Thermal, purity, and solubility properties of cholesteryl esters and their thermal behavior in lipid-water systems.

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THERMAL, PURITY, AND SOLUBILITY PROPERTIES OF
CHOLESTERYL ESTERS AND THEIR THERMAL BEHAVIOR
IN LIPID-WATER SYSTEMS

A Dissertation Presented
by
Gershon Jerry Davis

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THERMAL, PURITY, AND SOLUBILITY PROPERTIES OF
CHOLESTERYL ESTERS AND THEIR THERMAL BEHAVIOR
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A Dissertation Presented
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Gershon Jerry Davis

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(Chairman of Committee)

(Head of Department)

(Member)

(Member)

December 1969
To My Family
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CHAPTER VI

1. Cholesteryl Esters of C_{18} Aliphatic Acids, Temperatures of Transition vs. Unsaturation
SUMMARY

PREFACE

This investigation consisted of a thermodynamic evaluation of phase behavior and structure in systems involving cholesterol and its esters. The goal was to develop thermodynamic data and structural information on biological systems related to membrane organization and specifically to the nature of atherosclerotic plaques which lead to heart disease.

The experimentation was divided into four major areas of research: quantitative determination of ester purity, measurement of ester heats and temperatures of transition, characterization of solubility properties for cholesteryl esters, and studies on ternary phospholipid-cholesteryl ester-water systems. Each subject will be summarized under its own heading.

PURITY PROCEDURE

A new method is presented for the calculation of the purity of organic, non-polymeric samples from Differential Scanning Calorimeter (DSC-1B) recorder melting traces. It is based upon a probable DSC mechanism of power transfer and measurement. Ambiguities in the one previous method offered by Perkin-Elmer, the DSC manufacturer, are reduced. The entire DSC recorder trace is employed rather than only the initial portion of the curve. By application of this information, a rapid and meaningful purity determination has been developed from information on a single DSC trace for samples which range from 95 to 99.9% purity. An accuracy within 15% of the actual impurity value can be anticipated.
CHOLESTERYL ESTERS OF SATURATED ALIPHATIC ACIDS

The heats and temperatures of transition for fourteen cholesteryl esters of saturated aliphatic acids have been evaluated using a Differential Scanning Calorimeter, DSC-1B. The transition data obtained in this study indicated that the lowest molecular weight saturated aliphatic esters of cholesterol to show mesophase behavior was the propionate, not the formate or acetate. Less pure samples may be supercooled in the melt so that mesophases may be observed for the two lowest molecular weight esters. New transition data for the odd esters of cholesterol from undecanoate to nonadecanoate have also been obtained. Cholesteryl eicosanoate was examined and found to have only one transition, both on heating and cooling, which may indicate that the highest members of this series, when relatively pure, do not display mesophase behavior.

SOME SOLUBILITY CHARACTERISTICS OF CHOLESTERYL ESTERS

In this study of some solubility characteristics of cholesteryl esters, n-pentanol is shown to be a more effective solvent than ethanol in which to remove impurities and/or crystalline structural defects from cholesteryl esters. These impurities can cause additional transitions on initial heating. n-Pentanol is shown to be a regular solvent for cholesteryl esters whereas ethanol is not. This could be a major reason why n-pentanol is better able to discriminate between compound and impurity than ethanol. Contrary to previous reports, after crystallization from n-pentanol, in contrast to ethanol, cholesteryl acetate and cholesteryl laurate have only one transition on first heating. A
detailed study of cholesteryl benzoate recrystallized from n-pentanol has confirmed that there are two transitions, one at 146.0°C, the second at 179.6°C. The heats of transition are found to be 14.7 cal/gm and 0.26 cal/gm, respectively.

THERMAL STUDIES ON LIPID-WATER SYSTEMS

Lecithin and sphingomyelin organize in the presence of water into bimolecular layers separated by water. This membrane-like organization can be followed thermally with a Differential Scanning Calorimeter. To these binary systems were added either cholesterol or a C_{18} fatty acid ester of cholesterol - stearate, oleate, or linoleate. The unsaturated esters are abundantly present in atherosclerotic plaques. The addition of cholesteryl esters reduces the entropy of transition in the order linoleate > oleate > stearate. This effectiveness in reducing the entropy of transition is surmized to be directly proportional to the participation of the cholesteryl ester in the membrane structure. All transition temperatures on cooling were within 40.2 ± 1.5°C, quite close to human environmental temperatures.
CHAPTER I

INTRODUCTION

Interest of Sponsor

This work was supported by the U.S. National Institutes of Health. In the United States, disorders of the heart caused approximately one-third of all deaths in 1964\(^1\). Coronary heart disease accounts for 80% of all cardiac deaths\(^1\). The term coronary disease is a general designation for all forms of myocardial (heart muscle) disorders resulting from insufficient coronary blood flow. In 99% of the cases, such insufficiency is due to atherosclerotic narrowing of the coronary arteries, with or without complications\(^1\).

Atherosclerotic narrowing is produced by atheromatous plaques consisting of fatty-fibrous deposits within the intima of the arterial wall which projects into the lumen of the vessel\(^1\). Cholesterol, both free and esterified, is the major lipid component of plaques in the human aorta intima in all stages of the development of atherosclerosis. The source of this cholesterol is one of the outstanding problems in atherosclerosis research\(^2\). Characterization of the physical properties of cholesteryl esters and related systems may provide information important to the resolution of this problem.

Characterization of Cholesteryl Esters

The first of two major areas of research pursued in this work was the measurement of thermal transitions of individual cholesteryl esters on a Differential Scanning Calorimeter, Perkin-Elmer Corporation.
Model DSC-1B. However, heats and temperatures of transition increase as sample purity increases. As a result of this fact, suitable recrystallization techniques were required to purify the cholesteryl esters and a reliable method of purity evaluation had to be devised. This led to experimentation on the following three problems:

1. Development of an improved procedure for determining the amount of impurity in simple, non-polymeric, organic compounds.

2. Measurement of the transition temperatures and heats exhibited by cholesteryl esters of saturated aliphatic acids from formate through eicosanoate.

3. Determination of some solubility characteristics of cholesteryl esters.

There are three chapters in the thesis which describe the results and conclusions derived from experiments in each of these three problem areas. The following technical articles have been written from this information:


Ternary Systems Containing Cholesteryl Esters

The application of Differential Scanning Calorimetry to problems in atherosclerosis is a relatively unexplored field of research. The basic idea here was to study the thermal properties of phospholipid-water systems containing cholesteryl esters. The compounds chosen for this study (lecithin, sphingomyelin, and saturated and unsaturated C₁₈ fatty acid esters of cholesterol) are the major components in the abnormal human plasma lipid associated with atherosclerosis. Previous DSC studies have been made on lipid-water systems in which one of the components was cholesterol in order to shed light on the possible role of cholesterol in biological membranes. Other experimenters have studied the transition temperature (41°C) for dipalmitoyl lecithin, a major lipid in the lung membrane, in water by differential thermal analysis and discussed the implication of the transition being close to body temperature.

The fourth chapter following the Introduction presents the results and conclusions from DSC studies of lipid-water systems containing cholesteryl esters. In this chapter, the relationship between atherosclerosis and the formation of biological membranes is suggested. A technical article has been written from Chapter V by G. J. Davis and R. S. Porter entitled "Thermal Studies on Lipid-Water Systems by Differential Scanning Calorimetry with Reference to Atherosclerosis." A revised manuscript has been prepared on this subject for journal submission.
In Chapter VI is presented the heats and temperatures of transition of the individual cholesteryl esters of C\textsubscript{18} aliphatic acids. The biological importance of these esters has been emphasized in Chapter V.

Temperature Calibration of the DSC

The DSC, in common with other thermometric devices, requires calibration to determine the correspondence of program temperature to true temperature\textsuperscript{6}. This correspondence is obtained by setting a ten-turn potentiometer called the Average Temperature Calibration Dial. The most convenient method is to leave this dial in a fixed position and to measure the program temperature for a number of highly pure compounds whose temperature is known accurately through another method. This temperature determination requires an adjustment procedure, described by the Perkin-Elmer Corporation\textsuperscript{6} for a 99.999\% pure indium sample. The difference between the true melt temperature and the program temperature is the correction to the program temperature. A plot of correction to the program temperature versus program temperature\textsuperscript{6} gives a parabolic curve which can be applied to correct the program temperature for samples whose true melt temperature is not known. This correction curve should be applied to only those heating rates for which it was devised.

A major disadvantage of the Perkin-Elmer procedure is that by fixing the Average Temperature Calibration Dial at one setting, temperature corrections as large as 5\textdegree{}C are not uncommon. An alternate
procedure utilized by this experimenter was prepare a plot of sample temperature versus Average Temperature Dial setting, shown in Figure I-1, by determining the accurate setting to give the true melting point of a number of relatively pure compounds of known melt temperature. These compounds were four Fisher triple point standards, adipic acid, benzoic acid, naphthalene, and p-nitrotoluene. Indium from Perkin-Elmer and stearic acid and octadecane from Applied Science Laboratories were also used. In Figure I-1, the lower scale refers to a second range on the DSC, especially useful for lower temperature determinations, which subtracts 100°C from the program temperature reading. The values plotted in Figure I-1 are applicable for both the 1.25 and 2.5°C/min. heating rate. A second plot, Figure I-2, of temperature change per 100 average temperature dial units versus specific program temperature (information provided from the Perkin-Elmer instruction manual) allows for the following correction procedures when the Average Temperature Dial setting does not match the program temperature in Figure I-1:

1. Find in Figure I-1 the proper Average Temperature Dial setting for the particular program temperature under consideration.

2. Subtract the Average Temperature Dial Setting used in the experiment from that determined in (1).

3. Multiply this difference by the temperature change/100 Average Temperature Dial Units for this program temperature found in Figure I-2.
4. Add this value to the program temperature to obtain the true temperature.

For this somewhat more complex method, the temperature correction is generally less than 1°C.

References for Chapter I

FIGURE I-1

TEMPERATURE CALIBRATION CURVE FOR DSC for heating and cooling rates of 1.25 & 2.5 °C/min.

STANDARD UPPER SCALE

- INDIUM
- ADIPIC ACID
- BENZOIC ACID
- NAPHTHALENE
- STEARIC ACID
- p-NITROTOLUENE
+ OCTADECANE

Sample Temperature (°C)

Average Temperature Dial Setting
FIGURE I-2

TEMPERATURE CORRECTION FACTOR FOR AVERAGE TEMPERATURE DIAL SETTING

Temperature Change /100 Av. Temp. Dial Units

Temperature (°C)

0 100 200 300 400
CHAPTER II
APPLICATION OF THE DIFFERENTIAL SCANNING CALORIMETER TO PURITY MEASUREMENTS

Introduction

Several hundred papers have been published which involve the use of the Differential Scanning Calorimeter (DSC). Over forty papers involving the DSC were given at a single meeting in 1968\(^1\). Several of the authors at this meeting, G. T. Driscoll, et al., and R. D. Ennulat, and scientists publishing elsewhere\(^2,3\) have utilized the DSC to determine sample purity by a method published by Perkin-Elmer, the DSC manufacturer\(^4-6\). G. T. Driscoll, et al.,\(^1\) have demonstrated that this method can give potentially misleading results. The purpose of this chapter is to detail the ambiguities of the Perkin-Elmer method\(^4,5\) and to offer an alternative procedure for estimation of the purity of organic, non-polymeric samples by DSC.

Perkin-Elmer\(^4\), in its purity method, recommends that measurements be made on the initial portion of the DSC recorder melting trace only, preferably in the range from 10 to 50% of the area enclosed by the trace and the baseline. Driscoll, et al.,\(^1\) give an example of a purity determination by this method in which the mole per cent impurity calculated can be changed by more than a factor of ten by varying the lower and upper limits of this range. What is therefore needed is an improved method for purity calculation by DSC which employs the entire recorder melting trace and which does not involve an arbitrary choice
of only a part of the forward section of the DSC trace. Such a procedure is outlined in this paper.

As stated by the manufacturer, except for samples of very high purity, a significant amount of melting occurs before the melting curve rises detectably above the baseline. In this purity method, Perkin-Elmer implies that this totally unrecorded energy is a value which can be adjusted until the plot of sample temperature vs. reciprocal of the mole fraction of sample melted fit along a straight line. However, with relatively pure samples, the addition of this unrecorded energy to the energy measured by the DSC trace results in heat of melting values which are too high. Although the estimation of how much of the actual sample transition heat has not been recorded by the DSC is a principle problem, the value of this lost heat can be quite definite. Consider the compound, naphthalene, whose heat of melting is well established at 35.0 calories per gram for a theoretically 100% pure, crystallizing compound. Mastrangelo, et al., have determined the heat of melting of naphthalene containing various mole fractions of thionaphthene by adiabatic calorimetry. Their data are presented in the first two columns of Table II-1. As a simple mathematical model, the energy required to melt the sample can be attributed totally to the naphthalene present and the thionaphthene can be assumed to be dispersed in the naphthalene so as not to form crystals. At 0.9444 mole fraction naphthalene, this model can be shown to be applicable since a $\Delta H$ of 35.0 calories per
gram, as shown in column three of Table II-1, for naphthalene permits an accurate calculation of the transition heat. Thence from 0.9444 to 0.9958 mole fraction naphthalene, the sample heat of fusion in cal/gm, ΔH, can be treated as the following linear function of the mole fraction naphthalene, Na:

\[ ΔH = 35.0 N_a \]  

(II-1)

In this laboratory, a Fisher triple point naphthalene sample was diluted with 0.05 mole fraction of 1,2 diphenyl ethane with which it forms an ideal solution in the melt⁸. The resultant sample was melted at a heating rate of 2.5°C per minute in the DSC which gave a recorder trace corresponding to 21.0 calories per gram. From the mathematical model, 1.8 calories per gram would be the loss from the reduction in mole fraction of naphthalene and 12.2 calories per gram the energy unrecorded by the instrument. The unrecorded energy was 87% of the difference between the energy of the highly pure naphthalene and the energy of the impure sample, as determined by DSC. Similar results were obtained with Fisher triple point benzoic acid samples containing from 0.01 to 0.04 mole fraction naphthalene when run on the DSC at a heating rate of 2.5°C per minute. Their unrecorded energy varied from 85 to 89% of this difference when the sample heat was treated as a linear function of the mole fraction of benzoic acid present. A third system consisting of Fisher triple point naphthalene samples containing 0.01 to 0.04 mole fraction p-nitrotoluene melted
at a rate of 2.5°C/min. had unrecorded energy of from 83 to 88% of this difference when the sample heat was treated as a linear function of the mole fraction of naphthalene present. In the purity method to follow, for samples melted at a heating rate of 2.5°C per minute, 87% of the difference between the heat of melting of a highly pure sample and that of an impure sample, as determined by DSC, was estimated to be unrecorded heat. This estimate of per cent heat loss may eventually be shown to decrease a per cent or two as the sample size is increased from 3 to 6 mg.

