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Polymorphism in Cholesteryl Esters: Cholesteryl Palmitate†‡§

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Abstract—The thermal transitions in cholesteryl palmitate have been evaluated and compared to previously reported data. A sample synthesized from carefully purified cholesterol and palmitic acid using *p*-toluene sulfonic acid compared directly with previously reported data. A sample of recrystallized ester from a commercial source showed a slightly depressed solid to mesophase transition temperature. However, the mesophase proved to be smectic, commonly reported as monotropic with respect to the solid phase. Both smectic to cholesteric and cholesteric to isotropic liquid transitions were very sharp, indicating a very pure material. It is postulated that a specific impurity, an antioxidant, is responsible for this previously unreported effect. This effect of related compound interaction has been reported for nematic mesophase forming compounds. Thin layer chromatography, microscopy, depolarized light intensity analysis, differential scanning calorimetry and NMR spectroscopy data are discussed.

1. Introduction

The formation of mesophases of the smectic and cholesteric type has long been recognized as a property of cholesteryl esters.[1] The mesophases formed are frequently determined by the direction of the process, i.e., heating or cooling. For example, cholesteryl myristate

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exhibits a solid, smectic cholesteric and isotropic liquid on both heating and cooling. Cholesteryl nonanoate exhibits a solid, cholesteric and isotropic liquid phase on heating, but on cooling, a smectic mesophase appears between the cholesteric and solid phase. The smectic mesophase is said to be monotropic with respect to the solid phase. Cholesteryl laurate exhibits no mesophases (see exception below) on heating, but on cooling, a cholesteric and smectic mesophase appears between the isotropic liquid and solid phase. The smectic and cholesteric mesophases are monotropic with respect to the solid phase. In the monotropic cases the phase rule and laws of thermodynamics are not violated, since each phase is always in equilibrium with the next higher temperature phase stable at the transition temperature. Since 1962, the thermodynamic properties of these interesting materials have been studied in this series of papers. Valuable insights and contributions have been made by other workers during this same time period.[2-5]

In part VIII of this series it was noted that significant variations occurred in the transition heats and temperatures of the solid phase depending on the way in which the solid phase had been formed: from solution, from the isotropic melt or from the mesophase.[6] Solid phase polymorphy was further explored for cholesteryl propionate in part XX.[7] This compound was found to form two solid phases with melting points at 95.2° and 98.0° depending on the solvent used for recrystallization. In addition, the high melting phase was found to form predominantly on very slow cooling ($< 1°$/min.) of the mesophase. Interconversion between solid forms without the presence of the mesophase was not noted after repeated efforts. The crystal morphology of the two solid phases is probably fundamentally different in the propionate case, since the higher melting form is predominantly prismatic and the lower melting form spherulitic.[7]

In part XXII of this series cholesteryl heptadecanoate was found to exhibit a high and low melting form.[8] It was not possible to separate the two forms in very pure material. However, the introduction of impurities produced predominantly the lower melting form. The impurity content need be only 1.5% to produce the low melting form.[8] The very pure heptadecanoate ester gave only the high melting form when recrystallized from the mesophase.

This study was concerned with the investigation of the possible solid phase polymorphy or selective impurity depression of cholesteryl palmitate. This ester has been reported by a number of workers,[2,3,6,9,10] see Table 1, to undergo a transition to the cholesteric mesophase on heating of the solid followed by a cholesteric → isotropic liquid transition. On cooling the isotropic liquid, the cholesteric mesophase forms. At lower temperatures the cholesteric mesophase undergoes a transition to the smectic mesophase followed by formation of the solid phase, somewhat super-cooled. These data indicate that the smectic mesophase is monotropic with respect to the solid and cholesteric phases. The cholesteric mesophase has been reported as being monotropic with respect to one solid phase as well.[6] Therefore, the existing literature evidence indicates the strong possibility that cholesteryl palmitate may have a complex solid phase polymorphy. Co-laterally, the next lower member, cholesteryl myristate, has been reported without exception to be completely reversible in all mesophases, i.e., the smectic and cholesteric mesophases form on both heating and cooling.[5,6,9,10] Cholesteryl laurate would behave as does cholesteryl myristate were it not for the spontaneous conversion of the low melting solid form near 85 °C to a higher melting form (~95 °C). This renders both the cholesteric and smectic mesophase monotropic.[11,12] Davis and Porter have succeeded in isolating the pure low temperature solid phase of the laurate ester.[12] Their sample, prepared by careful precipitation from *n*-pentanol, apparently lacked nuclei of the high temperature form, thus preventing the usual[20] solid phase recrystallization.

