THE DISSOLUTION OF CHOLESTEROL MONOHYDRATE CRYSTALS IN Atherosclerotic Plaque Lipids

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Summary

Uncomplicated human atherosclerotic plaques often contain large amounts of cholesterol esters and solid cholesterol monohydrate crystals. If such plaques are to regress the crystalline cholesterol would have to dissolve and be transported out of the arterial wall. Since cholesterol is quite insoluble in water, dissolution of plaque crystals might occur through lipids in the plaque, specifically, the cholesterol esters. As part of a study on feasibility of plaque regression, we have studied a specific step involving the dissolution of cholesterol monohydrate into cholesterol ester oil. With specific considerations of the composition and physical state of the cholesterol ester solvent, the size and form of cholesterol monohydrate crystals, the agitation rate, the temperature and the presence of water, we have found that cholesterol esters are an efficient solvent of cholesterol monohydrate crystals. The rate of dissolution was fast reaching the rate of saturation in 1 h. We conclude dissolution of cholesterol monohydrate into cholesterol ester oil is not a rate-limiting step in reversal of the atherosclerotic plaque. We suggest that transport of dissolved cholesterol from cholesterol ester oil may limit the removal. If transport of dissolved cholesterol could be enhanced, cholesterol monohydrate crystals could be rapidly dissolved to facilitate reversal of atherosclerotic lesions.

Key words: Atherosclerosis — Cholesterol — Gallstones — Lipoproteins — Regression atherosclerosis

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lipid deposits in human atherosclerotic lesions are composed primarily of 3 classes, cholesterol ester, cholesterol, and phospholipid [1]. Recent physi-
chemical studies have shown that advanced human plaques contain 3 lipid
es, a cholesterol ester oil droplet phase, a phospholipid bilayer phase (both
ated with free cholesterol), and a cholesterol monohydrate crystal phase.
If plaques are to undergo regression, the accumulated lipids, in particular
alline cholesterol, would have to be mobilized. Since cholesterol is insol-
in water [4], dissolution of plaque crystals into a lipid component of
ues which solubilizes cholesterol could be an important early step in cho-
rol removal [2].

ever, the rate of dissolution would have to be sufficiently rapid for
he regression to be therapeutically feasible. We have studied the in vitro
dution rates of cholesterol monohydrate crystals into cholesterol ester oil,
the major cholesterol esters of plaques, cholesteryl linoleate and choles-
olate [3,5] and into the total cholesterol esters isolated from the
es of 3 human aortas. We have established that dissolution of crystalline
sterol monohydrate in cholesterol ester oil is rapid and thus would not be
ate limiting step in the regression of advanced atherosclerotic lesions.

Materials

olesterol and cholesterol esters were obtained in greater than 99% purity
NuChek Prep, Elysian, MN. Thin layer chromatography [5] using 200 µg
established their 99% purity. Their melting points and liquid crystal trans-
s, determined using a Perkin Elmer DSC 2B microcalorimeter, agreed with
C of literature values [7,8,9]. Cholesterol esters from human necropsy
es were isolated, purified and characterized as previously described [3].
ester sample had the following fatty acid composition by gas—liquid chro-
ography (12% DEGS column): C_{18:2} *, 35%; C_{18:1}, 31%; C_{16:0}, 15%; C_{16:1},
_{20:4}, 3%, C_{18:0}, 2%; C_{20:2}, 2%; C_{16:0}, 1%; C_{20:3}, 1%. Radioactive choles-
monohydrate crystals (specific activity approx. 10^{4} cpm/mg) were pre-
by co-crystallizing [4-^{14}C] cholesterol (New England Nuclear, Boston,
and cholesterol from 95% ethanol. The X-ray powder diffraction pattern
es was identical to the previously reported diffraction pattern of
sterol monohydrate [3,10]. The crystals contained 1 mole of water per
of cholesterol, as determined gravimetrically. The crystals are flat rhoms
an edge angle of 79° which range in size from 400—800 µm and are quite
ar in morphology to crystals in human plaques (Fig. 1).

Experimental

study the dissolution of cholesterol monohydrate, glass tubes (5 mm ID)
aining approximately 200 mg of the cholesterol ester oils were fitted with

The first subscript refers to the number of carbon atoms and the second to the number of
bonds in the fatty acid.
Fig. 1 Cholesterol monohydrate crystals crossed polars, 37°C. A: crystals used for dissolution experiments. The size ranges from 400–800 μm in length; B: crystals from atherosclerotic plaques. The polarized microscopic and X-ray powder diffraction characteristics of these crystals were identical to authentic monohydrate.
Dissolution of cholesterol monohydrate in cholesterol esters as a function of time. ▲ — ▲, dissolution of cholesterol monohydrate in anhydrous cholesteryl linolate at 37°C; ■ — ■, dissolution of cholesterol monohydrate in cholesteryl linolate with 3% cholesteryl olate, at 40°C in the presence of a phosphate; ○ — ○, dissolution of cholesterol monohydrate in cholesterol esters isolated from human atherosclerotic plaques, at 37°C.

After stoppers and placed in a thermostatically regulated water bath. [4-14C]-cholesterol monohydrate crystals (approximately 3 times the amount necessary to saturate the oil) were added to the tube and the components continuously stirred by bubbling nitrogen into the bottom of the tube through a needle inserted through the stopper at a rate of 1 bubble/sec. At intervals, (10, 30, 60, 120 min, 3, 4, 6–24 h) the tube was removed from the bath and centrifuged at the same temperature to obtain a clear oil phase which contained no solids by microscopic examination. A weighed aliquot of the oil was assayed for radioactivity and the results expressed in weight percent cholesterol monohydrate in the oil. The dissolution of cholesterol monohydrate was studied in systems as well as in the presence of water. In the latter case, water was added to the tubes and bubbles of water-saturated nitrogen were used to solubilize the components. Dissolution was studied in 3 liquid cholesterol ester systems: (1) pure cholesteryl linolate at 37°C with and without added water, (2) a mixture of pure cholesteryl linolate 97% and pure cholesteryl olate 3% at varied temperature with and without added water *, (3) isolated human p'aque esters at 37°C with added water.