The important property of a DSC trace, which is not known with reasonable certainty, is the location on the trace for the temperature where the sample can be considered to have just finished melting. A clue to its location comes from studies of melting point depression by impurity. Experiments have been made in this laboratory on systems of naphthalene diluted with 1,2 diphenyl ethane and cholesteryl myristate diluted with toluene. Tremendous broadening of the temperature range for the DSC melting peak trace is observed with increasing sample impurity. However, an excellent straight line relationship was achieved between the reciprocal of the melt temperature, when designated as occurring at the curve peak, and the log of the mole fraction of the major component, as shown in Figures II-1 and II-2. This means conformance with the Van't Hoff equation. The heat of transition calculated from the Van't Hoff equation for these systems is compared to the known value for naphthalene and cholesteryl myristate in Figures II-1 and II-2 and found to be in error by -12.3% and -2.7%
respectively. This new procedure for the determination of sample purity by DSC is based on a trial and error method to estimate the location, near the curve peak, of where the sample has just finished melting.

Function of the Electrical Loops in the DSC

Any valid method for determinations of sample purity requires an understanding of instrument operation. Transition temperatures and heats are determined from the DSC by measuring the differential power required to maintain the same temperature program on two nominally identical and symmetrically placed cups. One cup holds the weighed samples in a sealed planchet; the second cup is the differential reference. Electrical power is supplied to the reference and sample holders through the operation of two essentially separate electrical control loops. An average temperature loop maintains the cups at a preset heating rate. The power for this loop is supplied equally to both cups and is not recorded. This loop operates by comparing the average temperature of the two cups to that demanded by the programmer, and the power required always is provided equally to both cups as needed. The temperature program is marked out on the abscissa of the DSC recorder.

A separate electrical loop is used to handle temperature differences between the cups. This second loop senses the temperature difference between the sample and reference cups and supplies the differential power needed to compensate for the difference; this is the power input indicated on the DSC recorder ordinate. Before and
after a sample transition (for conditions where the temperature of
the sample cup does not change relative to the reference cup tempera-
ture), i.e., no transition or heat capacity difference, the differen-
tial power supplied to both cups is the same. During an endothermic
transition, however, differential power is subtracted from the
reference cup circuit and added to the sample circuit. The DSC
chart ordinate changes by an amount proportional to the temperature
difference between sample and reference cups converted to units of
power, dq/dt or q, as the average temperature of the cups, T_p', is
programmed on the abscissa at a rate dT_p'/dt or T_p'. An exothermic
transition merely reverses the electrical process. Importantly, each
change in the differential temperature loop, as during the melting
of the sample, causes the average temperature loop to add compensating
power, divided equally between cups, to maintain the preset heating
rate. Note that the power supply for the average temperature loop
is an order of magnitude larger than that of the differential tempera-
ture loop.

The functions of the electrical loops described above could
provide a simple theoretical basis for a purity method. Unfortunately,
there appears to be a threshold temperature difference, recognized to
be several thousandths of a degree by the manufacturer, below which
differential heat is not transferred and q is not recorded. There
are at least two conclusions concerning the operation of the electrical
loops which influence potential methods for sample purity. In melting
a highly pure sample, for example, the power put in at the start of
the transition, to bring sample and reference cup temperatures together, is provided presumably by the average temperature loop. This energy addition and the energy required below the threshold response of the differential loop mentioned in the previous paragraph are both made by the average temperature loop and neither are recorded. The energy put in during the initial portion of a transition by the average temperature loop must eventually be transferred to the part of the recorded $q$ curve that occurs after the sample has undergone the maximum melting rate, which is the region where the temperature of the sample cup must catch up to the temperature of the reference cup.

For relatively impure samples, a large fraction of the sample can be melted over a wide temperature range before the threshold temperature difference is reached and the differential temperature loop responds. The average temperature loop provides whatever extra power is required to maintain $T_p$; this power is not recorded and little, if any, of it appears in the latter portion of the transition curve.

Immediately after the sample is completely melted, the average temperature loop returns to its operating level prior to the transition; however, the sample and reference cups are still at different temperatures. The differential temperature loop must continue to transfer energy until these temperatures are equalized. Thus, the recorded energy transferred by the differential temperature loop after melting is energy that had been added by the average temperature loop during melting.
Analysis of the DSC Recorder Trace

Figure II-3 presents a trace, exactly as it occurred on the recorder graph of a model DSC-1B, for the crystal-mesophase transition of cholesteryl propionate, sample obtained from Applied Science Laboratories, Incorporated, State College, Pennsylvania. The area between the curve and the baseline, AGC in Figure II-3, is a measure of the transition heat. The abscissa of Figure II-3 represents time and/or temperature. For example, at a $T_p$ of 2.5°C/min., each mark on the abscissa means 1.0°C or 0.4 minutes.

The level of impurity in a sample can be calculated from DSC data and the Van't Hoff equation which relates sample temperature, $T_s$, to sample mole fraction, $F$, melted at $T_s$:

$$T_s = T_o - (RT_o^2N/\Delta H) (1/F) \quad (II-2)$$

where

- $\Delta H = \text{heat of fusion of the pure major component}$
- $R = \text{gas constant}$
- $N = \text{mole fraction of impurity}$
- $T_o = \text{melting point of the pure major component}$

A plot of $T_s$ vs. $1/F$ will be a line of slope $-RT_o^2N/\Delta H$. A knowledge of $\Delta H$ obtained from the area under the DSC curve or from the literature permits a calculation of $N$, the mole fraction of impurity. The above equation was developed from Raoult's Law and in most cases
it can be expected to hold only for high purity samples, >95%. Also implicit in the equation is the assumption that the impurity must be insoluble in the solid yet completely soluble in the melt.

A sample temperature, \( T_s \), can be derived from any point on the DSC trace \( AGC \). Take point E as an example. Drop a line from E perpendicular to the abscissa intersecting the baseline at H. The line EH is proportional to the temperature difference between the reference, \( T_r \), and the sample, \( T_s \). The relationship between the programmed temperature \( T_p \), \( T_s \), and \( T_r \) is presented in the following equation:

\[
T_s = T_p - \frac{1}{2} (T_r - T_s) \quad \text{(II-3)}
\]

The proportionality constant to determine the actual temperature represented by EH is derived from the shape of the DSC trace for an extremely high purity sample, e.g., 99.999% pure indium\(^4\), at the same heating rate of 2.5°C/min. If line EH is proportional to \( T_r - T_s \), then:

\[
R_o q = T_r - T_s \quad \text{(II-4)}
\]

where

\[
R_o = \text{the proportionality constant in } °C\text{-sec/millical}
\]

\[
q = \text{the actual value of EH in millical/sec}
\]
During the melting of the extremely pure indium sample, $T_s$, the actual sample temperature does not change. From the differentiation of Equations (II-3) and (II-4), $R_o$ can be evaluated for the indium sample since $\frac{d^2q}{dt^2}$ or $q$ is constant. $R_o$ is the valid proportionality constant for all distances between trace and baseline at a particular $T_p$ or heating rate. From Equation (II-4), the $T_r - T_s$ value of line EH can then be determined and substituted in Equation (II-3) to give $T_s$. All other points on trace AGC can be similarly converted to $T_s$ values. Each of these $T_s$ values then should be adjusted by reference to a temperature calibration correction curve similar to that presented by the manufacturer. This calibration curve is a plot of correction to the dial reading vs. dial reading, in degrees.

Generally samples change temperature throughout the tracing of the sample melting curve, AGC. Differentiation of Equation (II-4) would give:

$$R_o-q = T_r - T_s$$  \hspace{1cm} (II-5)

At the curve peak, $G$, $q$ must equal zero and $T_r$ equals $T_s$. This fact suggests that the sample must have completed melting before the curve peak is reached. Essentially all compounds analyzed in this laboratory appeared to have their melting point near but before the peak of the DSC trace.
New Purity Method Procedure

The method to be described for the calculation of \(1/F\), the reciprocal of the mole fraction of sample melted, is entirely different from that of Perkin-Elmer\(^4,5\). The following procedure is offered for estimating \(1/F\) at each \(T_s\):

 Arbitrarily choose a likely locale for the final sample melting point which will be prior to but near the peak, B, on the DSC trace; for example, choose point B in Figure II-3. The choice of this point is not critical because the plot of \(T_s\) vs. \(1/F\) must be a straight line with an intercept at \(1/F\) equals 1.00 at the \(T_s\) corresponding to point B (see previous temperature correction method). This point where \(1/F\) equals 1.00 corresponds to the melting of the last crystals, and, therefore, the theoretical melting point.

 Divide the curve into two sections with a line from B perpendicular to the abscissa which intersects the baseline AC at D in Figure II-3. Area ABD of Figure II-3 can subsequently be divided into arbitrarily chosen segments, such as area AEH, which can be considered as proportional to an uncorrected \(F\) value. The sample temperature, \(T_s\), at corresponding point E is determined as described previously. The uncorrected \(1/F\) value for an area AEH is:

\[
(1/F)\text{ uncorrect} = \frac{\text{Area AGC}}{\text{Area AEH}} \quad (\text{II-6})
\]
Area ABD represents energy added by the average power loop during the melting of sample, half to the sample holder and half to the reference holder. Therefore, energy of size \( \frac{1}{2} \text{ABD} \) is recorded by the differential temperature loop during the energy transfer represented by area BCD. Subtract \( \frac{1}{2} \) area ABD from area BCD to obtain an area P. This area P represents energy that was added by the average power loop before the differential power loop began to record and should be added to each chosen segment, such as area AEH. The value P implies that power transfer lags power input.

Calculate the trace area that a highly pure sample of equivalent weight would have and, from this value, subtract area AGC. Multiply the difference by 0.87. This is the area corresponding to the energy that was supplied by the average temperature loop, but not recorded by trace AGC. Call this value Q.

Area AEH should be further corrected by the addition of its share of the \( \frac{1}{2} \text{ABD} \) recorded by the differential temperature loop during the energy transfer represented by area BCD or \( \frac{1}{2} \text{AEH} \). The corrected \( \frac{1}{F} \) value for an area AEH would then be the following:

\[
\frac{(\text{Area AGC}) + Q}{3/2 (\text{Area AEH}) + P + Q} \quad (\text{II-7})
\]

The calculation of a complete series of \( 1/F \) values would be handled similarly.
Discussion of Results from the New Purity Method

Figure II-4 presents plots of \( T_s \) vs. \( 1/F \) for both uncorrected and corrected \( 1/F \) values. Note that the curvature is entirely removed with the corrected \( 1/F \) values and the line intersects the \( 1/F \) equals 1.00 line within 0.1 degree of its value at \( B \) from Figure II-3. This permits a direct calculation of \( N \), the sample impurity through the Van't Hoff relationship. The Perkin-Elmer method also linearizes the \( T_s \) vs. \( (1/F) \) uncorrected plot.

Estimates of the accuracy of the purity evaluations are presented in Table II-2 and II-3. Known amounts of naphthalene were added to benzoic acid (Table II-2) and \( \rho \)-nitrotoluene to naphthalene (Table II-3), all Fisher triple point samples. The samples were melted, recrystallized from the melt, and then remelted. The curves from the remelted samples were analyzed by this new method for purity. The data from both tables indicate that an accuracy within 15% of the actual impurity value can be anticipated in the 95 to 99.9% purity range.

This purity method is applicable to heating rates other than 2.5°C per minute by replacement of the 0.87 factor in determining \( Q \) with the one proper for that heating rate. For heating rates of 0.625 and 1.25°C per minute the factor is larger, that is, the ratio of the unrecorded energy to the actual energy loss due to impurities is even greater. Generally, these low heating rates are reserved for samples of high purity where \( Q \) is approximately equal to zero. The actual trace of a triple point benzoic acid sample from Fisher melted at a
heating rate of 0.625°C per minute is shown in Figure II-5. Its apparent melting point is marked by the letter "B." The plots of $T_s$ vs. $1/F$ of both uncorrected and corrected $1/F$ values for this benzoic acid sample are presented in Figure II-6. Curvature in the plot of sample temperature, $T_s$, vs. $1/F$ is removed by the procedure described before and a melt temperature, $T_m$, is determined.

A change in sensitivity or ordinate scale factor does not seem to effect the 0.87 Q factor significantly. Sample heats of transition measured at different sensitivities give equivalent results, within experimental error. The threshold temperature difference does not seem to be dependent on sensitivity.

A comparison can be made between the purity values estimated by the new method and the Perkin-Elmer method. The Perkin-Elmer method has been applied to the same benzoic acid-naphthalene system presented in Table II-2. The results are shown in Table II-4.

Note that the relative error by the Perkin-Elmer method is always negative, that is, the amount of impurity is always understated. The relative error can be as large as 30%. The Perkin-Elmer method appears to give impurity values 15 to 25% lower than the new method.