Indeed, it may be possible to form low melting crystal forms of all the even *n*-alkyl cholesteryl esters. Preliminary evidence indicates that the solid phase polymorphy of the cholesteryl esters may be at least as complex as the mesophase polymorphy. In part this apparent polymorphy may be due to selective impurities as previously reported for nematic systems.[13]

2. Experimental

Preparation of Samples

Cholesteryl palmitate from two sources was studied. A sample of cholesteryl palmitate from van Schuppen Chemicals, Veenendaal,

TABLE 1 A Comparison of van Schuppen and Synthesized Cholesteryl Palmitate Transitions with the Literature

	Solid→Smectic			Solid→Cholesteric			Smectic→Cholesteric			Cholesteric→Isotropic Liquid		
Sample	Tm[a]	ΔH[b]	ΔS[c]	Tm[a]	ΔH[b]	ΔS[c]	Tm[a]	ΔH[b]	ΔS[c]	Tm[a]	ΔH[b]	ΔS[c]
van Schuppen	75.47	13.27	38.0	—	—	—	76.74	0.463	1.32	81.42	0.317	0.894
Synthetic	—	—	—	77.51	13.69	39.1	[76.4	0.41	1.21]	82.00	0.317	0.892
Sell[10]	—	—	—	77	—	—	[75.5	—	—]	80.0	—	—
Gray[9]	—	—	—	79	—	—	[78.5	—	—]	83.0	—	—
Ennulat[2]	77.5	14.4	41.0	—	—	—	[78.1	0.39	1.09]	82.6	0.29	0.82
Porter *et al.*[6]	—	—	—	79.6	14.2	40.3	[64.0	0.36	1.07]	80.0	0.28	0.82
Davis[3]	—	—	—	77.3	14.0	40.0	[76.5	0.36	1.04]	81.6	0.28	0.78

All DSC temperatures from the present study are accurate to ±0.07 °C except as specified by an omitted place where the accuracy is ±0.1 °C.

(*a*) °C, temperature at endothermal minimum for DTA or DSC measurements; (*b*) Kcal/mole; (*c*) cal/mole/°K; [] monotropic transition.

Holland, was recrystallized from hot ethanol three times, washed with cold ethanol and dried for 24 hours at 10^{-6} Torr. This produced a fine needle-like product with no odor of acid or oxidized cholesterol. Thin layer chromatography (TLC) on silica plates in air using a 1 : 1 tetralin-hexane mobile phase produced only a single spot.[14] Thin layer chromatography under a nitrogen blanket produced two spots, one for the ester and a very small spot for the impurity, $R_f = 0.65$. This material will be referred to in this text as the van Schuppen sample.