Results

Measurement of the concentration of dissolved cholesterol monohydrate as a function of time was obtained using a variety of conditions. The data for some of these experiments are presented in Fig. 2 and the final solubilities are given in Table 1. Curve a shows the dissolution of cholesterol monohydrate in anhydrous cholesteryl linolate at 37°C; curve b shows dissolution of cholesterol monohydrate into a 97% cholesteryl linolate—3% cholesteryl olate mixture in the presence of a water phase at 40°C. Curve c is the dissolution of pure cholesteryl olate could not be studied at 37–40°C because it crystallized at these temperatures [7].
TABLE 1
SOLUBILITY OF CHOLESTEROL IN CHOLESTEROL ESTER OIL AND CHOLESTEROL OIL/WATER

<table>
<thead>
<tr>
<th>Oil phase</th>
<th>Aqueous phase</th>
<th>Temp. (°C)</th>
<th>Solubility (weight %) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesteryl linoleate</td>
<td>None</td>
<td>37</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>Cholesteryl linoleate</td>
<td>H₂O</td>
<td>37</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Cholesteryl linoleate—cholesteryl oleate (97/3)</td>
<td>H₂O</td>
<td>49 b</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Cholesteryl linoleate—cholesteryl oleate (97/3)</td>
<td>H₂O</td>
<td>49 b</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Cholesteryl linoleate—cholesteryl oleate (97/3)</td>
<td>H₂O</td>
<td>49 b</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Cholesteryl linoleate—cholesteryl oleate (97/3)</td>
<td>H₂O</td>
<td>43 b</td>
<td>4.7 ± 0.1</td>
</tr>
<tr>
<td>Cholesteryl linoleate—cholesteryl oleate (97/3)</td>
<td>H₂O</td>
<td>40</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Cholesteryl linoleate—cholesteryl oleate (97/3)</td>
<td>H₂O</td>
<td>(37) c</td>
<td>3.6</td>
</tr>
<tr>
<td>Aortic plaque cholesterol esters</td>
<td>H₂O</td>
<td>37</td>
<td>3.7 ± 0.1</td>
</tr>
</tbody>
</table>

a Mean of 3—5 determinations ± standard deviation.
b The solubilities at 37 and 40°C are the measured cholesterol or cholesteryl monohydrate concentrations after 24 h equilibration. Solubilities at higher temperatures were obtained by equilibrating under the same conditions at the desired temperature for at least 8 h.
c The solubility of cholesterol monohydrate in this mixture is obtained from linear extrapolation of solubilities at higher temperatures.

tion of cholesterol monohydrate into cholesterol esters isolated from human aortic atherosclerotic plaques in the presence of water at 37°C. Experiments using each set of conditions repeated several times with constant results. In such cases dissolution of cholesterol was rapid, the concentration after 1 h read almost at least 90% of the saturation value. The final concentration of cholesterol monohydrate in the anhydrous system (4.8%) was not altered by additional 3% cholesteryl oleate to cholesterol linoleate. In the aqueous system of cholesteryl linoleate—3% cholesteryl oleate solubility increases with temperature. Neither rate of dissolution nor the final concentration was significantly different for the plaque esters as compared to the synthetic esters at 37°C (3.6–3.8%). The solubility of cholesterol monohydrate in cholesteryl linoleate agrees with a previous report [11] and is similar to its solubility in natural fats and oils as described by Kritchevsky and Tepper [12].

Discussion

Cholesterol monohydrate is a major crystalline solid of many human arterial atherosclerotic plaques [2,3]. The purpose of this study was to estimate the rate of dissolution of cholesterol monohydrate crystals in an oil phase similar to oily droplets found in advanced atherosclerotic lesions. We tried to use experimental conditions which would resemble the in vivo conditions as closely as possible. As a first approximation the composition and state of the solvent cholesterol esters, the size and form of the cholesterol crystals, the temperature, the agitation rate and the presence of water were considered.

The oil droplets in atherosclerotic plaques exist predominately as oily droplets at 37°C [3,6]. Thus we used a variety of cholesterol esters in the liquid state as solvents. The cholesterol monohydrate crystals found in
rus plaques are rhomboidal plates having a maximum length of approximately 250 μm. The cholesterol monohydrate crystals used for these dissolution experiments were on the average larger than this ranging in size from 180 to 800 μm (see Fig. 1). This larger size was chosen specifically to underestimate the dissolution rate. The smaller aortic crystals have a larger surface area/unit weight, thus, since the dissolution rate is proportional to the surface area of the crystals we would expect the dissolution rate of plaque crystals to be greater than the experimentally measured rates, all other factors being equal.

Another variable in the rate of dissolution is the agitation rate. The oily droplets in the aortic intima are subjected to agitation about 60 times/min by pulsing of blood through the artery. In the model system, the oil is stirred by bubbling nitrogen through it with a rate of approximately 60 bubbles/min.

The difference in solubility of cholesterol in the presence and absence of water is due to the difference in the crystalline form of cholesterol in equilibrium with the cholesterol in solution. In the absence of water, cholesterol monohydrate crystals are converted to anhydrous cholesterol as determined by the peak in the X-ray diffraction pattern [3]. When a water phase is present, the dissolved cholesterol remains hydrated and the solubility at equilibrium is less than in the absence of water. This lowering of the solubility of cholesterol in nonpolar solvents in the presence of a water phase was previously observed by Stauffer and Bischoff [13] and confirmed by Jandacek et al. [11].

It can be concluded that the rate-limiting step in plaque reversal is not the rate of dissolution of cholesterol into the oily cholesterol esters of the plaque. How-in atherosclerotic plaques, the oily droplets are saturated with cholesterol. If a means were found to remove dissolved cholesterol from the droplets, cholesterol monohydrate crystals would dissolve into the oil. From our experiments we can predict that the dissolution of cholesterol monohydrate crystals could occur rapidly enough to allow facile removal if dissolved cholesterol could be extracted from the solvent. The situation is quite different from dissolution of cholesterol gallstones by bile salt—lecithin micelles [15].

The dissolution process is slowed by complex phenomena involving the interaction of cholesterol molecules with bile salt and lecithin molecules at the air—water interface.

Acknowledgements

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References


Structural Organization of the Lipoprotein HDLc from Atherosclerotic Swine. Structural Features Relating the Particle Surface and Core†

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ABSTRACT: The plasma lipoprotein HDLc from miniature swine fed a high-cholesterol, saturated-fat diet exhibits a thermal transition (temperature range 25–45 °C) of its core-located cholesterol esters. This transition from an ordered, smectic-like structure to a more disordered structure is similar to that described for human plasma low-density lipoprotein (LDL). Small-angle X-ray scattering measurements demonstrate that HDLc is a spherical particle (~180-Å diameter) intermediate in size between human LDL (~220 Å) and normal high-density lipoprotein (~100 Å). The electron-density profile of HDLc below the transition (10 °C) exhibits a single core-located electron-density peak associated with a region of overlapping steroid moieties of the cholesterol esters arranged in a layered structure in the particle core. This electron-density profile may be compared to that for human LDL (below the transition temperature) which exhibits two core-located electron-density peaks due to layered cholesterol esters. Thus, the smaller size of HDLc results in one fewer molecular units in the repeating, layered cholesterol ester organization. Comparison of the electron-density profiles for HDLc and LDL demonstrates a common structural feature. The region of overlapping steroid moieties juxtaposed to the surface-locatd phospholipids and apoproteins is positioned at a constant distance from the particle surface in HDLc and LDL. This constant structural feature relating the core-located cholesterol esters and the particle surface suggests a common interaction between the phospholipid and proteins at the surface and the initial layer of cholesterol esters in the particle core.

Human plasma low-density lipoprotein (LDL1) undergoes a reversible thermal transition between 20 and 45 °C (Dealbaum et al., 1975, 1977). This transition is associated with a change from an ordered to a more disordered organization of the cholesterol esters localized in the core of the quasi-spherical LDL particle. Recently, we have used models of the molecular organization of the cholesterol esters in LDL to interpret the X-ray small-angle scattering profiles obtained from these assemblies and, hence, derive information on the molecular packing of the cholesterol esters below and above the transition (Atkinson et al., 1977). At 10 °C, the cholesterol esters are arranged in the core of the LDL particle in a radially repeating organization with a molecular packing similar to that of the smectic phase exhibited by the isolated esters. At 45 °C, this regular radial repeating organization is absent and the organization of the cholesterol esters is less ordered.