References for Chapter II


5. Perkin-Elmer Corporation, Thermal Analysis Newsletter, 6, Norwalk, Connecticut.


10. A. Gray, personal communication.
<table>
<thead>
<tr>
<th>Mole Fraction Naphthalene</th>
<th>$\Delta H$, cal/gm of Sample</th>
<th>$\Delta H$, cal/gm of Naphthalene</th>
</tr>
</thead>
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<tr>
<td>0.9958$^a$</td>
<td>34.92</td>
<td>35.0</td>
</tr>
<tr>
<td>0.9444</td>
<td>33.02</td>
<td>35.0</td>
</tr>
<tr>
<td>0.8095</td>
<td>29.00</td>
<td>36.1</td>
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</table>

$^a$ - Purity of original sample of naphthalene.
TABLE II-2

Summary of Benzoic Acid and Naphthalene Purity Results
By New Method Purity Procedure

Major Component - Benzoic Acid (Fisher Triple Point)

Impurity - Naphthalene (Fisher Triple Point)

<table>
<thead>
<tr>
<th>Mole % Added</th>
<th>Total % Impurity</th>
<th>% Experimental Impurity</th>
<th>Absolute Error %</th>
<th>Relative Error %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.24</td>
<td>0.24</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.77</td>
<td>1.01</td>
<td>1.12</td>
<td>+0.11</td>
<td>+10.9</td>
</tr>
<tr>
<td>1.07</td>
<td>1.31</td>
<td>1.24</td>
<td>-0.07</td>
<td>- 0.5</td>
</tr>
<tr>
<td>2.57</td>
<td>2.81</td>
<td>2.70</td>
<td>-0.11</td>
<td>- 3.9</td>
</tr>
<tr>
<td>3.91</td>
<td>4.15</td>
<td>3.85</td>
<td>-0.30</td>
<td>- 7.2</td>
</tr>
</tbody>
</table>

1 Experimentally determined from the recrystallized melt.
TABLE II-3

Summary of Naphthalene and p-Nitrotoluene Purity Results
By New Method Purity Procedure

Major Component - Naphthalene (Fisher Triple Point)

Impurity - p-Nitrotoluene (Fisher Triple Point)

<table>
<thead>
<tr>
<th>Mole % Added Impurity</th>
<th>Total % Impurity</th>
<th>% Experimental Impurity</th>
<th>Absolute Error %</th>
<th>Relative Error %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.38 (^1)</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.46</td>
<td>0.84</td>
<td>0.89</td>
<td>-0.05</td>
<td>- 6.0</td>
</tr>
<tr>
<td>1.10</td>
<td>1.48</td>
<td>1.67</td>
<td>+0.19</td>
<td>+12.8</td>
</tr>
<tr>
<td>2.66</td>
<td>3.04</td>
<td>3.17</td>
<td>+0.13</td>
<td>+ 4.3</td>
</tr>
<tr>
<td>4.32</td>
<td>4.70</td>
<td>4.89</td>
<td>+0.19</td>
<td>+ 4.0</td>
</tr>
</tbody>
</table>

\(^1\) Experimentally determined from the recrystallized melt.
### TABLE II-4

Summary of Benzoic Acid and Naphthalene Purity Results by Perkin-Elmer Purity Procedure\(^5\)

Major Component - Benzoic Acid (Fisher Triple Point)

Impurity - Naphthalene (Fisher Triple Point)

<table>
<thead>
<tr>
<th>Mole % Added Impurity</th>
<th>Total % Impurity</th>
<th>% Experimental Impurity</th>
<th>Absolute Error %</th>
<th>Relative Error %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.17(^1)</td>
<td>0.17</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.77</td>
<td>0.94</td>
<td>0.87</td>
<td>-0.07</td>
<td>-7.4</td>
</tr>
<tr>
<td>1.07</td>
<td>1.24</td>
<td>0.93</td>
<td>-0.31</td>
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</tr>
<tr>
<td>2.57</td>
<td>2.74</td>
<td>2.26</td>
<td>-0.48</td>
<td>-17.5</td>
</tr>
<tr>
<td>3.91</td>
<td>4.08</td>
<td>2.85</td>
<td>-1.23</td>
<td>-30.1</td>
</tr>
</tbody>
</table>

\(^1\) Experimentally determined from the recrystallized melt.
FIGURE II-1

NAPHTHALENE MELTING POINT DEPRESSION

major component: naphthalene
diluent: 1,2 diphenyl ethane

slope = 5.06 x 10^{-4}
$$\Delta H_{\text{VAN'T HOFF}} = 3,930 \text{ cal./mole}$$
$$\Delta H_{\text{ACTUAL}} = 4,480 \text{ cal./mole}$$
error = -12.3%
FIGURE II-2

CHOLESTERYL MYRISTATE MELTING POINT DEPRESSION

major component: cholesteryl myristate
diluent: toluene

$\Delta H_{\text{VAN'T HOFF}} = 10,900 \text{ cal./mole}$

$\Delta H_{\text{ACTUAL}} = 11,200 \text{ cal./mole}$

error = -2.7%

slope = $1.82 \times 10^{-4}$
CHOLESTERYL PROPIONATE CRYSTAL-MESOPHASE TRANSITION

\( \dot{T}_p = 2.5^\circ C./\text{min.}, \text{ scale}=4x \)

sample wt. = 5.306 mg.
CHOLESTERYL PROPIONATE PURITY DETERMINATION

SLOPE = -0.80
ΔH = 5.98 kcal./mole
PURITY = 98.25 %
INTERCEPT = T_m = 96.32 °C

○ 1/F uncorrected
× 1/F after correction

FIGURE II-4
FIGURE II-5

BENZOIC ACID
MELTING CURVE

\[ T_p = 0.625 \, ^\circ\text{C.}/\text{min.}, \text{scale} = 8 \times \]
\[ \text{sample wt.} = 2.959 \, \text{mg.} \]
**BENZOIC ACID PURITY DETERMINATION**

SLOPE = -0.075

\[ \Delta H = 4.27 \text{ kcal./mole} \]

PURITY = 99.90%

INTERCEPT = \( T_m = 122.27^\circ C \)

○ 1/F uncorrected

× 1/F after correction

**FIGURE II-6**
CHAPTER III

EVALUATION OF THERMAL TRANSITIONS IN SOME CHOLESTERYL ESTERS OF SATURATED ALIPHATIC ACIDS

Introduction

Esters of cholesterol differ from many other compounds whose transition from crystalline solid to isotropic liquid is a simple, single-step phase transformation. Cholesteryl esters have at least two "liquid crystal" mesophases, the smectic and the cholesteric, between the true solid and isotropic liquid. Multiple heat and temperatures of transition for eight cholesteryl esters of the saturated aliphatic acid series measured by Differential Scanning Calorimetry (DSC) have been reported previously. However, thermodynamic evaluation of this data showed that this cholesteryl ester series exhibited quite anomalous behavior. The purpose of the work will be to measure the heats and temperatures of transition for fourteen cholesteryl esters of saturated aliphatic acids by DSC, to analyze this data thermodynamically, and to attempt to explain the reason for some of this anomalous behavior.

Experimental

A. Cholesteryl Esters. The ester samples came from five sources - Applied Science Laboratories, State College, Pennsylvania; Analabs, Hampden, Connecticut; Eastman Chemicals, Kingsport, Tennessee; Aldrich, Cedar Knolls, New Jersey; and Matheson, Coleman, and Bell, East Rutherford, New Jersey. With the exception of some esters from Applied Science Laboratories, all were recrystallized from n-pentyl
alcohol, washed after the removal of the mother liquor in an ethanol-water solution, and vacuum dried at 50°C. The purity that each sample attained is recorded in the table presenting the thermal transitions. The purity analysis technique applied is based on the shape of the differential scan. The individual samples varied widely in mole per cent purity with all in the range of 96.0 to 99.6% pure. The best cholesterol available for esterification is obtained from animal sources and is only about 98% pure. This is thus a major limitation on the quality of available ester samples. In a previous paper by E. M. Barrall, et al., cholesteryl esters were thought to have less than 0.1% impurity, as indicated by spectrometric analyses. Two other analytical techniques for cholesteryl ester purity applied by Arnold gave values from 98.1 to 99.6% purity by one method and 91.9 to 99.5% by a second method. The values by Arnold are more consistent with the purities reported in this chapter.

B. Instrumentation. The samples were analyzed on a Differential Scanning Calorimeter, Model DSC-1B, manufactured by the Perkin-Elmer Corporation, Norwalk, Connecticut. The calorimeter had previously been calibrated for temperature at the applied heating rates of 1.25 and 2.50°C/min. in a temperature range of 50 to 156°C with p-nitrotoluene, naphthalene, benzoic acid, indium, stearic acid, and adipic acid. Heats of fusion were calculated by comparison with the area under the melting curve of an exceptionally pure sample of indium supplied by the Perkin-Elmer Corporation and stated as being 99.999% pure. The heat of elemental melting is well established at 6.80 cal/gm. As a check on the curve area, the heat
of transition of the indium was also computed from instrument parameters, as the chart ordinate represents cal/sec. and the abscissa, seconds; this method gave a heat of transition of 6.89 cal/gm for indium. Two to five mg samples were weighed on a Nettler balance to 0.001 mg and enclosed in an aluminum planchet. All samples were heated to complete melting, cooled to crystallization, and reheated to isotropic liquid. Duplicate runs were made on fresh samples. All values reported are from first heating or cooling, but, except for cholesteryl tridecanoate, all samples consistently repeated the same transitions and heats on reheating.

The accuracy of transition heat presented can be estimated from multiple runs on Fisher triple point benzoic acid that ranged from 35.0 to 35.6 cal/gm. A previous investigation by adiabatic calorimetry gave a value for benzoic acid of 35.2 cal/gm.\(^7\) Accepting this figure for the mean permitted the calculation of a standard deviation of 0.23 cal/gm. A 95% reliability requires two standard deviations of ± 0.46 cal/gm. Conversion of this figure to percent of sample heat gives an expected accuracy of ± 1.3%. This estimate is satisfactory for the sizable calorie change on heating the crystalline material through its initial transition because the ordinate scale can always be adjusted to give a large full curve on the chart. However, all mesophase-isotropic transitions had to be run on the scale of maximum sensitivity smaller than in normal melting. (Attenuation at different sensitivities were checked for error.) In addition, the specific heat before and after each mesophase transition is abnormally
high which alters the baseline slope. From a comparison of the meso-
phase data reported here for cholesteryl myristate with that of other
investigations, such as Barrall, et al., 8 and M. Leclerq, et al., 9 a
good accuracy of ± 0.02 cal/gm can be anticipated. Conversion of this
value to per cent would suggest accuracy ranging anywhere from ± 3.3%
for nonadecanoate to ± 11.8% for the smallest heat reported which is
for the cholesteric-isotropic liquid transition of cholesteryl pro-
pionate.

The precision of transition temperatures presented here can
be estimated from multiple tests made over a period of weeks on the
three transitions of a 98.1% purity cholesteryl myristate. The trans-
ition ranges for each were from 70.0 to 71.0°C, 77.3 to 78.2°C, and
82.8 to 83.7°C. The expected precision then is approximately ± 0.5°C.
These transition temperatures on cholesteryl myristate are compared
with those of Martire 10, Fridel 11, Gray 12, Arnold 3, and Barrall 2 in
Table III-1. The cholesteryl myristate sample of Martire, et al., 10
had a 98.8% purity, as determined by the Perkin-Elmer Corporation
method of analyzing the shape of the differential scan 13,14. This
sample and that of these investigations are likely comparable in
purity, since the purity analysis technique developed for this work
generally gives somewhat lower purity values on the same sample than
the Perkin-Elmer method 2. The transition temperatures reported by
Barrall, et al., are higher than those reported here, as are all
other cholesteryl ester melting temperatures reported in this same
The methods of calibration and operation of the DSC are likely the determining factors, rather than purity, for the higher transition temperatures reported previously by Barrall, et al.

Results

The temperatures and heats of transition of fourteen cholesteryl esters derived from saturated aliphatic acids of varying chain length are presented in Table III-2, columns one and two. Values for unesterified cholesterol are listed first, followed by the esters from the shortest, formate, of carbon tail length one, to eicosanoate, of carbon tail length twenty. Eight of these esters have been measured previously by E. M. Barrall, et al.,\textsuperscript{1,15} Their results are shown for comparison in columns three and four. The percent difference in $\Delta H$ between these new and the published values is presented in column five. The purity as estimated by the analysis of the DSC curve shape is presented in column six. The final two columns show the calculated entropies of transition and the sum of all transition entropies for each compound. The thermodynamics of each compound studied will be discussed separately.

A. Cholesterol. The series, in theory, can be considered to begin with an ester having a carbon tail length of zero, or the parent compound, an alcohol. However, the transition temperature column in Table III-2 clearly shows the significant difference of cholesterol from the remainder of the true ester series. That is, there is a melting temperature decline of 50°C between cholesterol and the first ester, the formate. The high melt temperature of cholesterol
demonstrates a structural stability to thermal energy that is lost on esterification. The cholesterol, received from Analabs, has one transition and exhibited no mesophases on either heating or cooling which is consistent with the literature\textsuperscript{16}.

B. **Cholesteryl Formate.** This ester exhibited only simple melting and melt recrystallization. It did not appear to form mesophases. Neither the original compound from Aldrich nor a sample recrystallized from n-pentyl alcohol showed an additional transition as suggested by E. M. Barrall, et al.\textsuperscript{1} One of these authors (EMB) has recently found that with somewhat less pure samples, the formate ester can be supercooled sufficiently to give a mesophase transition of 0.08 cal/gm at 57.1°C.

C. **Cholesteryl Acetate.** The original compound from Matheson, Coleman, and Bell exhibited the multiple transition behavior suggested by Barrall, et al.\textsuperscript{1} However, after n-pentyl alcohol recrystallization, only one transition appeared on melting, on melt recrystallization, and on remelting. This compound, like the formate, demonstrated no mesophase character.

D. **Cholesteryl Propionate.** In this work, this ester had the minimum carbon length for the observation of the cholesteric mesophase. The heat absorbed at the cholesteric-isotropic liquid transition is small but reproducible both on heating, cooling, and on reheating. The high purity of this sample originally obtained from Eastman Chemicals was achieved by two recrystallizations from n-pentyl alcohol. Despite its 99.6% purity, the transition temperature remains below that stated
by E. M. Barrall, et al. All data in the table are from melting curves of original crystals unless otherwise stated. Barrall, et al. detected an additional transition on first heating the crystalline material, i.e., two mesophases, one of which was a solid-solid transition, but this transition did not appear upon reheating. H. Arnold reports only two transitions at 99.6 and 114°C of 11.8 and 0.43 cal/gm respectively (one mesophase). This compound appears to form only a single mesophase which can be formed reversibly on either heating or cooling.

E. Cholesteryl Nonanoate. In this investigation, three transitions were observed. As with other experimenters, viz. M. Leclerq, et al., and E. M. Barrall, a small third transition on cooling from the melt, in this case of 0.14 cal/gm at 74.6°C, occurred. The sample studied here was obtained from Eastman Chemicals. It was recrystallized twice from n-pentyl alcohol.