A sample of the palmitate ester was synthesized from cholesterol and palmitic acid, both recrystallized from ligroin. The palmitic acid was obtained from Eastman Kodak and was recrystallized from ligroin two times. The cholesterol was obtained from van Schuppen Chemicals as a specially prepared high purity material (99.98 mole % by DSC). It is not known if van Schuppen Chemicals used this identical lot in the preparation of their ester. Into a flask equipped with a Stark tube (to monitor water production) was placed 0.064 mole cholesterol, 0.0722 mole palmitic acid, 1 g. *p*-toluene sulfonic acid and 150 ml ligroin. The mixture was refluxed 90 minutes until 95.5% of the cholesterol had reacted as indicated by the water in the Stark tube. The reaction mixture was transferred to a separatory funnel and washed successively with five 10 ml portions of hot 20% ethanol-water, five 10 ml portions of hot 26% ethanol-water, 2% sodium carbonate and additional ethanol/water until a blue litmus reaction was obtained. The ethanol/water mixture was used to facilitate the removal of the unreacted cholesterol (although some ligroin solubility is unavoidable). The ligroin phase was dried over anhydrous sodium sulfate. The dry ligroin phase was reduced to 50 ml. and allowed to stand at room temperature over night. This produced a fine suspension which was chilled (to coagulate the suspension), filtered, washed with cold ligroin, recrystallized twice from acetone and vacuum dried at 10^{-6} Torr. This product was put through the same recrystallizations as the van Schuppen sample to insure identical products as nearly as possible. This material will be referred to in this text as the synthetic sample.

Time average NMR spectra of both the van Schuppen and synthetic esters were identical. This technique is capable of detecting isolated proton resonances present to an extent of $> 0.05\%$ under

favorable conditions. This should be applicable for most non-cholesterol-based impurities.

Scanning Calorimetry

The technique employed for thermal and DSC purity measurements has been given elsewhere.[15-17] The scanning calorimeter traces were quite different for the two samples, Figs. 1 and 2. The van Schuppen sample had an indicated purity of 97.31 mole % and exhibited three transitions on heating (solid → smectic, smectic → cholesteric and cholesteric → isotropic liquid). The data are given in Table 1. The synthesized sample had an indicated purity of 99.52% and exhibited in the DSC only two transitions on heating (solid → cholesteric and cholesteric → isotropic liquid). The data are given in Table 1. Both samples showed three transitions on cooling.

Fractional Solution

The van Schuppen sample was submitted to the following fractional solutions and recrystallizations:

1. Wash with 100 cc acetone in ultrasonic bath—4–5 min.
2. Vacuum dry sample.
3. Wash with 100 cc ethanol in ultrasonic bath—4–5 min.
4. Vacuum dry.
5. Dissolve in 100 cc acetone/ethanol solution 50/50 by boiling on hot plate. Cap and bring to room temperature under fume hood.
6. Vacuum dry sample.
7. Dissolve in 100 cc acetone/ethanol solution 50/50 by boiling on hot plate. Cap and put in refrigerator to precipitate out crystals rapidly.
8. Vacuum dry at 10^{-8} Torr for 24 hours.

This treatment produced a material thermally *identical* with the synthetic sample. The success of partial solution of the crystal *surface* in the ultrasonic bath indicates that the impurities are sorbed on the crystal after growth is complete, i.e., during the digestion period. Partial solution probably takes advantage of the change in solubility with temperature between the desired ester and the impurity. Vacuum sublimation was not successful in removing the impurity.

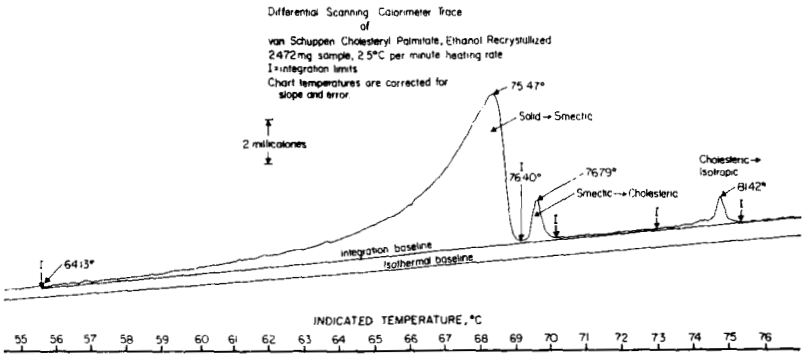

Figure 1.