The lipoprotein designated HDLc, which appears in the plasma of cholesterol-fed miniature swine, has α2 mobility on an apoprotein composition including the arginine-rich apo-A1 apoproteins (LDL apo-B is absent) (Mahley et al., 1

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3 Abbreviations used: LDL, low-density lipoprotein; HDL, high-density lipoprotein.
TABLE I: Percent Chemical Composition of HDLc Fractions.

<table>
<thead>
<tr>
<th></th>
<th>1a</th>
<th>2b</th>
<th>3c</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein</td>
<td>15.8</td>
<td>20.3</td>
<td>25.7</td>
</tr>
<tr>
<td>phospholipid</td>
<td>17.5</td>
<td>29.3</td>
<td>32.5</td>
</tr>
<tr>
<td>cholesterol ester</td>
<td>56.3</td>
<td>42.5</td>
<td>35.0</td>
</tr>
<tr>
<td>cholesterol</td>
<td>9.9</td>
<td>7.5</td>
<td>6.2</td>
</tr>
<tr>
<td>triglyceride</td>
<td>0.5</td>
<td>0.5</td>
<td>0.6</td>
</tr>
</tbody>
</table>

\[ a \quad 1.02 < d < 1.04 \text{ g/mL} \quad b \quad 1.04 < d < 1.06 \text{ g/mL} \quad c \quad 1.06 < d < 1.09 \text{ g/mL} \]

HDLc is a cholesterol ester rich particle and is intermediate in size between LDL and normal high-density lipoproteins (HDL). Unlike normal HDL, HDLc is bound by the cell-surface LDL receptor, internalized, and catabolized by cells (Mahley and Innerarity, 1977). In this communication, we are concerned with the description of the molecular packing of the cholesterol esters in this lipoprotein.

Differential scanning calorimetric and X-ray small-angle scattering evidence indicate that the cholesterol esters in this lipoprotein can, like LDL, also form an organized microdomain in the core of the lipoprotein (Tall et al., 1977). This organized domain similarly undergoes a cooperative transition to a more disordered state. However, since LDL and HDLc exhibit this order–disorder transition whereas normal HDL does not (Tall et al., 1977), HDLc may represent a lower size limit for lipoprotein particles in which the cholesterol esters can adopt an organized structure. Thus, a structural description of the cholesterol ester organization in HDLc and comparison with the organization in normal LDL may lead to information on the details of the interaction between the cholesterol esters and the other lipid and protein components. This may provide a description of the structural principles governing lipoprotein assembly.

Materials and Methods

Swine LDL and HDLc were isolated from plasma by ultracentrifugation at a density in the range 1.02–1.063 g/mL and purified by Geon-Phenol block electrophoresis as previously described (Mahley and Weisgraber, 1974). Lipoproteins were identified by paper electrophoresis and chemical composition (Mahley and Weisgraber, 1974).

X-ray scattering measurements were made on samples, (ca. 200 mg/mL concentration) sealed in 1-mm capillary tubes, using Cu Kα radiation from an Elliott GX-6 rotating anode generator. X rays were collimated using double-mirror focusing optics as previously described (Atkinson et al., 1977). Photographically recorded scattering patterns were quantitated using a Joyce-Loebel Model H13S microdensitometer. Measurements were made on HDLc fractions of density 1.02–1.04 (fraction 1), 1.04–1.06 (fraction 2), and 1.06–1.07 g/mL (fraction 3). Table I summarizes the compositional data for these HDLc fractions (Mahley et al., 1975; Tall et al., 1977).

Results and Interpretation

Scattering patterns (Figures 1a and 3a) for HDLc (Tall et al., 1977) show a series of well-resolved scattering maxima similar to the scattering observed from normal human LDL (Atkinson et al., 1977; Laggner et al., 1976; Tardieu et al., 1976; Muller et al., 1978). These maxima are indicative of the quasispherical morphology of the HDLc and LDL particles. The scattering profiles obtained for HDLc from each fraction were qualitatively similar. However, fraction 1 was more monodisperse than fractions 2 and 3, as evidenced by the sharpness of the scattering maxima. Therefore, analysis of the data obtained from fraction 1 is presented.

In contrast to the scattering profile for LDL, which shows five subsidiary maxima, the scattering pattern for HDLc at 10 °C (Figure 1a) exhibits four maxima at larger angular spacings, consistent with a particle size smaller than LDL. The scattering profile for LDL is characterized by the high relative intensity of the fifth subsidiary maximum at 1/36 Å⁻¹. In the case of HDLc, it is the fourth subsidiary maximum centered at 1/36 Å⁻¹ which exhibits a similar high relative intensity. At 45 °C, above the temperature of the thermal transition for HDLc (Tall et al., 1977), this maximum at 1/36 Å⁻¹ is absent from the scattering pattern. However, the three maxima at smaller angles remain unchanged, demonstrating that the spherical morphology is not disrupted at 45 °C. These changes in the scattering pattern correlate with the thermal transition observed calorimetrically, are reversible, and correspond to similar changes observed for human LDL (Tall et al., 1977).

Figure 1a,b illustrates the structure factor $|F(2s)|$ for HDLc at 10 °C and the spherically averaged electron-density distribution of the HDLc particle calculated by Fourier transformation of these data. The electron-density profile clearly demonstrates that the outer radius of the particle is ~90 Å, intermediate between HDL (~50 Å) and LDL (~110 Å). The electron-density distribution shows that the overall structural arrangement of a hydrocarbon core region surrounded by a shell of high electron density which is characteristic of normal HDL and LDL also applies in the case of HDLc. The surface location of the protein and polar phos-
pholipid head groups is clearly shown by the surface-located electron-dense peak centered at 80-Å radius. This outer peak is of similar thickness (20-25 Å) to that observed in normal LDL (Atkinson et al., 1977; Laggner et al., 1976; Muller et al., 1978).

Of particular importance is the single electron-density maximum centered at a radius of 42 Å, located in the core region of the distribution. This electron-density profile showing a single maximum of electron density in the core region may be contrasted with the electron-density distribution of LDL, which shows two electron-density maxima in the core region located at ~30- and ~60-Å radius (Figure 1c). The electron-density distributions for human LDL (Atkinson et al., 1977) and HDLc, however, show a striking similarity when the two profiles are compared with the surface-located protein/polar group peaks superimposed as shown in Figure 1b,c. The core electron-density maximum located ~40 Å from the surface protein/polar electron density peak (i.e., the peak at 60-Å radius in the profile for LDL and the peak at 42-Å radius in the profile for HDLc) is a common feature of the electron-density distribution of both LDL and HDLc at 10 °C.