F. Cholesteryl Undecanoate. This ester exhibits three transitions on heating, cooling, and reheating. This means the reversible formation of two mesophases likely to be identified as smectic and cholesteric. A quite pure sample of 99.6 mole % was received from Applied Science Laboratories. The DSC traces were unusually clear and sharp on the sample as received.

There are no known previous studies of either the heats or transition temperatures for this compound. This is the case for all the higher odd carbon length esterified acids.
G. Cholesteryl Laurate. This ester and the next in the series, the tridecanoate, demonstrate a similar form of instability, that is, the impure material exhibits on heating a reproducible exothermic recrystallization between two other endothermic crystallization processes. The laurate exhibits this behavior both on heating and on reheating. This unusual behavior has been described in detail by E. M. Barrall, et al.\textsuperscript{15} Importantly, after recrystallization of the Eastman cholesteryl laurate from n-pentyl alcohol, only one endothermic transition on heating occurs, but three endothermic transitions (smectic and cholesteric mesophase) form on cooling. The heat absorbed on melting is substantially smaller than what would have been estimated from a knowledge of the heats of transition of the nearest two even carbon esters, the decanoate and the myristate. H. Arnold\textsuperscript{3} reports a similarly small heat of 13.9 cal/gm. Transition temperatures by Barrall, et al.,\textsuperscript{15} Arnold\textsuperscript{3}, Gray\textsuperscript{12}, Sell, et al.,\textsuperscript{17} and these investigators are compared in Table III-3.

H. Cholesteryl Tridecanoate. This is the only ester for which endothermic transitions obtained on heating the recrystallized material could not be repeated on reheating the same melt recrystallized sample. Instead the tridecanoate displays on second heating two sizable endothermic transitions between an exothermic transition, similar to the laurate, as well as a final mesophase-isotropic transition. An initial attempt to purify the ester from n-pentyl alcohol failed when an unexpected ester degradation occurred at the standard drying
temperature of 50°C as detected by its DSC trace. None of the other esters dried in the presence of minute quantities of water at this temperature under vacuum displayed this instability. The three transitions from the original crystalline material, including two mesophases, are listed in Table III-2.

I. Cholesteryl Myristate. This ester is highly regular and exhibits three distinct and reversible transitions on heating, cooling, and reheating. The compound analyzed was a relatively high purity sample from Applied Science Laboratories. The compound is difficult to purify further by recrystallization with common alcohols. Cholesteryl myristate has been studied extensively by DSC and can be considered a reference compound. It has now become the most widely studied of all esters of cholesterol.

J. Cholesteryl Pentadecanoate. Almost a twin of cholesteryl myristate, this ester has three distinct transitions in heating, cooling, and reheating and at almost the same temperatures as the myristate. Note in Table III-2 that with the next four higher members of the series, the odd carbon ester takes on the same characteristics of its even number predecessor in the molecular weight series. These characteristics are in the number, reversibility of formation, and the monotropic nature of the mesophase formed.

K. Cholesteryl Palmitate. On first heating and reheating this ester, only two distinct transitions were observed. These were the crystal-cholesteric and cholesteric-isotropic liquid transitions. Barrall, et al., did not resolve these transitions into two separate
maxima in the heating curves. One of these authors (EMB) has performed experiments which indicate that this ester has three optically different crystalline forms. As in this and previous studies, the cholesteric-smectic transition appears only on cooling the sample. Again, a temperature depression of the major transition on cooling, as in cholesteryl laurate, appears to permit the appearance of this transition. The sample, supplied by Analabs, was unresponsive to purification by recrystallization in n-pentyl alcohol.

L. Cholesteryl Heptadecanoate. This ester exhibits phase behavior in common with its even predecessor, cholesteryl palmitate, and has similar transition temperatures. The cholesteric-smectic transition occurs only on cooling. The sample received from Applied Science Laboratories was only about 93.8 mole per cent pure but one recrystallization from n-pentyl alcohol markedly increased the purity to 98.4%.

M. Cholesteryl Stearate. Only one transition appears on first heating and on reheating this ester. However, two mesophases, a smectic and a cholesteric, are obtained on cooling at a rate of 2.5°C/min. If the cooling rate is slowed to 1.25°C/min, however, the cholesteric-smectic transition is lost in the transition to crystalline forms. The slower heating rate permits crystal formation at a higher temperature. Barrall, et al., did not resolve the cholesteric-smectic transition. The Analabs sample improved somewhat with recrystallization.

N. Cholesteryl Nonadecanoate. Again, this ester is a close replicate of the even carbon predecessor in the series. The common
features include the loss of the cholesteric-smectic transition on cooling by changing the cooling rate from 2.5 to 1.25°C/min; transition temperatures are also very similar. The sample came from Applied Science Laboratories in good quality and was tested as received.

0. Cholesteryl Eicosanoate. This ester exhibited only one transition on heating, cooling, and reheating. These features thus may likely mark the termination of the series where mesophases are formed by cholesterol esters of saturated aliphatic acids. The sample was tested as received from Analabs. The purity of the sample, as calculated from shape of the DSC trace, was estimated to be 97.7%.

Discussion of Results

The sum of transition entropies for each compound (see Table III-2, column eight) is plotted against number of carbons in esterified acid in Figure III-1. An inspection of data shows that from the nine carbon acid through the nineteenth, there appears to be a minor but real odd-even effect. The odd-carbon acid esters differ from the next in the series by 6.3 ± 0.8 cal/mole/°C in this range. The even carbon esterified acids from fourteen to eighteen are separated from each other by about 6.6 cal/mole/°C. Using the data for the ten carbon esterified acid from Barrall, et al.,15 of 20.8 cal/mole/°C, a difference is found from the fourteen carbon esterified acid of 13.4 cal/mole/°C. This is about the value that would be expected for regularity, i.e., twice 6.6 cal/mole/°C. Bondi18 has indicated that there can be a linear relationship between total entropy of fusion and the number of carbon atoms in a chain for a
homologous series, and that for n-paraffins, there is an odd-even effect. This part of the series can be correlated in a similar manner. The resulting equations are, given that $N_c$ is the chain length, $\Sigma \Delta S_T$, the total entropy, and $R$, the gas constant:

$$\begin{align*}
(N_c = 10, 14, 16, 18) & \quad \Sigma \Delta S_{T/R} = -6.04 + 1.66 N_c \\
(N_c = 9, 11, 13, 15, 17, 19) & \quad \Sigma \Delta S_{T/R} = -5.54 + 1.58 N_c
\end{align*}$$

The equation form is the same as for the n-paraffin series but the large negative constant is characteristic of the n-alkyl benzene series and indicates that initial members of the series will deviate from this regular behavior. The twelve carbon esterified acid, the cholesteryl laurate, is over 6 cal/mole/°C too low. This large deviation is not too well understood at present. The value given by Arnold\textsuperscript{3} for cholesteryl laurate is only 0.8 cal/mole/°C higher than that of these investigations. Arnold has indicated, by the second of his purity determination methods\textsuperscript{5}, that his cholesteryl laurate contained 8.1% impurity. Should the level of impurity be this high, such a sample could be dramatically lower in entropy than that of a high purity sample. Barrall, et al., indicate in their data\textsuperscript{15} that the total entropy increases from the third to the eighth carbon esterified acid reaching a value of 25.7 cal/mole/°C.

These investigators feel that the above odd-even effect is real, particularly since the total entropy data for the even carbon esters
is comparable to that of Barrall, et al.,¹ Arnold³, and M. Leclerq, et al.⁹ The purity of the samples and crystal morphology of the solid phase remains, however, the major problem in cholesteryl ester transition evaluations. The cholesteryl pentadecanoate, of 96.0% purity, is judged to have the largest error in the crystalline-smectic transition, probably low by 4 to 5%.

The smectic-cholesteric transition entropies from Table III-2, for both the measured and the reference data, are plotted against number of carbons in esterified acid in Figure III-2. An estimated curve has been drawn through the data so that its general trend can be followed. Odd-even effects cannot be established, possibly because there is a ± 0.03 cal/mole/°C deviation in each value that could be larger than the effect itself. The smectic-cholesteric entropy of transition becomes small at an esterified carbon length of 9. The sharp downward slope of the curve at length 9 may indicate that the smectic transition begins here rather than at length 7 as reported by Gray¹⁶.

The cholesteric-isotropic liquid transition entropies from Table III-2 for both the measured and the reference data are presented vs. number of carbons in esterified acid in Figure III-3. As in Figure III-1, two distinct upward sloping curves are formed with a break between cholesteryl octanoate and nonanoate. For reasons similar to that for the smectic-cholesteric transitions, odd-even effects are not discernible. While Barrall, et al.,¹ and Gray¹⁶ have indicated that cholesteryl formate and acetate have a mesophase
transition, this investigation determined that the first cholesteryl ester exhibiting a mesophase is the propionate. Gray\textsuperscript{16} reports that cholesteryl acetate has a cholesteric-isotropic liquid transition on cooling at 94.5°C, but a 99.4% pure cholesteryl acetate became crystalline at 102.5°C, 8°C above the mesophase transition reported by Gray.

The temperatures at which the major or largest transition entropy occurs is plotted against the number of carbons in esterified acid for lengths from 9 to 20 in Figure III-4. The minor odd-even effect is apparent in acid carbon lengths 13 to 19. The relatively high transition temperatures of the decanoate, undecanoate, and laurate may be related to the fact that all three exhibit mesophase behavior only on cooling from the melt. In Figures III-5 and III-6, the data for the temperature at which the smectic-cholesteric and cholesteric-isotropic liquid transitions appeared vs. number of carbons in esterified acid for lengths 9 through 20 is presented. The smectic-cholesteric transition temperatures form the general shape of a horseshoe with a maximum at cholesteryl laurate and agrees with the graphical presentation of Gray\textsuperscript{16}. In the case of the cholesteric-isotropic liquid transition, the suggested trend of the data is a downward sloping line from nonanoate to nonadecanoate.

Conclusions

1. In this work, cholesteryl propionate appears to be the first member of the series to exhibit mesophase behavior in the pure state. The first two pure esters, the formate and acetate, exhibited only one transition on both heating and cooling.
2. From the undecanoate to the nonadecanoate ester, all members have two distinguishable mesophases obtained both from heating and cooling. In optical studies on the even esters\textsuperscript{16}, these mesophases have been identified as smectic-cholesteric and cholesteric-isotropic liquid transitions. By analogy, it may be suggested that the mesophases of the odd esters may also be smectic-cholesteric and cholesteric-isotropic liquid transitions.

3. Cholesteryl eicosanoate has only one transition under all conditions. This may indicate that higher members of this series do not display mesophase behavior, although samples of lower purity may permit the appearance of mesophases\textsuperscript{19}.

4. There is a small but definite odd-even effect in the sum of transition entropies for each compound. This behavior is established for cholesteryl nonanoate up through cholesteryl nonadecanoate. Cholesteryl laurate exhibits deviating behavior in all tests run and in previous reports on this compound\textsuperscript{3,15}.

5. Similarly, there is a small but definite odd-even effect in the temperatures of the major calorimetric transition (crystal-mesophase) for compounds from tridecanoate through nonadecanoate.

References for Chapter III


TABLE III-1

Transition Temperatures for Cholesteryl Myristate

<table>
<thead>
<tr>
<th>Transition</th>
<th>Martire&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Friedel&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Gray&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Arnold&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Barrall&lt;sup&gt;1&lt;/sup&gt;</th>
<th>This Work&lt;sup&gt;1&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Crystal-Smectic</td>
<td>69.8</td>
<td>72</td>
<td>71</td>
<td>71.0</td>
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<td>Smectic-Cholesteric</td>
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<td>81</td>
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<td>77.8</td>
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<td>83</td>
<td>86.5</td>
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</tbody>
</table>

<sup>1</sup>Transition determined by DSC.

<sup>2</sup>Transition determined by optical technique.

<sup>3</sup>Transition determined by equilibrium calorimetry.
### TABLE III-2

**Cholesteryl Ester Transition Values**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Measured $\Delta H$</th>
<th>Reference $\Delta H^1$</th>
<th>% Diff $\Delta H$</th>
<th>% Purity$^3$</th>
<th>$\Delta S$ Cal/mole/°K</th>
<th>$\Delta S$ Total mole/°K</th>
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<td>99.4</td>
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<td>12.6</td>
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<td>26.3</td>
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<td>26.2</td>
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</tbody>
</table>
CAPTIONS FOR TABLE

1. E. M. Barrall, et al., \textsuperscript{1,8,15} are the source of these values.

2. All values reported in the above table are from DSC curves except the reference temperatures which are from DTA curves\textsuperscript{1}.

3. Purity estimates are derived from the shape of the DSC curves\textsuperscript{2}.

4. Barrall, et al., reported a second transition on cooling of 0.08 cal/gm at 57.1°C.

5. Barrall, et al., detected another transition at 81 - 87°C of 4.89 cal/gm.

6. Barrall, et al., reported another transition at 110°C of 0.43 cal/gm on first heating. The % ΔH difference is based on the sum of 12.5 cal/gm and this value of 0.43 cal/gm.

7. Barrall, et al., \textsuperscript{1} M. Leclerq, et al., \textsuperscript{9} and these investigators report an additional transition on cooling of 0.11 cal/gm at 66°C, 0.16 cal/gm at 76.5°C, or 0.14 cal/gm at 74.6°C, respectively.

8. The values reported for the C\textsubscript{11}, C\textsubscript{13}, C\textsubscript{15}, C\textsubscript{17} and C\textsubscript{19} saturated aliphatic acid have no known reference for comparison (until Ref. 19).

9. These values can only be obtained from the cooling curves.

10. Cry-Sm and Sm-Chol transition separable only from cooling curves. Chol-Sm transition equals 0.58 cal/gm at 75.6°C on cooling; ΔS equals 1.04 cal/mole °C.

11. Cry-Sm and Sm-Chol transitions separable only from cooling curves. Chol-Sm transition equals 0.69 cal/gm at 74.8°C on cooling; ΔS equals 1.26 cal/mole °C.
12. For comparison purposes, the measured ΔH for the Cry-Chol and Chol-Iso transitions are added together.

13. Barrall, et al., originally did not resolve this transition into two components as achieved here, although a recent result by one of the authors achieved this separation.