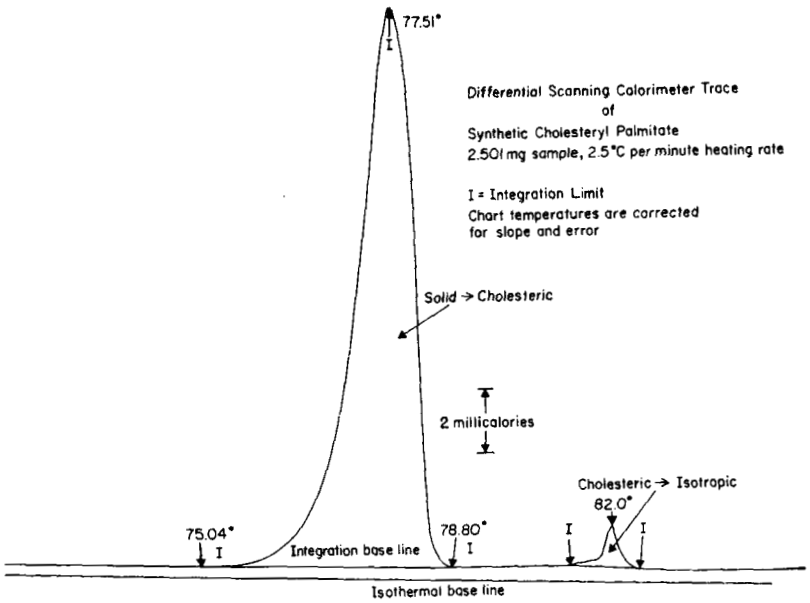

Figure 2.

Microscopy

The samples were observed and photographed with a Ziess Ultraphot II microscope between crossed polarizers. The samples were heated and cooled on a Mettler FP-2 stage equipped for both heating and cooling. The amount of light rotated (DLI) was measured by a simple photometer similar to that described previously[18-20] on the second melting of the sample.

3. Results

Microscopy and DLI

The unusual behavior of the van Schuppen and synthesized cholesteryl palmitate esters was first noted on routine microscopic examination of the samples. This examination showed a well defined smectic structure was formed on heating the van Schuppen sample, see Figs. 3a, b and c. In addition, a transient smectic form appeared

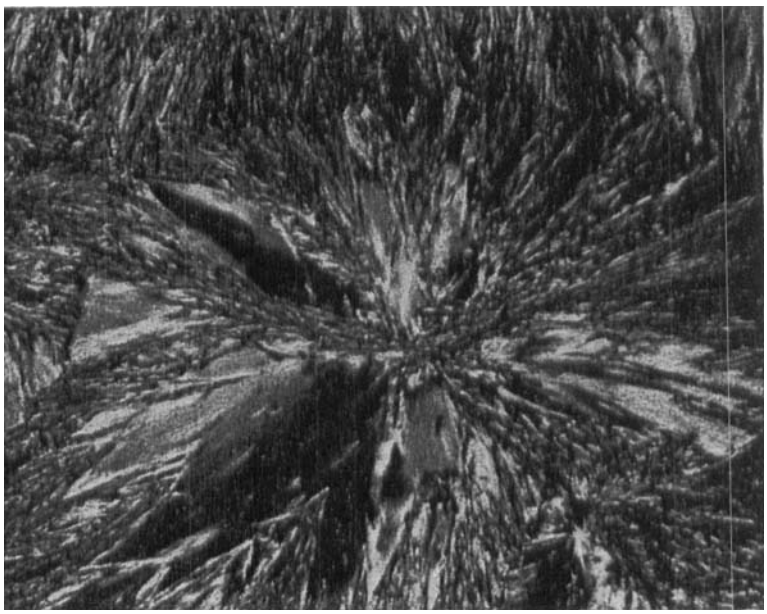

Figure 3a. Photomicrograph of van Schuppen cholesteryl palmitate. Solid phase at 50 °C, 200 ×, 1 mm thick sample between coverslip and slide, cross polarizers.