In the case of LDL below the thermal transition, we have proposed (Atkinson et al., 1977) (see also Laggner et al., 1976, 1977; Muller et al., 1978) that the two electron-density peaks in the core region of the profile arise from regions of superimposed steroid moieties of the cholesterol esters packed in a radial-layered structure. The molecular packing of the cholesterol esters and the resulting electron-density distribution of the model proposed for normal human LDL are shown in Figure 2a. The organization of the cholesterol esters in this model is based on the molecular pair organization observed for the packing in crystalline cholesteryl myristate (Craven and DeTitta, 1976) modified to represent the major HDLc cholesterol esters, cholesteryl oleate, and linoleate (Tall et al., 1977). The perturbations and modifications of the crystalline packing of cholesteryl myristate involved "melting" of the hydrocarbon chains of the esters, with a concomitant decrease in the length of the hydrocarbon region, together with translations of molecular pairs parallel to the molecular long axis (Atkinson et al., 1977).

The difference in radius of ~20 Å between HDLc and LDL corresponds to approximately the length of a single cholesterol ester molecule in this perturbed packing, suggesting that the smaller size of HDLc results from one less molecular unit in the radial-repeating organization. As illustrated in the model shown in Figure 2b, this size difference may be accommodated by the removal of the first molecular layer located at the center of the particle in the model for LDL. Thus, whereas in the model for LDL, the regions of overlapping steroids are centered at 30- and 60-Å radius, the removal of the first molecular layer would give regions of overlap at ~10- and ~45-Å radius.

The X-ray scattering curve calculated for this model distribution of electron density (Figure 2b) with electron-density maxima at ~10 and 45 Å together with a surface-located peak of electron density corresponding to the protein and phospholipid polar groups is shown in Figure 3a compared with the scattering of HDLc at 10 °C. The calculated scattering profile is in good agreement with that observed experimentally, both reproducing the positions and relative intensities of the subsidiary maxima and accurately reproducing the high relative intensity of the fourth maximum at 1/30 Å⁻¹. The electron-density profile of the model calculated from the theoretical scattering data at the same resolution obtained experimentally (~20 Å) is shown in Figure 3b. This electron-density d
bution is also in good agreement with that derived from the experimental data (Figure 3b), particularly in the region of the outer protein/polar group shell and the electron-density peak at 42-Å radius.

Resolution artifacts at a radius <20 Å due to the limited angular range of the experimental data preclude a direct confirmation of the layer of superimposed cholesterol moieties in this region. However, calculations for a model (Figure 2b) in which the electron-density peak close to the origin was omitted (i.e., containing only the peaks at ~45 and 80-Å radius) showed that removal of this central region of high electron density results in a scattering profile (Figure 3a) in which the third rather than the fourth subsidiary maximum has a high relative intensity. The experimental data for HDL_c clearly shows a high relative intensity of the fourth subsidiary maximum at \( \bar{V}_{10} \) Å\(^{-1}\). Thus, the model calculations provide indirect evidence for a region of high electron density at the center of the particle. However, the packing in this region (\( R < 20 \) Å), which contains less than 5% of the volume of the core region, is undoubtedly highly perturbed due to the small radius of curvature.

Discussion

The similarities in the thermal behavior of the cholesterol esters in swine HDL_c and human and swine LDL suggest a similar structural organization of the esters in both lipoprotein classes (Tall et al., 1976). The qualitative features of the X-ray scattering profiles for HDL_c and LDL, particularly the enhanced relative intensity of the scattering maximum at \( \bar{V}_{10} \) Å\(^{-1}\) (and its disappearance above the calorimetric transition), direct comparison of the electron density profiles of HDL_c and LDL, together with the model calculations further substantiate a common structural arrangement of the cholesterol esters.

The electron-density distribution for HDL_c exhibits a single peak in the electron density of the core region in contrast with the distribution for LDL which exhibits two peaks. These electron-density maxima in the core region of LDL have previously been ascribed to regions of register steroid moieties of the cholesterol esters, organized in a radially layered organization (Atkinson et al., 1977). The single electron-density peak in the core region of HDL_c undoubtedly arises from a similar overlap of steroid moieties of the cholesterol esters. The small size of HDL_c results simply in fewer molecular layers in the radial organization.

More important, however, is the observation that the distance between the electron-density maxima due to the overlap of steroid groups juxtaposed to the surface-located protein/polar-group region is similar in HDL_c and LDL (\( \sim 45 \) Å peak to peak). This common feature suggests a similar structural arrangement and interactions between the outer layers of cholesterol esters and the layer of protein and phospholipids at the surface of HDL_c and LDL. The model calculations indicate that the distance between the outer edge of the steroid peaks (\( R = 50 \) Å for HDL_c and \( R = 75 \) Å for LDL) and the inner edge of the protein/polar surface peak (\( R = 65 \) Å for HDL_c, \( R = 90 \) Å for LDL) is 15–20 Å and accommodates the hydrocarbon chains of the phospholipids. This indicates that the first layer of overlapping steroid regions beneath the surface of the lipoprotein abuts the phospholipid acyl chains. With the steroid nucleus located at this position relative to the surface of the particle, either the \( C_17 \) isooycetyl side chain or the fatty acid chain of the cholesterol ester may interdigitate with the fatty acid chains of the phospholipid. Which of these alternatives applies cannot be determined at the resolution of this study. However, interdigitation of the cholesterol ester fatty acid chain amongst those of the phospholipid would necessarily expose a large apolar area at the particle surface. This apolar area could be adequately shielded from the aqueous environment by the surface-located apoproteins.

A similar argument may apply in the case of the cholesterol ester organization of normal HDL. The interaction and location of a single layer of cholesterol moieties abutting the phospholipid acyl chains in HDL, however, would account for the 50-Å radius of HDL. Thus, for normal HDL the particle size will not accommodate additional repeating layers of cholesterol esters necessary for an organized domain capable of undergoing thermal rearrangement.

This orienting effect of the surface organization on the core-located cholesterol esters is likely to persist above the thermal transition. Thus, above the thermal transition these surface constraints may result in residual radial alignment of the cholesterol esters, resulting in structural arrangement resembling a nematic or cholesteric phase (i.e., alignment of molecular long axes but not layering of molecules) rather than an isotropic liquid (Tall et al., 1977).

The demonstration of this common structural feature in the molecular organization of HDL_c and LDL points to a "common" interaction between the surface-located phospholipid and protein components and the initial layer of the core-located cholesterol esters. This interaction stabilizing the surface of the lipoprotein particle may apply for all cholesterol ester carrying lipoproteins. The subsequent molecular organization of the cholesterol esters in the bulk of the core of HDL_c, LDL, and perhaps normal HDL may then be described at low temperatures by the number of regularly organized layers.