### TABLE III-3

Transition Temperatures for Cholesteryl Laurate

<table>
<thead>
<tr>
<th>Transition</th>
<th>Gray¹</th>
<th>Arnold²</th>
<th>Barrall³</th>
<th>Sell⁴</th>
<th>This Work³</th>
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<tr>
<td>Crystal-Isotropic Liquid</td>
<td>93</td>
<td>91.3</td>
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<td>Isotropic Liquid-Cholesteric</td>
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<td>81.4</td>
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¹ Transitions determined by optical technique.

² Transitions determined by equilibrium calorimetry.

³ Transitions determined by DSC.

⁴ Transitions determined by DTA.
ALIPHATIC ESTERS OF CHOLESTEROL
TOTAL TRANSITION ENTROPIES

\[ \sum \Delta S, \text{ TOTAL TRANSITION ENTROPY, (CAL/MOLE/°K)} \]

- **THIS WORK**
- **BARRALL ET AL**

FILLED SYMBOLS FOR EVEN CARBON NUMBERS

NUMBER OF CARBONS IN ACID
FIGURE III-2

ALIPHATIC ESTERS OF CHOLESTEROL
SMECTIC-CHOLESTERIC TRANSITION
TRANSITION ENTROPIES

\[ \Delta S, \text{ transition entropy, (cal/mole/}^{\circ}\text{K)} \]

- ○ THIS WORK
- □ BARRALL ET AL

\[ \begin{align*}
\Delta S & < 1.0 \\
\Delta S & < 0.8 \\
\Delta S & < 0.6 \\
\Delta S & < 0.4 \\
\Delta S & < 0.2 \\
\Delta S & < 0.0
\end{align*} \]

- ○ FROM HEATING
- □ CURVES
- ○ FROM COOLING
- □ CURVES

NUMBER OF CARBONS IN ACID
FIGURE III-3

ALIPHATIC ESTERS OF CHOLESTEROL
CHOLESTERIC-ISOTROPIC LIQUID TRANSITION
TRANSITION ENTROPIES

○ THIS WORK
□ BARRALL ET AL

▲ FROM HEATING CURVES
▼ FROM COOLING CURVES

ΔS, TRANSITION ENTROPY, (CAL/MOLE*K)

0.0 0.2 0.4 0.6 0.8 1.0 1.2

0 2 4 6 8 10 12 14 16 18 20

NUMBER OF CARBONS IN ACID
FIGURE III-4

ALIPHATIC ESTERS OF CHOLESTEROL TEMPERATURE FOR MAJOR TRANSITION

- THIS WORK
- BARRALL ET AL

FILLED SYMBOLS FOR EVEN NUMBERS

TRANSITION TEMPERATURE (°C)

NUMBER OF CARBONS IN ACID

9 10 11 12 13 14 15 16 17 18 19 20

60.0 65.0 70.0 75.0 80.0 85.0 90.0
ALIPHATIC ESTERS OF CHOLESTEROL
TEMPERATURE FOR SMECTIC-
CHOLESTERIC TRANSITION

- THIS WORK
- BARRALL ET AL

FILLED SYMBOLS FOR VALUES
FROM COOLING CURVE

TRANSITION TEMPERATURE (°C)

NUMBER OF CARBONS IN ACID
FIGURE III-6

ALIPHATIC ESTERS OF CHOLESTEROL TEMPERATURE FOR CHOLESTERIC-ISOTROPIC LIQUID TRANSITION

95.0
90.0
85.0
80.0
75.0
70.0

TRANSITION TEMPERATURE (°C)

8 9 10 11 12 13 14 15 16 17 18 19 20

NUMBER OF CARBONS IN ACID

○ THIS WORK
● BARRALL ET AL

FILLED SYMBOLS FOR VALUES FROM COOLING CURVE
CHAPTER IV
SOME SOLUBILITY CHARACTERISTICS
OF CHOLESTERYL ESTERS

Introduction

The importance of obtaining high purity esters of cholesterol for the study of their inherent properties, such as heats and temperatures of transition, cannot be overemphasized. Experience has demonstrated that impurities can create transitions, as for instance the appearance of a mesophase where none truly exists in a purer sample, or can inhibit the formation of mesophases which would be present in a purer sample. An example of transitions created by impurity is presented in Figure IV-1. Published Perkin-Elmer Differential Scanning Calorimetry data on cholesteryl oleate show five transitions on heating from -3 to +57°C (270 to 330°K)\(^1\), whereas a 98.8 per cent* pure sample from Applied Science Laboratories tested in this study on a Perkin-Elmer Differential Scanning Calorimeter (DSC) gives only a single transition on heating over the same temperature interval. An Imperial Chemical Industry, Limited, study of cholesteryl oleate by DSC indicated three endothermic transitions on heating\(^2\). On cooling from the isotropic melt, the Applied Science cholesteryl oleate showed mesophase transitions at 46.4°C and 41.8°C of 0.18 cal/gm and 0.37 cal/gm, respectively. Perkin-Elmer reported

*All purities quoted in this chapter were estimated from the shape of the DSC traces. This method is extremely sensitive to impurities.
these mesophase transitions on cooling at 37°C and 32.5°C of 0.7 cal/gm and 1.2 cal/gm, respectively\(^1\). The transitions of the Applied Science sample displayed on heating from the crystalline solid and cooling from the isotropic melt indicates that cholesteryl oleate is a monotropic liquid crystal.

An example of mesophase removal by impurity can be illustrated with data on cholesteryl myristate. A 93 per cent pure sample, obtained from Eastman Chemicals, gives only a suggestion of the two well-established smectic-cholesteric and cholesteric-isotropic liquid mesophase transitions for purer materials.

Cholesteryl benzoate is another important case of an ester whose reported transitions have been affected by impurities. The earliest investigators claimed two transitions (meaning one mesophase)\(^3\); a recent work has indicated four\(^4\). This solubility study will help explain why experimenters have expressed varied views on supposedly pure compounds.

The purity problem is understandable when one considers that cholesterol is a natural animal product which has to be separated from many similar steroids. Esterified cholesterol samples supplied by a variety of manufacturers vary widely in purity, from 92.0 to 99.5%. The virtually exclusive purification practice involves solvent recrystallization. The solvent most widely used is ethanol. This purification procedure was followed at first, but only small improvements in cholesteryl ester purity were achieved, as measured by DSC trace shape.
For more definitive studies, a more effective recrystallization process became necessary. Thus, a new purification solvent had to be chosen and a better procedure developed.

Development of Recrystallization Procedure

A. Theory. The choice of an effective solvent for recrystallization is often stated as being a matter of trial and error. However, certain solvent characteristics, such as a high temperature coefficient of solubility for the substance to be purified, an absence of solvation, and an affinity for impurities are generally indicated as being desirable. A high temperature coefficient of solubility permits either a good yield or a selective recovery of higher purity crystals, without excessive temperature change. The above characteristics would be fulfilled by a solvent which forms a regular solution with the esters of cholesterol, yet has a polarity different enough so that impurities of opposite polarity remain in solution.

Regular solutions are defined as those that possess ideal entropy of mixing, just as for ideal solutions, but have a positive enthalpy of mixing. In other words, a regular solution is one in which there is sufficient thermal energy to overcome the tendency of solute and solvent to segregate due to different molecular fields. From this concept, the following relationship is set forth by Hildebrand and Scott for regular solutions, assuming negligible heat capacity effects.
\[
\log \left( \frac{1}{x_2} \right) = \frac{\Delta H_m^f}{4.575} \left( \frac{T_m - T}{T} \right) + \frac{V_2}{4.575} \left( \delta_1 - \delta_2 \right)^2 \phi_1^2
\]

(IV-1)

where

- \( x_2 \) = mole fraction of solute dissolved in solvent
- \( \Delta H_m^f \) = molar heat of fusion of solute in cal/mole
- \( T_m \) = melt temperature of solute, °K
- \( T \) = temperature of solution, °K
- \( V_2 \) = molar volume of solute, c.c.
- \( \delta_1 \) = solubility parameter of solvent (cal/c.c.)^{1/2}
- \( \delta_2 \) = solubility parameter of solute (cal/c.c.)^{1/2}
- \( \phi_1 \) = volume fraction of solvent

The first term in the equation represents the change in entropy when a solute and solvent are mixed together for an ideal solution in which the heat of mixing is zero and the molar volumes of the solute and solvent are the same. However, it is quite common for volume differences to be at least as large as a factor of three and ideality is still followed, e.g., Iodine-SnI\(_4\).\(^5\) The second term represents the internal energy change from the variation in solvent-solvent, solvent-solute, and solute-solute potential energy interaction. These effects are derived from London forces but exclude such interactions as hydrogen bonding.
B. Experiment. The concept of regular solution behavior was applied to the solubility of cholesteryl myristate in four common solvents - ethanol, toluene, acetone, and n-pentanol. Of particular interest was whether any of these four solvents functioned as a regular solvent, as solved by Equation (IV-1), in dissolving cholesteryl myristate.

The procedure applied to determine the mole fraction of solute $x_2$ dissolved in the solvent was as follows:

1. Heat solvent to boiling in presence of an excess of ester.
2. Cool solution and the excess of solute to 28°C.
3. Filter off the excess of insoluble solute.
4. Determine weight of filtrate.
5. Evaporate to dryness under vacuum.

The equilibrium solubilities for each solvent at 28°C were as follows:

1. Toluene: $x_2 = 0.138$
2. Acetone: $x_2 = 0.00076$
3. n-pentanol: $x_2 = 0.0026$
4. Ethanol: $x_2 = 0.00008$

Note that precision and accuracy are considerably reduced at the lower concentrations. The solubility of cholesteryl myristate in ethanol should be considered to be equal to or less than the 0.00008 figure stated.
The solubility of cholesteryl myristate in toluene was so high that calculations by Equation (IV-1), using only the first term on the right hand side as would be done for an ideal solution, predicted the solubility within 1.8 per cent. This result indicated that the solubility parameter of cholesteryl myristate had to be within ± 0.35 of the 8.9 value for toluene. With this information, the second term of Equation (IV-1) could now be utilized to determine whether one of the other three solvents produced a regular solution. However, Equation (IV-1) requires the molar volume of the cholesteryl ester, in this case the myristate ester. This was estimated as follows:

1. The molar volume of cholesterol was calculated from its molecular weight of 386.66 and density of 1.0676 to be 362 c.c.

2. The molar volume of myristic acid was calculated from its molecular weight of 228.36 and density of 0.84396 to be 270 c.c.

3. As an estimation of the molar volume of cholesteryl myristate, cholesterol was assumed to combine with the myristic acid by losing one molar volume of water of 18 c.c. The molar volume of cholesteryl myristate would then be 362 + 270 - 18 = 614 c.c.

This estimation is considered accurate only to ± 10%. Such an error in \( V_2 \), however, would lead to only a ± 3% error in \( \delta_2 \).

n-Pentanol has a solubility parameter of 10.9. Substitution of this value and the measured solubility of the myristate ester in n-pentanol into Equation (IV-1) gives a calculated solubility parameter of 8.9 for cholesteryl myristate. This value is so close, within experimental error, to the 8.9 ± 0.35 suggested by toluene that
n-pentanol can be considered to function as a regular solvent. A similar substitution into Equation (IV-1) for acetone predicts a solubility parameter for cholesteryl myristate which is much too low, and for ethanol, a solubility parameter which is much too high. Acetone and ethanol appear to be highly non-regular solvents.

With a value of 8.9 taken as the solubility parameter for cholesteryl myristate, acetone should dissolve 0.04 mole per cent and ethanol only $10^{-9}$ mole per cent as regular solvents. The fact that ethanol appears to have about a $10^5$ higher solubility capability than Equation (IV-1) would predict either that ethanol will be relatively insensitive as a purification solvent or worse, that ethanol is a poorer solvent for certain impurities than for the ester itself.

The solubility parameters for cholesteryl nonanoate and cholesteryl propionate, as determined from their solubility in n-pentanol, are 8.85 and 8.75, respectively. The similar solubility parameter values for these three esters of widely differing acid chain lengths indicates that the whole series of esters of aliphatic acids have close to the same solubility parameter.

**Purification Procedure**

With the identification of n-pentanol as a regular solvent for cholesteryl esters, the following recrystallization procedure was applied to obtain purified esters of cholesterol:

1. For a known volume of n-pentanol, add cholesteryl ester in the amount of 140 to 150 per cent of saturation by weight based on calculations from Equation (IV-1).
2. Heat to dissolve; this temperature will be well below the n-pentanol boiling point of 138°C.

3. Filter the hot solution through a standard No. 1 filter paper to remove undissolved particles.

4. Reheat to approximately 80°C to insure solution.

5. Permit recrystallization to occur during cooling to room temperature. (For samples with low transition temperatures, recrystallization may be carried out at lower temperatures. 5°C has been used.)

6. Filter off mother liquor.

7. Wash crystals with a solution of ethanol and water in a ratio of 3:1 to 9:1 by volume, depending on ester acid chain length; the longer the chain length, the less water is used. The wash solution is important for the removal of residual mother liquor containing impurity. These wash solutions are chosen to be relatively poor solvents for the crystals but not so incompatible that the crystals congeal and reduce wash efficiency. The water is necessary to avoid dissolving a substantial portion of the crystals to be washed. Note that as the size of the acid chain length increases, the solubility of the cholesteryl ester in ethanol decreased, and less water is needed.

8. Slurry crystals in the above wash solution. Crystals and water must be mingled to insure quantitative removal of impurities.

9. Refilter through standard No. 1 filter paper.

10. Place recrystallized sample in clean container and dry at 50°C and 28 inch Hg vacuum (a lower temperature with a higher vacuum would be more desirable if proper equipment is available).
The above procedure emphasizes the recovery of high purity esters at the expense of yield since the sample size required for the DSC analysis of transition heats and temperatures is only 3 to 4 mg per run. All samples were tested on a Perkin-Elmer Differential Scanning Calorimeter, Model DSC-1B.

Purification Results

A. Cholesteryl Laurate. In Figure IV-2, DSC traces of three cholesteryl laurate samples, areas normalized to 2.000 mg, are shown as they occurred upon heating from 67 to 107°C (340 to 380°K) at a rate of 2.5°C per minute. Note that the sample as received and the ethanol recrystallized compound both display an endotherm followed by an exotherm prior to the major transition. Another investigator⁷ has reported a similar result for samples recrystallized from boiling ethanol. With the n-pentanol recrystallized sample, only the major transition occurred, indicating the ability of the n-pentanol recrystallization procedure to eliminate extraneous transition.