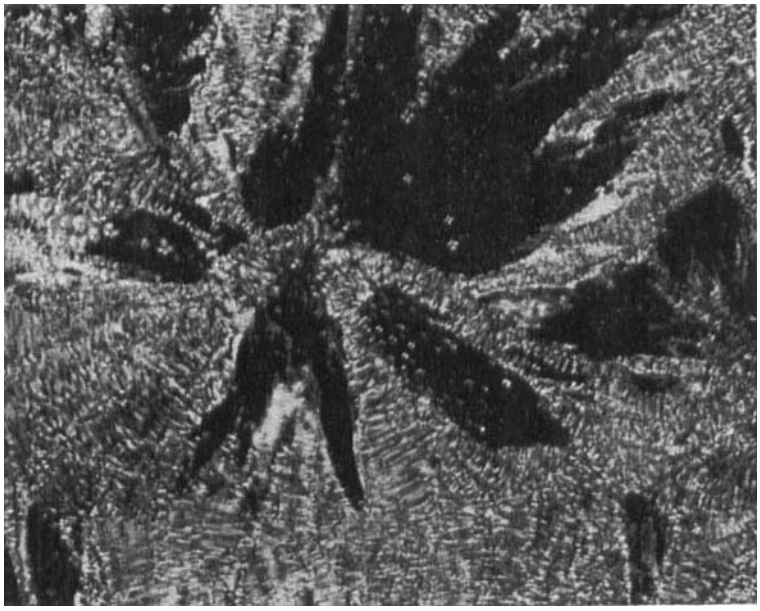

Figure 3b. Photomicrograph of van Schuppen cholesteryl palmitate. Smectic mesophase forming at 75.8 °C on heating, same field and conditions as Fig. 3a.

in the synthetic sample on heating. A smectic front passed across the sample as the solid converted to the cholesteric mesophase. This is illustrated in Fig. 4.

The smectic mesophase in the van Schuppen sample is shown in the DLI curve, Fig. 5. The smectic form appears as a decrease in the depolarized light intensity from 70.5 to 74.3 °C. Above 74.3 °C the DLI increases to a level slightly higher than the initial sample. At 76.3 °C the smectic mesophase streams and rapidly converts to the cholesteric texture. At 76.7 °C the cholesteric texture begins to "*open*" with dark areas (substrate-induced fast vibration direction aligned with the polarizer) growing larger. At 80.9 °C the cholesteric texture begins to stream rapidly and flash (see spike at 80.9 °C on Fig. 5). At 80.95° on the DLI temperature axis, the cholesteric texture transforms to the isotropic liquid and the DLI drops to instrument zero. The lower legend on Fig. 5 refers to optical observation during heating. A small trace (a haze) of the cholesteric meso-

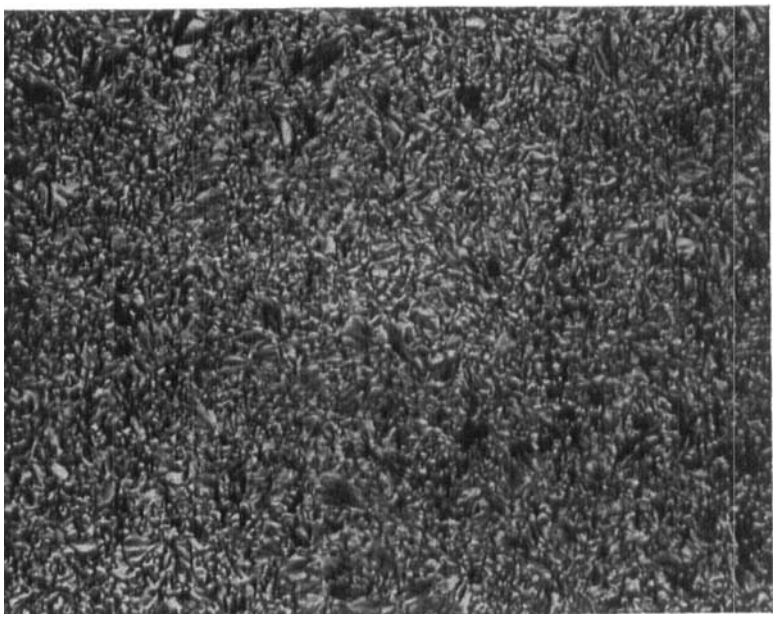

Figure 3c. Photomicrograph of van Schuppen cholesteryl palmitate. Fully perfected and thermally stable smectic mesophase formed on heating at 76.6 °C. Same field and conditions as Fig. 3a.