Acknowledgments

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Studies on the Structure of Low Density Lipoproteins Isolated from
Macaca Fascicularis Fed an Atherogenic Diet

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Abstract
Cynomolgus monkeys, Macaca fascicularis, fed cholesterol-containing saturated-fat diets develop increased levels of high molecular weight plasma low density lipoproteins (LDL), associated with accelerated atherosclerosis. To study the composition and structure of these abnormal particles, LDL from monkeys, fed atherogenic and control diets, were characterized chemically and examined by differential scanning calorimetry and low-angle X-ray scattering. LDL from animals on the experimental diet showed an increase in molecular weight (4.0 to 7.0 x 10^6, experimental diet compared with 3.0 to 3.7 x 10^6, control diet) associated with a large increase in cholesterol ester content and concomitant smaller increases in protein, phospholipid, and free cholesterol. There was a strong positive correlation between molecular weight and the number of saturated and monounsaturated cholesterol esters in the particle. In contrast, particle content of polyunsaturated cholesterol esters remained constant despite large changes in total particle cholesterol esters.

When examined by calorimetry and X-ray scattering, LDL from monkeys on both diets displayed a reversible transition of cholesterol esters from an ordered smectic-like (layered) structure to a more disordered state. For all animals on the experimental diet, the peak temperature of the cholesterol-ester transition (42-48°C) was above body temperature (39°C), but below body temperature on the control diet (34-38.5°C). In the experimental group, the transition temperature was correlated with the LDL molecular weight. However, after thermal disruption of LDL, liquid-crystalline transitions of LDL cholesterol esters were observed in the same temperature range as in the intact lipoprotein, which shows that changes in particle size had little effect on the cholesterol-ester transition temperature. Rather, the transition temperature was determined by the degree of saturation of the LDL cholesterol ester fatty acids and the LDL cholesterol ester triglyceride ratio, both of which correlated with increased LDL molecular weight.

The existence of smectic-like cholesterol ester in LDL at body temperature was clearly a discriminating feature between monkeys on control and experimental diets. Diet-induced changes in the lipid composition of precursor lipoproteins of LDL appeared to lead to the existence of smectic-like cholesterol ester in LDL above body temperature. The altered composition and structure of the core lipids of high molecular weight LDL probably account, in part, for the previously documented correlation between increased LDL molecular weight and atherosclerosis in this species.

Introduction
In an attempt to understand their role in the pathogenesis of atherosclerosis, the plasma lipoproteins have been studied in a number of non-human primate species receiving atherogenic diets (1-7). The hyperlipoproteinemia induced in Macaca fascicularis is characterized by increased concentrations of low density lipoprotein (LDL) and decreased concentrations of high density lipoprotein, a profile resembling the atherogenic pattern of humans. In addition,

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1Abbreviations used in this paper: HDL, high density lipoprotein; HDLc, HDL isolated from cholesterol-fed swine; LDL, low density lipoprotein.

studies of LDL chemical composition and size in M. fascicularis and in Rhesus monkeys have indicated that these animals respond to atherogenic diets by developing cholesterol ester-enriched LDL particles of increased molecular weight (4–6). The LDL molecular weight of the more atherosclerosis-prone adult male M. fascicularis greatly exceeded that of the adult females of this species (7).

In a study of 40 M. fascicularis monkeys fed atherogenic and control diets, it was found that the average percent stenosis of the coronary arteries correlated more strongly with LDL molecular weight than with other variables (8), including total plasma cholesterol, LDL cholesterol, LDL cholesterol ester:protein ratio, and molar concentration of LDL. Factors that might explain the relationship between LDL atherogenicity and molecular weight probably include abnormalities of protein or lipid structure associated with increased size of LDL.

Recent studies, with techniques such as differential scanning calorimetry and low-angle X-ray scattering, have helped to elucidate lipid structure and lipid/protein interactions in the plasma lipoproteins (9–15). Human plasma LDL displays a broad, reversible thermal transition which encompasses body temperature associated with the transition of its core-located cholesterol esters from a smectic-like (layered) to a more disordered state (11, 15). In studies of normal human lipoproteins it was found that the peak temperature of this transition varied from 28 to 37°C and was related to the LDL cholesterol ester:triglyceride ratio and, to a lesser extent, the degree of saturation of the LDL cholesterol ester fatty acids (11). Because the peak temperature of the cholesterol-ester transition corresponds with the temperature at which about one-half of the cholesterol esters are ordered in a smectic-like state, and because the transition occurs over a broad temperature range, those individuals who have a peak temperature near body temperature probably have some ordering of their LDL cholesterol esters. Although the importance of the lipid organization of human LDL is unknown, studies of LDL from miniature swine fed atherogenic diets showed that their LDL cholesterol ester was more ordered at body temperature than that of normal human LDL, which suggests that the physical state of the LDL cholesterol esters might have a role in determining atherogenicity of LDL (13).

To investigate the interrelations between chemical composition, LDL molecular weight, and the physical state of LDL cholesterol esters, we have undertaken a detailed chemical and physical study of LDL from groups of M. fascicularis monkeys fed atherogenic and control diets. Our specific aims were (a) to examine the relationship between chemical factors known to influence the structure of LDL core lipids (cholesterol ester:triglyceride ratio and cholesterol ester fatty acid composition) and LDL molecular weight; and (b) to determine the effect of increased size of LDL on the structural organization of LDL core lipids.

METHODS

A group of 40 adult male M. fascicularis were conditioned to their new laboratory environment throughout a 90-day quarantine period. At the end of this period, a test diet containing 40% of calories as fat with a cholesterol level of 0.70 mg/kcal (0.3% of diet) was fed for 60 days. At the end of this feeding period, the animals fasted for 24 h, a 30-ml blood sample was taken from each of the animals, and the group was then placed on a monkey chow diet (Ralston Purina Co., St. Louis, Mo.). After plasma cholesterol concentrations had returned to prechallenge levels, eight of the animals were fed a control diet for 60 days of the same composition as the test diet, except that the cholesterol level was 0.03 mg/kcal (0.015%). Blood samples were collected from these animals after a 24-h fasting period. All animals were immobilized with ketamine-HCl, 10 mg/kg, before blood sample collection. Blood collections and subsequent handling were carried out as described previously (16).

Plasma lipoproteins were isolated by centrifugation and separated by agarose column chromatography. Before examination by calorimetry or X-ray diffraction, samples were concentrated by vacuum dialysis. Lipoprotein cholesterol and protein distribution were determined after chromatographic separation (17). Measurement of cholesterol content was carried out by the method of Rudel and Morris (18) and protein was assayed with the procedure of Lowry et al. (19), with bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, Mo.) as the standard. Measurement of the molecular weight of the LDL was routinely carried out during chromatography with a standard [3H]LDL marker as described previously (5). In addition, eight selected samples were subjected to molecular weight determination by the analytical ultracentrifuge method of Nelson et al. (20). The agreement between the two methods was excellent (Analytical ultracentrifugation, 5.24 ± 0.30 [× 10^15] vs. Column method 5.2 ± 0.31 [× 10^15], mean ± SEM). LDL isolated as peak III of the column elution profile, were then subjected to detailed chemical analysis, as described previously (5). Briefly, lipids were extracted, individual lipid classes separated by thin-layer chromatography, and determinations of the amounts of triacylglycerol (21) and free and esterified cholesterol (18) were performed. Phospholipid phosphorus (22), total cholesterol, and protein content was determined directly in the lipoprotein solution.