On cooling from the isotropic melt, cholesteryl laurate displays an isotropic liquid-cholesteric transition at 87.2°C of 0.31 cal/gm and a cholesteric-smectic transition at 80.2°C of 0.40 cal/gm. Gray also reports first one transition at 93°C on heating cholesteryl laurate and the above two mesophase transitions at 90°C and 83.5°C on cooling⁸. This data indicates that cholesteryl laurate is a monotropic liquid crystal.
There is some concern about the use of the term "monotropic liquid crystal" to indicate that cholesteryl esters, such as the laurate and oleate, show mesophase behavior only on cooling from the isotropic liquid. The reason that this effect occurs is that the temperature that the isotropic liquid organizes to form the crystalline solid is considerably lower than the temperature necessary to melt or disorganize the crystals. For these "monotropic liquid crystals," the mesophase transitions lie between these two temperatures and will appear only on cooling the sample from the isotropic liquid.

B. Cholesteryl Benzoate. The effect of twice recrystallizing cholesteryl benzoate from n-pentanol is presented in Figure IV-3. These are the normalized traces of an "as received," a first, and a second recrystallized sample, as they were heated from 127 to 187°C (400 to 460°C) at 5°C per minute. After the first recrystallization, the major transition gave three peaks, approximately equal in area to the one previous peak. This same effect was found with an ethanol recrystallized benzoate ester. This behavior also has been previously obtained on samples recrystallized three times from boiling ethanol with the spurious inference that there were four transitions on heating cholesteryl benzoate. However, the trace of the second n-pentanol recrystallized sample shows a return to just two transitions. Each of the three samples presented in Figure IV-3 gave only two transitions on cooling and reheating, as did the ethanol recrystallized samples of the present investigators and in an earlier study. In the experience of this investigator, a cholesteryl ester transition
is not meaningful unless the transitions that appear when a crystalline sample is heated also occur on heating that sample from the recrystallized melt. The second transition is quite small, about 0.26 cal/gm, and is spread out over 2.5°C at a heating rate of 5°C per minute, a wider temperature span than the typical cholesteryl ester of an aliphatic acid. This second transition appears to be extremely sensitive to heating rate. At a DSC heating rate of 2.5°C per minute, the second transition could not be detected unless the size of the sample under test was increased from 1.6 to 2.6 mg.

Cholesteryl benzoate transition heats and temperatures for this investigation are presented in Figure IV-3. The two transition temperatures, after the second recrystallization, of 146.0 and 179.6°C agree reasonably well with those listed in the International Critical Tables of 146.0 ± 1.0°C and 178.5 ± 0.3°C. The first heat of transition ever reported for the change of cholesteric mesophase to isotropic liquid was a value of 0.32 cal/gm (not a calorimetric measurement) by Schenck on cholesteryl benzoate in 1905 and is relatively close to the 0.26 cal/gm value of this study. Cholesteryl benzoate was the first liquid crystal ever confirmed.

C. Other Cholesteryl Esters of Aliphatic Acids. The presence of impurities in cholesteryl esters can either create transitions or decrease heats and temperatures of transition. In Table IV-1, presenting a comparison of cholesteryl ester transition values, cholesteryl acetate is an example of the first type caused by impurities. The samples as received had two substantial transitions separated
in temperature by 30 - 35°C on first heating. After the first recrystallization in n-pentanol, only a single transition appeared at 114.6°C whose transition heat was approximately the sum of the two original transition heats. A sample as received was recrystallized from ethanol. A DSC trace of this recrystallized sample had the same two transitions as the original uncrystallized compound. Previously published DSC data on ethanol recrystallized cholesteryl acetate also indicated two transitions\textsuperscript{11}. The single transition value represents an improvement over the two transition values.

The other three esters in Table IV-1, n-propionate, n-nonanoate, and n-heptadecanoate, exhibited increased transition heats and temperatures at higher purities after recrystallization from n-pentanol. At 93.2 per cent purity and lower, the reported cholesteric-isotropic liquid transition for the n-propionate ester was apparently eliminated. The major heats of transition of these esters (the crystal-mesophase transitions), as recorded by the DSC, were increased anywhere from 20 to 80 per cent.

Conclusions

1. Heretofore, ethanol has been the most widely used solvent for the recrystallization of cholesteryl esters. n-Pentanol has been shown here to be a more effective solvent than ethanol in which to remove impurities and/or crystalline structural defects from cholesteryl esters that can cause additional transitions on initial heating. In addition, higher purity cholesteryl esters have been observed to exhibit significantly increased heats and temperatures of transition.
2. n-Pentanol has been shown here to be a regular solvent for cholesteryl esters, whereas ethanol is not. This could be a major reason why n-pentanol is better able to discriminate between compound and impurity than ethanol.

3. After recrystallization from n-pentanol, in contrast to ethanol, cholesteryl acetate and cholesteryl laurate have only one transition on first heating. This is contrary to previous reports.\(^7\)\(^1\)\(^1\)

4. A detailed study of cholesteryl benzoate has confirmed that there are two transitions, one at 146.0°C, the second at 179.6°C. The heats of transition are found to be 14.7 cal/gm and 0.26 cal/gm, respectively.

References for Chapter IV

2. Personal Communication from Imperial Chemicals.


TABLE IV-1

A Comparison of Cholesteryl Ester Transition Values

All Measured Recrystallizations from n-Pentanol

<table>
<thead>
<tr>
<th>Sample</th>
<th>Measured $\Delta H$</th>
<th>% Purity</th>
<th>Reference $\Delta H^2$</th>
<th>% Diff.</th>
<th>Ref. Temp. $^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Temp. °C</td>
<td>Cal/gm</td>
<td>Est. $^4$</td>
<td>Temp. °C</td>
<td>Cal/gm</td>
</tr>
<tr>
<td>n-ACETATE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lower Trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As Received</td>
<td>79.5</td>
<td>3.5</td>
<td></td>
<td>81-87</td>
<td>4.89</td>
</tr>
<tr>
<td>1st Recry.</td>
<td>None</td>
<td>None</td>
<td>99.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Higher Trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As Received</td>
<td>111.2</td>
<td>8.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Recry.</td>
<td>114.6</td>
<td>11.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n-PROPIONATE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry.-Chol. Trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As Received</td>
<td>89.9</td>
<td>7.1</td>
<td>93.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Recry.</td>
<td>97.2</td>
<td>13.3</td>
<td>99.6</td>
<td>99.</td>
<td>12.5$^6$</td>
</tr>
<tr>
<td>Chol.-Iso. Trans</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As Received</td>
<td>None</td>
<td>None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd Recry.</td>
<td>113.0</td>
<td>0.17</td>
<td></td>
<td>115.3</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Measured ΔH</td>
<td>% Purity</td>
<td>Reference ΔH</td>
<td>% Diff.</td>
<td>Ref. Temp.</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------</td>
<td>----------</td>
<td>--------------</td>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>n-NONANOATE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry.-Chol. Trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As Received</td>
<td>77.0</td>
<td>9.6</td>
<td>96.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Recry.</td>
<td>77.8</td>
<td>11.4</td>
<td>98.5</td>
<td>80.8</td>
<td>10.2</td>
</tr>
<tr>
<td>Chol.-Iso. Trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As Received</td>
<td>90.5</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Recry.</td>
<td>91.7</td>
<td>0.25</td>
<td></td>
<td>93.0</td>
<td>0.22</td>
</tr>
<tr>
<td>n-HEPTADECANOATE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cry.-Chol. Trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As Received</td>
<td>75.4</td>
<td>18.6</td>
<td>93.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st Recry.</td>
<td>76.3</td>
<td>22.8</td>
<td>98.4</td>
<td>(8)</td>
<td>(8)</td>
</tr>
<tr>
<td>Chol.-Iso. Trans.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>As Received</td>
<td>78.3</td>
<td>0.43</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1st Recry.</td>
<td>79.7</td>
<td>0.52</td>
<td></td>
<td></td>
<td></td>
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</table>
CAPTIONS FOR TABLE

1. Cholesteryl Acetate supplied by Coleman, Matheson, and Bell; cholesteryl propionate and cholesteryl nonanoate by Eastman Chemicals; and cholesteryl heptadecanoate by Applied Science Laboratories.

2. Barrall, et al., obtained these temperatures by DTA and heats by DSC. The measured transition temperatures are somewhat lower than these values. This result is not attributed to purity but to differences in instrumentation and calibration methods.

3. Gray recorded these temperatures for purified synthetic samples of the esters (rather than commercial esters) after measurement by optical microscopy in conjunction with a microscope heating stage.

4. Purity estimate refers to measured samples.

5. Gray reports a cholesteric transition at ca. 90°C on cooling. The 99.4% pure cholesteryl acetate of the present investigators had already recrystallized at 102.5°C on cooling.

6. Barrall, et al., detected another transition at 110°C of 0.43 cal/gm on first hearing. The % difference in ΔH is based on the sum of 12.5 cal/gm and this value of 0.43 cal/gm.

7. Barrall, et al., Leclercq, et al., and the present investigators found an additional transition on cooling of 0.11 cal/gm at 66°C, 0.16 cal/gm at 76.5°C or 0.14 cal/gm at 74.8°C, respectively. Gray noted this monotropic smectic transition at 77.5°C.

8. R. D. Emmulat reports a crystal melting temperature of 78°C and a cholesteric-isotropic liquid temperature of 80.6°C.
FIGURE IV-1

CHOLESTERYL OLEATE DSC TRACES

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APPLIED SCIENCE LABORATORY SAMPLE-LOT 762-19
HEATING RATE: 25°C/MIN SAMPLE WT: 2084 MG

T = 325° K
ΔH = 11.6 CAL/GM
PURITY = 98.8%
CHOLESTERYL LAURATE DSC TRACES

HEATING RATE: 2.5 °C/MIN  SAMPLE WT.: 2.000 MG

CHOLESTERYL LAURATE - AS RECEIVED

FIRST RECRYSTALLIZATION - ETHANOL

FIRST RECRYSTALLIZATION - n-PENTANOL
FIGURE IV-3

CHOLESTERYL BENZOATE DSC TRACES

HEATING RATE: 5°C/MIN. SAMPLE WT.: 2000 MG.

CHOLESTERYL BENZOATE - AS RECEIVED

\[ \Delta H_1 = 142 \text{ CAL/GM} \]

\[ T_1 = 149.1^\circ C \]

PURITY = 97.1%

\[ \Delta H_2 = 0.23 \text{ CAL/GM} \]

\[ T_2 = 176.8^\circ C \]

FIRST RECRYSTALLIZATION - n-PENTANOL

\[ \Delta H_1 = 147 \text{ CAL/GM} \]

\[ T_1 = 146.2^\circ C \]

\[ \Delta H_2 = 0.24 \text{ CAL/GM} \]

\[ T_2 = 180.6^\circ C \]

SECOND RECRYSTALLIZATION - n-PENTANOL

\[ \Delta H_1 = 147 \text{ CAL/GM} \]

\[ T_1 = 146.0^\circ C \]

PURITY = 99.1%

\[ \Delta H_2 = 0.26 \text{ CAL/GM} \]

\[ T_2 = 179.6^\circ C \]
CHAPTER V

THERMAL STUDIES ON LIPID-WATER SYSTEMS
BY DIFFERENTIAL SCANNING CALORIMETRY
WITH REFERENCE TO ATHEROSCLEROSIS

Introduction

Thermal studies on lipid-water systems provide information which have implications in biological membrane organization in connection with atherosclerosis. The origin of lipids in atherosclerotic lesions is not known but its possible association with abnormal plasma lipids has been suggested\(^1\). Plasma lipids are, for the most part, a mixture of triglycerides, cholesterol and its fatty acid esters, and phospholipids, of which the main components are lecithin and sphingomyelin\(^1\). These circulate as lipoproteins which can be separated (by electrophoresis, ultracentrifugation, or chemical fractionation) into two main divisions - the alpha or high density lipoproteins and the beta or low density lipoproteins. It is the latter group which may be associated with increased deposits in clinical atherosclerosis\(^1\).

If the lipids in atherosclerotic plaques are compared with those in different classes of low density lipoproteins, their composition is found to be closest to the \(S_f\) 0-12 or true \(\beta\)-lipoprotein\(^2\). The \(S_f\) 0-12 lipoprotein contains 15 to 20 per cent protein\(^2\). The lipid content of \(S_f\) 0-12 lipoprotein has been analyzed to be in terms of percentages of total lipid, 58.2% cholesteryl ester, 20% phospholipid,
and 10.2% triglyceride\(^3\). The most abundant fatty acids of the cholesteryl esters are, in percentage of total fatty acid, oleic (18:1) at 24.1% and linoleic (18:2) at 46.8%\(^3\).

Lipoproteins are complexes of lipid and protein that have the solubility characteristics of protein, i.e., are soluble in water or aqueous salt\(^4\). The lipid links together with the protein, in most instances, by the association of the non-polar regions of the lipid molecules with similar regions of the protein molecule, such as the hydrocarbon tails of the fatty acid moieties of lipids with the hydrophobic amino acid side chains of the protein\(^4\). However, many lipoprotein fractions contain far too much lipid for direct association of all non-polar regions of lipid residues with non-polar regions of protein\(^4\). In such cases, the lipoprotein structure must also involve lipid-lipid interaction. It is hypothesized here that \(S_f 0-12\) \(\beta\)-lipoprotein is an example of a lipoprotein which has lipid-lipid interaction.

Lipoproteins can supply the major constituents for mammal membranes which are composed of lipids and proteins\(^5\). Carbohydrates and nucleic acids are minor components in most membranes\(^5\). Electron microscopic observation suggests that many different kinds of membranes have a similar molecular structure\(^6\). With adequate resolution, the membranes show a trilaminar structure consisting of two dense outer laminae separated by a faint interface\(^6\). Model studies on phospholipid-water systems of low water content indicate that the darkly stained laminae are charged and the faint inner layer is the
hydrocarbon region\(^7\). A trilaminar structure which would be hydrophilic on the membrane exteriors and hydrophobic internally could be formed by protein alone, lipoprotein, or lipid alone.