phase persists beyond the conclusion of the transition by photometer. This could be due to a small thermal gradient around the field.

The DLI curve of the synthetic sample, Fig. 6, shows a step in the DLI curve between the solid and cholesteric forms. During this step the transient smectic phase appears to the observer. No alteration was noted on either the van Schuppen or synthetic sample on repeated reheating.

Scanning Calorimetry

The DSC curve on the synthetic sample, shown in Fig. 2, resembles work reported previously as illustrated by the data in Table I in the number of transitions and transition temperature on heating. The solid → cholesteric mesophase transition agrees well with the observations by Sell[10] and Davis.[3] The cholesteric → isotropic liquid transition of the synthetic sample is above that of Sell[10] and Porter *et al.*[6] but agrees well with those of Davis,[3] Ennulat,[2] and

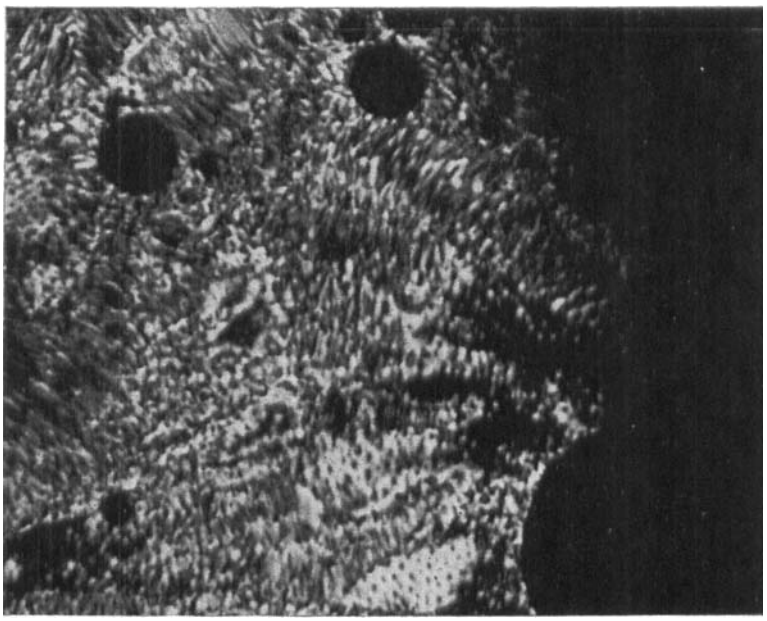

Figure 4. Photomicrograph of Synthetic cholesteryl palmitate. Smectic front (middle) is shown passing through the solid phase (bottom) as the cholesteric mesophase (top) is formed at 78.0 °C. The conditions are the same as those given for Figure 3a.