The cholesterol-ester fatty acid compositions were determined by gas-liquid chromatography, essentially according to Kuksi (23). The region of the thin-layer chromatography plate that contained the cholesterol esters was identified by staining an adjacent spot with rhodamine. The spots were scraped, extracted, and the esters were saponified. The fatty acids were then methylated with boron trifluoride in methanol (24). The methyl esters were then extracted into hexane, and separation of the fatty-acid methyl esters were carried out isothermally in a Bendix model 2500 Chromatograph (Bendix Corp., Environmental & Process Instruments Div., Baltimore, Md.) in 6-foot × ½-inch internal diameter glass columns packed with 10% EGSS-X on Gas-Chrom. P, 100–200 mesh (Applied Science Labs, Inc., State College, Pa.). Data were calculated and expressed as molar percentage values; standard National Institutes of Health fatty acid mixtures (Applied Science
Table I

Influence of Dietary Cholesterol on Distribution of Lipoprotein Cholesterol and Protein among
Size Populations of Plasma Lipoproteins

<table>
<thead>
<tr>
<th>Lipoprotein elution region on agarose column</th>
<th>Whole plasma*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td>n</td>
</tr>
<tr>
<td>-----------------</td>
<td>---</td>
</tr>
<tr>
<td>Cholesterol</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
</tr>
<tr>
<td>Test</td>
<td>36</td>
</tr>
<tr>
<td>Protein</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
</tr>
<tr>
<td>Test</td>
<td>36</td>
</tr>
</tbody>
</table>

* Determined on the samples used for lipoprotein isolation.
† Mean±SEM.

Labs, Inc.) were used to establish the relative weight responses of individual fatty acids.

Physical methods. Differential scanning calorimetry was performed on a Perkin-Elmer DSC-2 instrument (Perkin-Elmer Corp., Norwalk, Conn.), calibrated with cyclohexane, indium, and dimyristoyl lecithin, as previously described (9-12). 75-μl samples were sealed hermetically in sample pans and scanned at heating and cooling rates of 2.5 or 5°C/ min. Most experiments were performed at a sensitivity of 0.1 or 0.2 mcal/s.

X-ray scattering measurements were made on samples sealed in Lindeman glass tubes (Lindeman Corp., Indianapolis, Ind.) with CuKα radiation from an Elliott GX-6 rotating anode X-ray generator (Marconi-Elliott Avionics, Ltd., Borehamwood, England), with Franks or toroidal mirror optics (15).

Hot-stage polarizing light microscopy was performed with a Zeiss NL polarized light microscope (Carl Zeiss, Inc., New York) as previously described (25). Samples were examined under glass coverslips at heating and cooling rates of 1-2°C/ min. The smectic phase of cholesterol esters was identified by its positive sign of birefringence, and the cholesteric phase by its negative sign (25).

Statistical analysis were performed as described in Snedecor and Cochran (26), and differences are at the P < 0.01 level, unless otherwise indicated.

RESULTS

The agarose column elution profiles of the plasma lipoproteins resembled that described previously.2 The control samples contained more material absorbing at 280 nm in the region IV high density lipoproteins (HDL) than in the other lipoprotein fractions. The hyperlipoproteinemia induced by the test diet was characterized by a shift in lipoprotein distribution so that LDL of region III contained by far the most 280-nm-absorbing material, while the amount of material in the HDL region was decreased compared with the control samples. The average size of the test-diet plasma LDL was larger, as demonstrated by the shift to a lower elution volume at the center of peak III. To quantitate the shift in lipoprotein distribution induced by dietary cholesterol, the total cholesterol and protein content in each of the regions of the column elution profile was measured (see Table I). Significant increases in the amount of protein and cholesterol in regions I (very low density lipoprotein), II (intermediate density lipoprotein), and III (LDL) occurred, and significant decreases in the region IV (HDL) were present.

Samples from nine animals that encompassed the range of LDL molecular weight variation observed among those that received the test diet, were selected for detailed studies. Samples from eight monkeys on the control diet were also studied in detail (Tables II-V, and Figs. 1-5). Four of the nine animals examined on the atherogenic diet were also examined on the control diet; that is, they acted as their own controls. There was a significant increase in LDL molecular weight induced by dietary cholesterol; i.e., control 3.3±0.10×106 vs. atherogenic 5.3±0.33×106 (Table II). Also, there was a higher percentage of protein (P < 0.05) and phospholipid, and lower percentage of cholesterol ester in the low molecular weight LDL of the control diet group (Table II). The mean composition within the particle was calculated from the percentage composition and the molecular weight data as shown in Table III. Free cholesterol and phospholipid increased in proportion to molecular weight, while the phospholipid:free-cholesterol ratio decreased. The most striking increase was in the number of cholesterol ester molecules per particle. Because there was a small decrease in the triglyceride content of LDL on the experimental diet compared with the control, there was an increase in the cholesterol ester:triglyceride ratio in the experimental group (mean±SEM = 206±52).


compared with the controls (41±9.9, P < 0.05). On the test diet there was an increase in the protein content of higher molecular weight LDL.

The average diameter for each particle was calculated, with the formula for the volume of a sphere to relate volume and diameter, and with the partial specific volume of each component to calculate volume. These calculations assume that high molecular weight LDL has similar structure to normal LDL, i.e., with a core of apolar lipid and a surface coat of phospholipid, protein, and free cholesterol (15). The diameters increase in proportion to molecular weight from a value of 207 Å at a molecular weight of 2.98×10^6 to a value of 277 Å at 6.97×10^6 (Table IV). The volume of the core of the LDL particle was estimated by adding that of cholesterol ester and triglyceride which occupy the core. When the core diameter was subtracted from that of the whole particle and the difference divided by 2, the thickness of the cholesterol-phospholipid-protein coat was obtained. This value (22 Å) was constant throughout the range of LDL molecular weights. Thus, all of the size difference in the LDL particles resulted from an increase in core diameter. The LDL cholesterol ester fatty-acid composition is shown in Table V. There was a trend in cholesterol ester fatty-acid composition with increasing molecular weight: the higher the molecular weight, the higher the percentage of cholesteryl oleate (18:1) and stearate (18:0), and the lower the percentage of cholesteryl linoleate (18:2) and arachidonate (20:4) (see Table V). The relation between LDL molecular weight and particle content of cholesterol esters of different saturation is shown in Fig. 1. In LDL-particles of increasing molecular weights there was a fixed amount per LDL particle of cholesterol ester which contained polyunsaturated fatty acids (Δ2 fatty acids) such as cholesteryl linoleate and arachidonate. However, there was an increased content of saturated and monounsaturated cholesterol esters proportional to LDL molecular weight. The correlation coefficient (r) between LDL molecular weight and the amount of monounsaturated cholesterol esters was r = 0.98, while that for saturated cholesterol esters was r = 0.99.

Physical data. In the scanning calorimeter, both control and experimental LDL displayed a broad, reversible transition of enthalpy 0.77±0.08 cal/g cholesterol ester (mean±SD), which resembled that previously described for human LDL (10) (Fig. 2). There was a progressive decrease in the peak temperature of this transition with decreasing molecular weight (atherogenic LDL of molecular weight 6.97×10^6 is shown in Fig. 2a and b, 5.18×10^6 in Fig. 2c and d, and control-diet LDL of molecular weight 3.08×10^6 in Fig. 2e and f). On the test diet, the peak temperature of the cholesterol-ester transition ranged from 42 to 48°C and correlated with LDL molecular weight.