Given that \(\beta\)-lipoproteins contain excess lipids which interact with each other, these lipids have the capacity to form membranes. Systems of phospholipid-water\(^8\) and phospholipid-cholesterol-water\(^9\) have been shown to form into discrete bimolecular layers of lipid in which the polar regions of each phospholipid layer face outward into a water layer and the hydrocarbon chains "fill" the interior of the "sandwich." These bimolecular structures would fulfill the tri-laminae requirements of the electron microscopic observations\(^7\).

The experimentally observed phospholipid, dipalmitoyl-L-\(\alpha\)-lecithin, organizes, in the presence of an equivalent weight of water, into bimolecular layers separated by water with the hydrophilic groups on the surface separating the lipid and water layers\(^9\). This ordering can be studied thermally on a Differential Scanning Calorimeter (DSC) by the observation of a measurable endothermic transition at a temperature of 41°C on heating\(^9\). The addition of cholesterol to dipalmitoyl-L-\(\alpha\)-lecithin-water systems has been shown to reduce the heat of transition until at a mole ratio of 1:1 cholesterol to lecithin, no transition was observed\(^9\). The cholesterol appears to become a part of the lecithin structure, as noted by x-ray examination, up to this 1:1 mole ratio, but in doing so, causes a reduction in the cohesive forces between adjacent hydrocarbon chains of the lecithin\(^9,10\). These hydrocarbon chains contract in length\(^9\).
This study is particularly concerned with the effect of cholesteryl esters on phospholipid-water systems. A comparison is made with previous systems involving cholesterol. A goal is to see whether this information might help suggest a mechanism by which lipids accumulate in arteries during development of atherosclerotic lesions. The idea that the deposits from the plasma accumulate through membrane formation rather than by direct deposition will be considered. The phospholipids acquired for these experiments were dipalmitoyl-L-α-lecithin, bovine lecithin, and bovine sphingomyelin. From a physical-chemical standpoint, sphingomyelin is similar to lecithin, since it has a hydrophobic region of two long-chain fatty acyl groups and a highly polar region consisting of a phosphoryl choline zwitterion as does lecithin. The cholesteryl ester series of the C₁₈ fatty acids, stearic (18:0), oleic (18:1), and linoleic (18:2), was chosen to be used in conjunction with the phospholipids because of the presence in S₉₀⁻₁₂ β-lipoprotein of large quantities of cholesteryl oleate and linoleate.

Experimental Procedure

The dipalmitoyl-L-α-lecithin, bovine lecithin, bovine sphingomyelin, cholesterol, and cholesteryl esters of stearic, oleic, and linoleic acid used in this study are 99% pure according to the supplier, Applied Science Laboratories, State College, Pennsylvania. The phospholipids and cholesteric compounds were weighed directly into the liquid sample pans provided for DSC testing. A six place Mettler balance weighed the samples to 0.001 mg. Spectroscopic grade benzene
was added to the pan to dissolve and mix the phospholipid and cholesterol compound in situ. The benzene was evaporated under vacuum at 35°C. The sample was reweighed as a function of time to insure that all benzene was removed. Distilled water in the amount of twice the phospholipid sample weight was added into the planchet by syringe and the sample lid clamped on. The space between the crimped sample lid and the pan was sealed with Eastman 910 adhesive. Each sample contained one phospholipid and one cholesterol compound. The sealed planchet containing the sample was placed in an oven at a temperature of 70°C for one hour before testing. This temperature is 25°C above that necessary to disperse the phospholipid in the water. The temperature choice also insured that the cholesteryl esters would not pass through their major transition between 27 and 57°C. The phospholipids used in this study have crystalline melting points well above 70°C.

Transitions within the samples observed on heating (all endothermic) and on cooling (all exothermic) were measured on a Perkin-Elmer Differential Scanning Calorimeter, DSC-1B. The calibration of the DSC for temperatures and heats of transition has been described elsewhere. Equilibrium conditions for the samples were demonstrated by repeating the heating and cooling cycle three times over a period of one hour with observation of effectively the same heats and temperatures of transition in the first and third cycle. The samples were heated and cooled at a constant rate of 2.5°C per minute. The ordinate scale was on the maximum sensitivity of one calorie per second. An accuracy in the heat of transition determination of ± 5% for the
dipalmitoyl-L-α-lecithin and ± 10% for the bovine phospholipids is estimated. The precision in temperature measurements is ± 0.5°C for the dipalmitoyl-L-α-lecithin and ± 1.5% for the bovine phospholipids, natural materials containing a mixture of compounds.

Results

A. Dipalmitoyl-L-α-Lecithin in Aqueous Systems. The temperatures and heats of transition for the dipalmitoyl-L-α-lecithin alone in water and with various mole fractions of cholesterol or cholesteryl ester in water, both in heating (endotherm) and cooling (exotherm), are presented in Table V-1. The entropy of transition per mole of lecithin for the heating and cooling of each sample has been calculated from its respective transition energy and temperature. The transition energies were relatively constant over several cycles of heating and cooling the same sample. This process is considered to be entirely reversible since measured energy on heating and cooling was, within the accuracy of the experimental technique, the same. For this reason, the entropy values from the heating and cooling of each sample were averaged. This average entropy value is shown in the final column of Table V-1. These average transition entropies per mole of lecithin are plotted as a function of the mole fraction of the cholesteric compound present in the sample, on a water-free (dry) basis, in Figure V-1. A representative DSC cooling curve for each cholesteric compound used in the dipalmitoyl-L-α-lecithin-water system fraction is shown to scale in Figure V-2. The cholesteric compound was used in concentrations between 0.32 and 0.40 mole.
A rapid reduction in the transition heat with the addition of cholesterol to the dipalmitooyl-L-α-lecithin-water system is observed in Figure V-1. This is similar to the results obtained by Ladbrooke, Williams, and Chapman\textsuperscript{9} for the dipalmitooyl-L-α-lecithin-cholesterol-water system, though the heat of transition values from this study are about 15 per cent lower than those of these investigators. The temperature of transition changed less than a degree over the entire range of compositions tested. The transition on heating occurred at a temperature about 1.5°C higher than that on cooling. Ladbrooke, Williams, and Chapman\textsuperscript{9} reported about 10°C temperature decline with increasing cholesterol content. Two possible reasons for this large temperature effect are a wider curve spread with increasing cholesterol content than experienced by the present investigators and a different method of temperature calculation. In this study, the peak temperature, corrected for difference between sample temperature and program temperature, the method recommended by Perkin-Elmer, the manufacturer\textsuperscript{15}, is reported, instead of the temperature the DSC trace leaves the baseline. The peak temperature is the most significant and reproducible value. This observation is supported by Figure V-2 which shows that all the transitions have the same temperatures but widely varying heats. An extensive analysis of DSC curve behavior for endothermic transitions at a heating rate of 2.5°C/min is presented in a complete and separate study by these investigators\textsuperscript{16}. 
In contrast to cholesterol, cholesteryl stearate has remarkably little effect either on the entropy or temperature of transition, even at 0.50 mole fraction or a 1:1 mole ratio of ester to lecithin. A plot of transition entropy per mole of lecithin vs. mole fraction of cholesteryl stearate in Figure V-1 indicates that perhaps a 5% drop in entropy occurred at a 1:1 mole ratio.

Cholesteryl oleate also has a small effect on transition entropy of the system, but somewhat more than the lecithin-cholesteryl stearate-water systems. The transition entropy decreased about 15% at a 1:1 mole ratio. Cholesteryl linoleate has a greater effect, reducing the transition entropy about 30% at a mole fraction of 0.35 or a 1:2 mole ratio. The presence of either cholesteryl oleate or linoleate has little effect on transition temperature.

B. Bovine Phospholipids in Aqueous Systems. No thermal transition could be detected by the DSC for the bovine lecithin-water system between the temperatures of 5 and 55°C. However, the bovine sphingomyelin-water system exhibited a thermal transition about 75% as large in energy as the dipalmitoyl-L-α-lecithin-water system and at the same transition temperature. This is not a crystalline-liquid transition since sphingomyelin melts at a temperature considerably higher than 70°C\textsuperscript{13}, but may be similar to the type of organization exhibited by the lecithin-water system\textsuperscript{9,10}. The breadth of the trace extended over a temperature span of 12°C, about twice that of the dipalmitoyl-L-α-lecithin, reflecting the difference between a natural and a single synthetic compound.
The above result is not unexpected since the bovine lecithin has two adjustable acyl groups and the bovine sphingomyelin only one. Spingomyelin usually has as one of its two acyl groups the hydrocarbon chain provided by sphingosine, though some sphingomyelin contains dihydrosphingosine rather than sphingosine. With two variable groups, bovine lecithin is likely to contain many more different molecules which have a broader range of physical properties than bovine sphingomyelin. These many different molecules in bovine lecithin could spread the transition energy over such a broad temperature range that the transition could go undetected by the DSC. However, the possibility exists that the transition temperature for this material is lower than +5°C.

The temperatures and heats of transition for bovine sphingomyelin alone in water and with various mole fractions of cholesterol or cholesteryl esters in water both in heating (endotherm) and cooling (exotherm) are presented in Table V-2. Given that the temperature range for all samples tested, either on heating or cooling, were separated by less than 3°C, the actual temperature variation for these systems (see Table V-2) should be considered as small as that of the dipalmitoyl-L-α-lecithin systems. The molecular weight of sphingomyelin was estimated at 770 from data supplied by Applied Science Laboratories. Bovine sphingomyelin is particularly rich in the saturated C_{24} amides though about ten per cent of the adjustable acyl group is the saturated C_{16} and C_{18} amide.
The average entropy of transitions of the sphingomyelin-cholesteric compound-water systems have been calculated in the same manner as the comparable dipalmitoyl-L-α-lecithin systems. These values are shown in the final column of Table V-2. These average transition entropies per mole of sphingomyelin are plotted as a function of the mole fraction of cholesteric compound present in the sample (on a dry basis) in Figure V-3.

The results displayed in Figures V-1 and V-3 indicate that cholesterol reduces the transition entropy of bovine sphingomyelin and dipalmitoyl-L-α-lecithin in water sharply; moreover, the transition entropy change with cholesterol concentration is quite similar in the two systems. In contrast, cholesteryl stearate has no ability to change the entropy of transition of either phospholipid. Cholesteryl oleate and linoleate are somewhat more effective in reducing the entropy of transition of bovine sphingomyelin with increasing mole fraction than with dipalmitoyl-L-α-lecithin. The most important observation, though, is that the curves in Figures V-1 and V-3 for each cholesteric compound are so similarly positioned.

Discussion of Results

A. Significance to Membrane Formation. When dipalmitoyl-L-α-lecithin and bovine sphingomyelin organize structurally in water on cooling to 41°C, heat must be removed from the sample causing a reduction in the entropy of the system. The magnitude of the entropy loss is a measure of the stability of the system since the same amount of entropy must be returned to the sample on heating to disrupt the
organization. As cholesterol is added to the lecithin or sphingomyelin system, the entropy of transition is reduced until at measurements at a 1:1 cholesterol to phospholipid ratio no energy of transition is recorded by the DSC.

The above information would suggest that in the limit of a 1:1 mole ratio, a cholesterol-lecithin or cholesterol-sphingomyelin structure is quite unstable. However, unpublished studies by D. M. Small indicate that the 1:1 structure is stable up to 100°C.17 Organization of the phospholipid structure did not increase the overall stability of the system measured. But for the cholesterol to participate in the phospholipid structure, as described by other investigators9,10, the crystal structure of the cholesterol must be disrupted. The heat absorbed by the breakdown of the crystal structure would offset the heat given off in the co-organization of the water molecules and acyl groups of the phospholipid. (Water molecules in contact with acyl chains cannot co-hydrogen bond and acyl chains separated by water cannot form van der Waal's forces with one another.) At a 1:1 mole ratio of cholesterol to phospholipid, the sum of these heat changes is zero. This fact could indicate a reversible transition between crystalline cholesterol with unorganized phospholipid and the cholesterol-phospholipid membrane-like structure.

In contrast to the behavior of cholesterol, cholesteryl stearate has little measurable effect on the entropic change that occurs as lecithin and sphingomyelin in water are cooled through the transition
temperature. The same heat is given off, within five per cent, whether the cholesteryl stearate is present or not.

The equilibrium temperature of 70°C applied to all samples causes the cholesteryl oleate and linoleate to melt. Once melted at 70°C, the cholesteryl oleate and linoleate will not revert to crystalline form at temperatures between 27 and 57°C. The cholesteryl oleate and linoleate can pass through their mesophase transitions in the 27 to 57°C range, but these heats are, at the maximum mole fraction of sample present, no more than 4 to 6 per cent of the heat reported for their ternary transition. The shape of the cholesteryl oleate ternary transition in Figure V-2 at 46°C does suggest the isotropic liquid to cholesteric transition expected at this temperature from the previous data of the present investigators. Since cholesteryl oleate and linoleate cannot be in crystalline form at 27 to 57°C, a change in the entropy of the system can be directly related to the stability of the phospholipid structure formed.

There is always present the biological possibility of phospholipids (in the blood plasma) organizing to form membranes. The sphingolipids have been reported to be major lipid constituents of most mammalian cell membranes. Sphingomyelin, even when composed of mixtures of molecules containing different acyl groups, can organize at temperatures close to human biological environment. It may be significant that the only sphingolipid in erythrocyte plasma membrane in appreciable percentage is sphingomyelin. In contrast, lecithin containing unsaturated bonds may have liquid crystalline to crystalline transitions in water many degrees below the biological environment.
$S_f$ 0-12 lipoprotein, in which lipid-lipid interaction is likely, contains substantial quantities of cholesteryl oleate and linoleate. A shift in the ratio of these two esters could have a significant effect on the stability of the structure formed with phospholipids, in particular sphingomyelin. Should the ratio of cholesteryl linoleate to oleate decline, structures of greater stability may form. The saturated or monounsaturated $C_{18}$ esters of cholesterol would most likely be excluded from the membrane structure owing to the length of their acyl chain. The diunsaturated fatty esters of cholesterol, the linoleate, could participate in the formation of the membrane-like structure, imparting to the structure a relatively high thermal instability. This could occur because, as has been reported, the amount of unsaturation in the fatty acid is important in determining its molecular shape. For instance, saturated and trans-saturated fatty acids are essentially straight, but at cis double bonds, the chain is bent. The higher the unsaturation, the more bent a fatty acid chain can be; in arachidonic acid, the molecule can be curved into a U. The magnitude of the entropy decrease during the organization of the sphingomyelin or dipalmitoyl-L-α-lecithin by esters of cholesterol is likely inversely proportional to their ability to participate in the membrane structure and can be expected to be, in order of decreasing effectiveness: linoleate > oleate > stearate. In other words, cholesteryl esters are not incorporated into the lecithin structure as readily as cholesterol, the solubilities being in the order stearate < oleate < linoleate.
B. Significance to Atherosclerosis. E. B. Smith, et al.,\textsuperscript{3} have been able to distinguish between perifibrous lipid of the human aorta intima that thickens with age from abnormal fat-filled cell thickening. Particularly noticeable in comparing the chemical composition of these two types of thickening, for the under 30, 30-40, and 40-49 age groups, is the decrease in the per cent cholesteryl linoleate and increase in the per cent cholesteryl oleate in the fat-filled membranes or cells compared to perifibrous lipid\textsuperscript{3}. An explanation may lie in the potential ability of cholesteryl linoleate to inhibit the formation of stable phospholipid structures from the plasma. The presence of increased cholesteryl oleate could accelerate the slower natural structure formation process in the presence of cholesteryl linoleate. The cholesteryl oleate, itself, could be enclosed inside the forming structure.