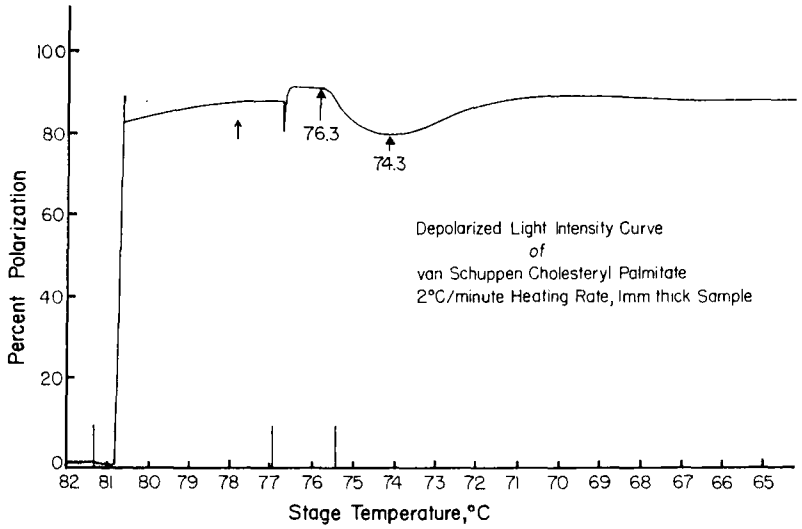

Figure 5.

Gray.[9] The palmitate sample originally studied by Porter, Barrall, and Johnson[6] was probably impure, ~96%, by present standards. The cooling thermogram of the synthetic sample agrees in number and temperature of transitions with previous work. It is also interesting to note that there is generally excellent agreement between all previous workers who measured transition entropy—even mesophase entropies. This is particularly noteworthy considering the small size of these transitions and the well recognized effects of certain impurities on transition range and temperature.[2,7,8]

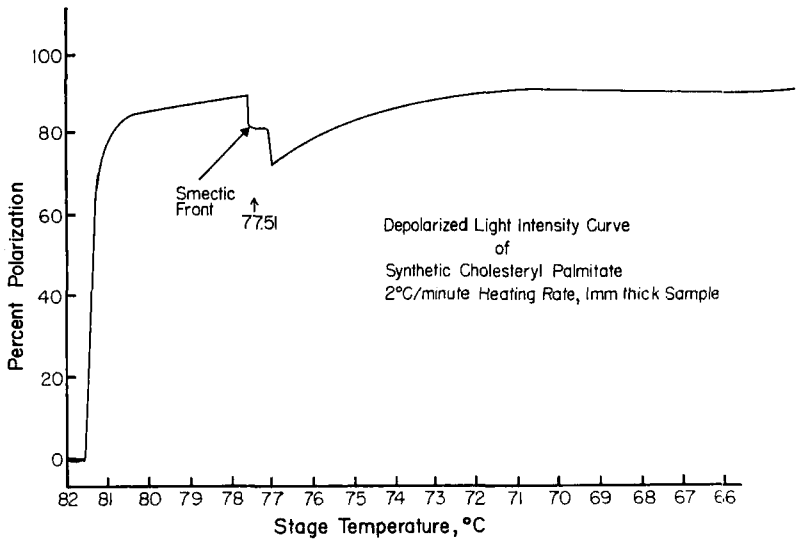

Figure 6.

The van Schuppen sample reproducibly gives a unique heating thermogram, Fig. 1. The smectic phase appears thermally, without ambiguity, well separated from the large thermal event. Separate portions of impure sample were recrystallized once from ethanol, acetone, hexane and gave identical results. The smectic → cholesteric transition temperature and entropy compare closely with those for the synthetic sample and the data reported by Davis.[3] In addition, the transition occurs at a lower temperature than that observed by Gray[9] and Ennulat.[2] The cholesteric isotropic liquid transition temperature is in agreement with that observed by Davis.[3] The

purity of the sample calculated from the solid → smectic transition was found to be 97.31 mole %. Similar calculations on the smectic → cholesteric and cholesteric → isotropic liquid transitions gave purities of 99.53 mole % and 99.99 mole %, respectively. This unusual variation suggests that an impurity is present which is insoluble in the solid phase (excluded from the lattice), but about equally soluble in the smectic, cholesteric and isotropic liquid phases. In a previous study of cholesterol heptadecanoate,[8] the impurity present in that sample (the unreacted acid) was found to be soluble only in the isotropic liquid. Ennulat[4] has obtained similar data on cholesteryl nonanoate.