### Table II

**Comparison of LDL Mass Composition among Normal and Hyperlipoproteinemic M. fascicularis**

<table>
<thead>
<tr>
<th>Diet</th>
<th>n</th>
<th>MW* (×10^6)</th>
<th>FC</th>
<th>CE</th>
<th>TG</th>
<th>PL</th>
<th>Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>3.29±0.10†</td>
<td>8.5±0.27</td>
<td>43.4±1.27</td>
<td>2.0±0.39</td>
<td>23.3±0.55</td>
<td>22.8±1.04</td>
</tr>
<tr>
<td>Test</td>
<td>9</td>
<td>5.31±0.33</td>
<td>8.8±0.15</td>
<td>51.1±0.60</td>
<td>0.4±0.13</td>
<td>18.9±0.43</td>
<td>20.7±0.55</td>
</tr>
</tbody>
</table>

*Abbreviations: MW, average molecular weight of LDL particle; FC, free cholesterol; CE, cholesterol ester; TG, triacylglycerol; PL, phospholipid; Pro, protein.
† Mean±SEM.
Table III
Relationship of LDL Mean Number of Molecules within Particle to Molecular Weight

<table>
<thead>
<tr>
<th>Diet</th>
<th>MWI</th>
<th>FC</th>
<th>CE</th>
<th>TG</th>
<th>FL</th>
<th>Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>molecules/LDL particle</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.98§</td>
<td>556</td>
<td>1,697</td>
<td>132</td>
<td>945</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>3.06§</td>
<td>664</td>
<td>1,909</td>
<td>41</td>
<td>880</td>
<td>32</td>
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<tr>
<td></td>
<td>3.07§</td>
<td>674</td>
<td>1,993</td>
<td>86</td>
<td>891</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>3.08</td>
<td>677</td>
<td>1,996</td>
<td>51</td>
<td>861</td>
<td>31</td>
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<tr>
<td></td>
<td>3.37</td>
<td>809</td>
<td>2,057</td>
<td>104</td>
<td>1,126</td>
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<tr>
<td></td>
<td>3.46</td>
<td>857</td>
<td>2,271</td>
<td>84</td>
<td>1,112</td>
<td>30</td>
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<tr>
<td></td>
<td>3.54</td>
<td>868</td>
<td>2,510</td>
<td>28</td>
<td>1,018</td>
<td>29</td>
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<tr>
<td></td>
<td>3.74§</td>
<td>840</td>
<td>2,765</td>
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<td>1,081</td>
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<tr>
<td>Test</td>
<td>3.98§</td>
<td>853</td>
<td>2,910</td>
<td>8</td>
<td>1,049</td>
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<td>4.22</td>
<td>972</td>
<td>3,138</td>
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<td>1,130</td>
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<td>979</td>
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<td>41</td>
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<td>3,846</td>
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<td>47</td>
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<td></td>
<td>5.18</td>
<td>1,150</td>
<td>4,108</td>
<td>33</td>
<td>1,274</td>
<td>40</td>
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<tr>
<td></td>
<td>5.64</td>
<td>1,269</td>
<td>4,230</td>
<td>10</td>
<td>1,460</td>
<td>48</td>
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<td></td>
<td>5.92</td>
<td>1,437</td>
<td>4,714</td>
<td>84</td>
<td>1,288</td>
<td>46</td>
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<tr>
<td></td>
<td>6.29§</td>
<td>1,432</td>
<td>4,856</td>
<td>46</td>
<td>1,498</td>
<td>52</td>
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<tr>
<td></td>
<td>6.97</td>
<td>1,710</td>
<td>5,693</td>
<td>66</td>
<td>1,655</td>
<td>50</td>
</tr>
</tbody>
</table>

* Molecular weights used to calculate these data were: FC, 387; CE, 667; FL, 775; TG, 906; Pro, 25,000.
1 Abbreviations are the same as in Table II.
§ Indicates the four monkeys who served as their own controls.

(r = 0.77, Fig. 3). Although there were only small amounts of LDL available from monkeys on the control diet, analysis of five samples of LDL (three individual samples and two pooled on the basis of similarity in molecular weight) also indicated a correlation between transition temperature and molecular weight (r = 0.83, Fig. 3). There was a notable hiatus between transition temperatures obtained for LDL from animals receiving control and test diets which occurred at the body temperature of the monkey (39°C).

When heated to higher temperature, LDL displayed a small irreversible endotherm of enthalpy which was ≈1 cal/g protein (e.g., Fig. 2a). This value is similar to the lipoprotein denaturation endotherm observed previously for human LDL (10). The peak temperature of the lipoprotein denaturation endotherm ranged from 79 to 87°C and correlated with particle molecular weight (r = 0.78). After lipoprotein denaturation a double-peaked endotherm was observed in the same temperature range as the cholesterol-ester transition of the intact lipoprotein. However, this enthalpy, 1.1 ±0.05 cal/g cholesterol ester (mean ±1 SD), had a value significantly greater than the corresponding enthalpy in the intact lipoprotein (P < 0.01). Despite variation in size, the enthalpy of the cholesterol-ester endotherm of the intact lipoprotein was consistently ≈70% of the

Table IV
LDL Particle Diameter and Molecular Weight Relationships

<table>
<thead>
<tr>
<th>Diet</th>
<th>Molecular weight</th>
<th>Particle diameter</th>
<th>Diameter of core</th>
<th>Thickness of coat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Control</td>
<td>2.98</td>
<td>207</td>
<td>161</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>3.06</td>
<td>209</td>
<td>163</td>
<td>23</td>
</tr>
<tr>
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<td>21</td>
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<tr>
<td></td>
<td>3.08</td>
<td>210</td>
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<td>22</td>
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<td></td>
<td>3.37</td>
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<td>169</td>
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<td>3.46</td>
<td>219</td>
<td>174</td>
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<td></td>
<td>3.54</td>
<td>220</td>
<td>178</td>
<td>21</td>
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<td></td>
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<tr>
<td>Mean</td>
<td>3.98</td>
<td>229</td>
<td>186</td>
<td>21.5</td>
</tr>
</tbody>
</table>

* Mean thickness of coat (Control + Test): 22.1 ±0.2.

enthality of the corresponding endotherm in the denatured lipoprotein, which indicates that the LDL cholesterol-ester transition did not vary in enthalpy with molecular weight (Fig. 3). Polarized light microscopy of

Table V
Cholesterol Ester Fatty-Acid Composition of LDL Particles of Varying Molecular Weight

