity between the anisotropic droplets and the cholesteryl ester suspensions in smectic state. The cholesteric phase has not been found in atherosclerotic lesions. The probable explanation of this fact is the property of triglycerides to remove this mesophase in mixtures with cholesteryl esters.

Besides isotropic and mesomorphic droplets there is also an abundance of solid lipid material in advanced plaques. From a chemical point of view the advanced plaques can be

divided into "cholesteryl ester type" and "cholesterol type".[3]

Typical for the "cholesteryl ester type" lesion is a high cholesteryl ester value and moderate cholesterol, triglyceride and phospholipid values. The plaque is made up of amorphous material which primarily melts between 40 and 50 °C. The melting points of saturated, high melting, cholesteryl esters which occur rather abundantly are thus depressed by unsaturated, low melting, cholesteryl esters and triglycerides.

The "cholesterol type" lesions have high cholesterol values and contain a rather great amount of cholesteryl esters. The triglyceride and phospholipid values are low. Typically enough these plaques contain crystals of cholesterol monohydrate and varying amounts of amorphous cholesteryl esters. The "cholesterol type" plaques typically melt between 50 and 60 °C; thus at a higher temperature than the "cholesteryl ester type" plaques but at a much lower than the melting point of cholesterol. This fact obviously is a result of the melting point depressing effect of the cholesteryl esters and triglycerides on cholesterol.

The anhydrous cholesterol shows a reversible endotherm at 38 °C both in pure form and in mixtures with other lipids. A possible significance of this transition in the atherogenesis has been proposed.[12] This hypothesis is opposed by the fact that the cholesterol in atherosclerotic plaques occurs in the form of cholesterol monohydrate which does not show the 38 °C endotherm. In this study has been shown that cholesterol can crystallize in the form of cholesterol monohydrate from a mixture with triolein. It is thus still unclear whether the cholesterol crystals in atherosclerotic deposits originate from membrane structures oversaturated with cholesterol or from an oily phase.

From a physicochemical point of view one can thus conclude that the most fatal factors for a deposition of solid lipid material in the arterial wall are excessive incorporation of cholesterol and/or saturated, high melting cholesteryl esters giving rise to "cholesterol type" or "cholesteryl ester type" plaques alternatively.

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