E. B. Smith, et al.,\textsuperscript{21} in a more recent technical article describe an amorphous lipid structure in the human aorta which is extremely high in cholesterol. It is conjectured here that this amorphous behavior may be due to the presence of the cholesterol causing the phospholipid to change between ordered and disordered phospholipid structure. In the same article by E. B. Smith, et al.,\textsuperscript{21} also provided is a table which shows the per cent distribution of the main phospholipid fractions in fatty plaques, fibrous plaques, and amorphous lipid. The percentage of sphingomyelin in these plaques varies from 30 to 80% of the total phospholipid. This percentage is remarkably high since only from 10 to 25% of the lipid phosphorous in plasma is in the form of sphingomyelin.\textsuperscript{12}
It is suggested here that the reason for this difference may lie in the ability of sphingomyelin to form membrane structure at biological environmental temperatures.

References for Chapter V

5. Ibid, 276.


17. Personal communication by D. M. Small.


<table>
<thead>
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<th>Mole Fraction Cholesteric Compound (Dry Basis)</th>
<th>Transition</th>
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<th>$\Delta S$ Cal/°K/ Mole Lecithin</th>
<th>$\Delta S_{av}$ Cal/°K/ Mole Lecithin</th>
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1At a mole fraction of 0.500 for cholesterol, no transition was detected on the DSC.
TABLE V-2

Thermal Transitions of Sphingomyelin (Bovine) - Water

Systems Containing Cholesteric Compounds

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103
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<td>3.62</td>
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Figure V-1.

(Dipalmitoyl)-L-α-Lecithin Cholesteric Compound Transition Entropies (in water)

ΔS\text{av} transition entropy (cal/K-mole lecithin)

Mole fraction Cholesteric compound (dry basis)

Cholesteric compound:
- None
- Cholesterol
- Cholesteryl Stearate
- Cholesteryl Oleate
- Cholesteryl Linoleate
FIGURE V-2

DSC COOLING CURVES OF DIPALMITOYL-L-α-LECITHIN-WATER SYSTEMS CONTAINING CHOLESTERIC COMPOUNDS SCALED TO 1.000 mg LECITHIN COOLING RATE 2.5 °C./MIN.

0.320 MOLE FRACTION CHOLESTEROL

0.401 MOLE FRACTION CHOLESTERYL LINOLEATE

0.366 MOLE FRACTION CHOLESTERYL OLEATE

0.329 MOLE FRACTION CHOLESTERYL STEARATE

POWER EVOLVED (milliJoule/sec.)

TEMPERATURE (°K)
FIGURE V-3

SPHINGOMYELIN (BOVINE)-CHOLESTERIC COMPOUND
TRANSITION ENTROPIES (IN WATER)

\[ \Delta S^{\text{eq}} \text{(TRANSITION ENropy (CAL/K-MOLE SPHINGOMYELIN))} \]

CHOLESTERIC COMPOUND
- NONE
- CHOLESTEROL
- CHOLESTERYL STEARATE
- CHOLESTERYL OLEATE
- CHOLESTERYL LINOLEATE

MOLE FRACTION CHOLESTERIC COMPOUND (DRY BASIS)
CHAPTER VI

THERMAL TRANSITIONS OF CHOLESTERYL ESTERS OF C\textsubscript{18} ALIPHATIC ACIDS

Introduction

The studying of physical properties for cholesteryl esters of C\textsubscript{18} aliphatic acids is of both fundamental and practical interest. The physical chemistry is of interest because of the mesophase or liquid crystal behavior that can be potentially exhibited by analogy with other esters of cholesterol. Moreover, systematic studies on these esters are rare. From the practical view, the cholesteryl ester content of human β-lipoprotein is 24.1% in cholesteryl oleate (18:1) and 46.8% in cholesteryl linoleate (18:2)\textsuperscript{1}. Recent studies at the Massachusetts Institute of Technology have indicated that measuring the amounts of cholesterol-bearing β-lipoprotein may be more important than measuring the amount of blood cholesterol itself in characterizing coronary heart disease\textsuperscript{2}. β-lipoprotein contains cholesterol predominantly in the esterified state containing only about 11.5% free cholesterol compared with 58.2% esterified cholesterol\textsuperscript{1}.

Experimental

The cholesteryl stearate (18:0), oleate, and linoleate esters tested in this study were obtained from Applied Science Laboratories, State College, Pennsylvania. The linoleate (18:3) ester came from Mann Research Laboratories, 136 Liberty Street, New York. The cholesteryl stearate and linoleate were recrystallized from n-pentyl alcohol, washed after removal of the mother liquor in an ethanol-water solution and
vacuum dried at 25°C. The recrystallization solvent was selected after a careful screening of different solvent efficiency for purification of cholesteryl esters. The samples were analyzed for heats and temperatures of transition on a Differential Scanning Calorimeter, Model DSC-1B, manufactured by the Perkin-Elmer Corporation, Norwalk, Connecticut. The DSC calibration procedure has been described in a previous technical article. The heats and temperatures of transition measured are markedly dependent on level of purity. An estimate of sample purity has, therefore, been made for each ester by a technique based on the shape of the DSC curves.

Results

The temperatures and heats of transition of the four cholesteryl esters of C₁₈ aliphatic acids are presented in Table VI-1 columns one and two. An estimate of ester purity by the method given in Reference 5 is listed in Table VI-1, column three. The purities are as high as possible considering that the best purity material was purchased and subsequently multiply recrystallized from an efficient purification solvent. The esters are tabulated according to acid unsaturation starting with the totally saturated stearate and ending with the triply unsaturated linolenate. The trend of temperatures of transition for the cholesteryl esters of C₁₈ aliphatic acids are plotted against the number of unsaturated bands in Figure VI-1. All of the esters exhibited three transitions: the crystal-isotropic liquid on heating, the isotropic liquid-cholesteric, and the cholesteric-smectic on cooling. The mesophases were thus monotropic. The specific mesophase types were
not identified optically and are labeled by analogy with the mesophases observed for saturated esters of cholesterol. Each type of transition is connected by a dotted line in Figure VI-1.

The general trend for each type of transition shows the temperature of transition decreasing with increasing unsaturation. This decline in temperature of transition is particularly sharp in going from zero to one unsaturation, amounting to about a 30°C drop for the major and the two mesophase transitions.

The heats of transition for these esters exhibit less regular behavior, see Table VI-1. The data may be subsequently subject to some revision because of the hypersensitivity of transition behavior to ester purity. One observation of interest is that the isotropic liquid to cholesteric heats of transition for the three unsaturated esters are all the same within measurement precision, 0.18 ± 0.02 cal/gm.

References for Chapter VI
<table>
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<tr>
<th>Name</th>
<th>Measured Temp. °C</th>
<th>ΔH Cal/gm</th>
<th>% Purity Estimate</th>
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</thead>
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<tr>
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<td>(3)</td>
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<td>Chol-Sm Trans</td>
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<td>0.68</td>
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1 All mesophase transitions occurred only on cooling the sample from the melt.

2 Purity estimates are derived from the shape of the DSC curves.

3 An endothermic followed by an exothermic change occurred prior to the transition which interfered with the calculation of transition heat.
FIGURE VI-1

CHOLESTERYL ESTERS OF C_{18} ALIPHATIC ACIDS TEMPERATURE OF TRANSITION vs UNSATURATION

- X CRYSTAL - ISOTROPIC LIQUID TRANSITION
- ■ ISOTROPIC LIQUID - CHOLESTERIC TRANSITION
- ○ CHOLESTERIC - SMECTIC TRANSITION

Temperature, (°C)

Number of Unsaturated Bonds
CHAPTER VII

FUTURE WORK

Thermal Studies on Other Lipid-Water Systems

Other fatty acids of the cholesteryl esters present in significant amounts in $S_f$ 0-12 lipoprotein* are, in weight percentage of total fatty acid, palmitic (16:0) at 13.7%, arachidonic (20:4) at 5.2%, and palmitoleic (16:1) at 4.2%; the only other measurable fatty acid is myristic (14:0) at 1.0%². Investigation of the effect of acid chain length for the cholesteryl ester on lecithin and sphingomyelin organization in water could be determined by comparison of the myristate, palmitate, and stearate. These three cholesteryl esters are available from Applied Science Laboratories.

Two other phospholipids present at measurable levels in atherosclerotic lesions are lysolecithin and phosphalidyl-ethanol amine³. These two phospholipids could be examined by the same procedure applied here to lecithin and sphingomyelin. Both phospholipids can be purchased from Applied Science Laboratories.

Binary Systems of Cholesteryl Esters

In a recent paper, the temperatures of transition of binary systems prepared from cholesteryl oleate and linoleate were reported⁴. Binary mixtures containing from 35 to 100% cholesteryl oleate formed unstable monotropic cholesteric and smectic mesophases from which cholesteryl oleate crystallizes. Mixtures containing less than 30% cholesteryl

*S₉ 0-12 lipoproteins are a class of low density lipoproteins called β-lipoproteins which are the components of abnormal plasma that are most closely associated with increased deposits in clinical atherosclerosis.¹
oleate form much more stable mesophases and no separate crystallization of cholesteryl oleate and linoleate occurs from these mesophases. At cholesteryl linoleate mixtures above about 90%, mesophase transitions occur in the region of body temperature, 37°C.

The next stage in the above work would be the measurement of heat of transitions as well as temperatures, and the calculation of entropies for different mixtures of cholesteryl oleate and linoleate. Mixtures having unique properties could be substituted for the cholesteryl ester in the previously described ternary system. For instance, the mixture containing 65% cholesteryl linoleate and 35% cholesteryl oleate, just at the cholesteryl linoleate level necessary to prevent crystallization, is similar to their relative percentage in $S_f^{0-12}$ lipoprotein.

Surface Properties of Lipid Droplets and Bilayers

Phospholipids, such as lecithin, are known to form membrane-like bilayers with water in water. Cholesterol is a lipid which can also become a part of the bilayer. However, from Chapter V, cholesteryl linoleate and oleate have much less ability to participate in the bilayer structure. Mixtures of cholesteryl linoleate and oleate appear to have the capacity to form lipid droplets at body environmental temperature.

Recently, a paper has been written suggesting that non-polar lipid droplets could enter the bilayer if its surface free energy on the oil-water interface were about 10 ergs/cm² or higher. This procedure would offer an alternative method of lipid accumulation in
atherosclerosis to the concept of entrapment as the membrane was formed. The first step in exploring this index would be to set up an apparatus which would be capable of measuring the surface tension of cholesteryl ester droplets in water at 37°C temperature.

Cholesteryl Esters of Saturated Aliphatic Acid Series

Recently, published data by Ennulat on the cholesteryl ester series of saturated aliphatic acids has raised several questions about the properties of this series. Particularly important is the question of whether the total entropy of transition and cholesteric-isotropic liquid entropy of transition increase sharply as the number of carbons in the acid increases from 4 to 8, as shown in Figures III-1 and III-3. Ennulat reports that these transition entropies do not increase so sharply. This investigator did not test the cholesteryl esters from butyrate to octanoate. Thus additional experimentation should be carried out to show whether the transition behavior reported in Figures III-1 and III-3 is accurate.

The article by Ennulat also suggests that cholesteryl formate, acetate, and eicosanoate have observable mesophases. The information available at present suggests that these transitions only appear in samples that are less pure than the ones tested in this study. This idea should be checked by actually adding defined impurity to pure samples followed by a determination of transitions after proper sample melting and recrystallization.
"Co-Crystallization" in Binary Systems of Cholesteryl Esters

The melting point depression behavior of cholesteryl myristate containing various mole fractions of toluene, described in Chapter II, is predicted accurately by the Van't Hoff relationship. This occurrence is not unexpected since toluene is an ideal solvent for cholesteryl myristate, even though its molar volume is about 1/6 of that of the ester (see Chapter IV). However, a different kind of behavior could be obtained if the additive to cholesteryl myristate would have a similar molecular weight and shape, as well as a nearly identical solubility parameter. Such an additive could "co-crystallize" with the cholesteryl myristate and depress its melting point much less than would be expected from the Van't Hoff relationship.

An example of a "co-crystallite" with cholesteryl myristate might be cholesteryl pentadecanoate. Here the only difference between the two compounds is a CH₂ group in a molecular weight of 597. Their heats and temperatures for all transitions are extremely close in value and the pentadecanoate is known to take on the same characteristics as its even number predecessor, the myristate (see Chapter III). For the same reason cholesteryl heptadecanoate may co-crystallize with the palmitate and the nonadecanoate with the stearate. Should these binary systems be shown to co-crystallize, further experimentation could be conducted to determine if cholesteryl esters which differ by two CH₂ groups can "co-crystallize," such as the cholesteryl myristate with the palmitate. This work can be extended, of course, to include binary mixtures of cholesteryl esters separated by more than two CH₂ groups.
The odd acid chain length esters of cholesterol, the pentadecanoate, heptadecanoate, and the nonadecanoate, are not reported as being present in $S_f 0-12$ lipoprotein$^2$. These esters could actually be there in significant quantities but may be extremely difficult to separate out from their even acid chain length predecessors.

References for Chapter VII