4. Conclusions

The sample of cholesteryl palmitate prepared in our laboratory from carefully purified cholesterol and palmitic acid compares very closely in all ways with material described by at least three other groups.[2,3,9] However, a sample of the palmitate ester obtained from van Schuppen Chemicals was somewhat different. This material shows a clearly defined sharp smectic mesophase on heating as well as the previously reported cholesteric mesophase. Differential scanning calorimetry indicates that the solid to smectic transition is relatively broad and characteristic of material with purity no higher than 97.31 mole %. However, the mesophase transitions are very sharp and characteristic of material of at least 99 mole % purity. In addition, thin layer chromatographic techniques, which have been demonstrated to be sufficiently sensitive and efficient to separate as little as 0.05% unreacted acid, alcohol, and cholesterol, do not show a trace of impurity. Contamination of the sample with other esters with the same retention time is also ruled out by the same method. A previously completed study of adsorbed solvent phenomena indicates that the van Schuppen sample is solvent free.[7] An earlier study of cholesteryl heptadecanoate has shown that as little as 0.1% unreacted starting material or solvent greatly broadens and shifts the mesophase transitions to lower temperatures by several degrees.[7,8,12] Such is not the case with the van Schuppen sample. For the above reasons, it is necessary to search for an impurity which is insoluble in the solid phase but which is equally soluble in the

smectic cholesteric and isotropic liquid phases. Since it is the entropy of mixing that results in peak broadening and transition point depression, this would be absent in true solutions. The effects of impurity solubility have been described in detail previously.[7,12] This postulated impurity, ~2% of the sample, was found only after exhaustive analysis by thin layer chromatography. The impurity is an antioxidant added to the ester by van Schuppen. This material was overlooked in first trials by thin layer chromatography due to oxidation during the chromatographic process. Only when oxygen was carefully excluded did a recognizable spot develop with an R_f of 0.65. The NMR in deoxygenated solvent showed an " Ionol " type antioxidant.

Therefore, it appears that by introduction of the proper impurity it is possible to move the solid → mesophase transition to a lower temperature. This shift reveals the presence of a normally monotropic mesophase on heating. Similar work with impurities and two component systems has been reported in detail by Dave *et al.*[13,21,22] for nematic mesophase forming materials. The van Schuppen palmitate ester behavior can best be explained by a selective impurity rather than be separate crystal forms of the solid.

Indeed, monotropic mesophases would not appear on cooling were it not for the tendency of the transition to the solid to supercool. Earlier work has shown that the monotropic propionate ester will not form a mesophase at all on cooling if the cooling rate is less than 0.5°/minute. The solid nucleates at a temperature below the melting point but above the cholesteric mesophase formation temperature.[23]

This study has demonstrated the role of a very selective impurity in depressing the transition temperature of a single phase. The mesophases, normally regarded as very sensitive to impurity, were left relatively unaffected. The demonstrated possibility of such an impurity further complicates the solid phase properties of the cholesteryl ester mesophase forming materials.

Acknowledgements

The authors wish to thank Mr. T. T. Horikawa of IBM Research, San Jose, for running the NMR spectra of both van Schuppen and synthetic palmitate esters, Mrs. Barbara Dawson for the thin layer

chromatography of the samples, and Mrs. Margene Yeaton for typing the manuscript.

Comment

When this paper was first presented, the authors suggested that the then unidentified impurity was an ester of the cis isomer of cholesterol. However, upon consultation in Holland by one of us (M.J.V.) with van Schuppen Chemical, we learned of the addition of an " antioxidant." Careful anaerobic thin layer chromatography and NMR in nitrogen saturated solvent then demonstrated the antioxidant in all samples of the palmitate ester which showed a smectic mesophase on heating. Oxidation during the routine NMR studies apparently removed the antioxidant and caused it to smear out during the thin layer chromatography. The fractional solution studies indicate that the antioxidant is adsorbed on the surface of the crystals during the normal " digestion " period. The authors are grateful for the generous cooperation of van Schuppen Chemicals in this search for the elusive " impurity."

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