<table>
<thead>
<tr>
<th>Diet</th>
<th>MW* (×10^n)</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.98</td>
<td>1.0</td>
<td>13.3</td>
<td>1.7</td>
<td>4.5</td>
<td>31.2</td>
<td>44.6</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>3.06</td>
<td>1.0</td>
<td>14.2</td>
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<td>37.5</td>
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<td>3.07</td>
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<td>1.6</td>
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<td>1.7</td>
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<td>35.8</td>
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<td>1.4</td>
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</tr>
<tr>
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<td>3.54</td>
<td>1.0</td>
<td>12.3</td>
<td>2.7</td>
<td>5.6</td>
<td>43.2</td>
<td>33.2</td>
<td>2.0</td>
</tr>
<tr>
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<td>0.7</td>
<td>12.1</td>
<td>3.7</td>
<td>7.1</td>
<td>41.7</td>
<td>32.6</td>
<td>2.0</td>
</tr>
<tr>
<td>Test</td>
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<td>15.6</td>
<td>3.3</td>
<td>8.1</td>
<td>47.9</td>
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<td>45.5</td>
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<td></td>
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<td>0.7</td>
<td>14.8</td>
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* Average molecular weight of LDL particle.

heat-denatured LDL showed that the peaks of this transition corresponded to smectic-cholesteric and cholesteric-liquid transitions of the liberated cholesterol esters (25). The temperatures determined by microscopy were within 1°C of the peak temperatures noted by differential scanning calorimetry.

The main determinant of LDL transition temperature was the saturation of cholesterol ester fatty acids \((r = 0.94\), controls; \(r = 0.74\), experimental, Fig. 4). Cholesterol ester fatty-acid composition was also correlated with the lipoprotein denaturation temperature \((r = 0.76)\). LDL from some animals in the control group had notably lower transition temperatures than test animals with similar cholesterol ester fatty-acid saturation. The lower transition temperatures in the controls are attributed to the lower cholesterol ester:triglyceride ratio of the control group.

**X-ray scattering.** Irrespective of LDL molecular weight, the X-ray scattering profiles of LDL that was isolated from control and test animals, recorded at 10°C (below the thermal transition), exhibited a series of scattering maxima which included a maximum of high relative intensity at 1/36 Å⁻¹. This maximum at 1/36 Å⁻¹ was absent from the scattering profiles recorded for all LDL samples above the thermal transition.

The scattering profile (Fig. 5a) obtained for the control LDL \((3.08 \times 10^6 \) daltons) closely resembled that previously reported for human LDL \((11, 15)\), exhibiting five, subsidiary scattering maxima with the fifth maximum at 1/36 Å⁻¹ having a high relative intensity. The high molecular weight LDL from animals on the test diet exhibited scattering profiles in which the positions, number, and relative intensities of the scattering maxima showed variations compared with the control LDL and human LDL. For example, the scattering profile for LDL of molecular weight 5.6 \(\times 10^6\) shown in Fig. 5b exhibits six subsidiary maxima at closer angular spacings than the maxima observed for the control LDL, consistent with a larger particle size.

In the case of human LDL and HDL isolated from
cholesterol-fed swine (HDL) (14) the high relative intensity of the 1/36 Å⁻¹ maximum in the scattering profiles observed below the thermal transition has been shown (15, 27) to arise from a radially layered arrangement of the cholesterol esters in the core of the particle. This radially layered organization results in concentric regions in the core of the particle in which the cholesterol moieties of the cholesterol esters are packed in register (13, 27). The smaller size of HDL (90-Å radius compared to 110-Å radius for human LDL) results in a scattering profile which exhibits four subsidiary maxima, the fourth maximum located at 1/36 Å⁻¹ having a high relative intensity. This profile indicates that HDL has one fewer layered molecular units in the radially layered structure (27).

The observation that the sixth maximum is located at 1/36 Å⁻¹ and shows a high relative intensity for the larger molecular weight LDL, demonstrates that below the thermal transition the cholesterol esters are also organized in a regular radially layered packing. The particle volume, core volume, and core diameter calculations presented in Table IV indicate that the radial dimension of LDL of molecular weight >5 x 10⁶ is sufficient to accommodate an additional layered molecular unit in the core. The inserts to Figs. 5a and b show schematic representations of the cholesterol ester organization below the transition in the control LDL and in an LDL of molecular weight >5 x 10⁶, respectively. The representation of the packing in the control LDL is identical to that previously given for human LDL. The schematic for the packing in the high molecular weight LDL was derived from that of the control LDL by the addition of one layered molecular unit in the radial cholesterol-ester organization. Also shown in Figs. 5a and b are the theoretical scattering profiles for models which correspond to the cholesterol-ester packing shown in the schematics. As in the case of human LDL (15) the calculated scattering profile for the model of the control LDL is in good agreement with the profile obtained experimentally. Furthermore, the profile derived from the model of the high molecular weight LDL which contained an additional molecular layer of cholesterol ester is in reasonable agreement with the profile obtained from LDL of molecular weight 5.6 x 10⁶, exhibiting six subsidiary maxima with the sixth maxima at 1.36 Å⁻¹.
The degree of saturation of LDL cholesterol ester fatty acids was correlated with cholesterol ester transition temperature for animals on both control and test diets (Fig. 3). The variation of cholesterol ester triglyceride did not correlate well with transition temperature within the experimental group, because of the virtual absence of triglyceride in experimental-group LDL. In the control group LDL as well as in normal human LDL (11) both the cholesterol ester triglyceride ratio and cholesterol ester fatty-acid composition are important in determining the LDL cholesterol-ester transition temperature. However, when particle triglyceride is very low, as occurs in several species fed atherogenic diets (2, 4, 14, 28), and perhaps in some individuals with homozygous familial hypercholesterolemia (29), cholesterol ester fatty-acid composition is the main determinant of LDL transition temperature.

An unexpected finding was the lack of correlation between the enthalpy of LDL cholesterol-ester transition and particle molecular weight, despite examination of particles with a wide range of molecular weights, from 3 to $7 \times 10^9$. In fact, even smaller particles such as HDL, of swine (molecular weight, 1.9–2.3 $\times 10^9$) show an enthalpy of the particle cholesterol-ester transition (0.83 cal/g cholesterol ester) similar to the larger LDL. An analysis of the X-ray scattering data with Fourier-Bessel transform techniques indicates that particles of increased size retain the same layered organization of cholesterol esters described previously (15, 27), but that they have added layers of cholesterol ester within the particle core. The lack of dependence of the cholesterol-ester transition enthalpy upon particle molecular weight suggests that the constraining effect of the particle on the liquid crystal melt is exerted throughout the particle core and extends for at least two or three layers of cholesterol ester. A reduction in enthalpy, dependent upon a non-melting layer of cholesterol ester contiguous with the particle surface, or upon a disordered region of lipids at the particle center, would be reflected in a progressive increase in enthalpy with increasing molecular weight. The experimental results are thus consistent with the proposed model of LDL where the reduction in melting enthalpy is a result of the persistence above the transition of a radially oriented, nematic-like arrangement of cholesterol esters (11, 15, 30). The latter is the predominant structural organization of LDL cholesterol esters in normal humans and control monkeys.

With increased molecular weight of LDL, there was an increased content of saturated and monounsaturated cholesterol ester fatty-acid particles, and a constant contribution from diunsaturated and polyunsaturated cholesterol ester fatty acids (Fig. 1). Because lecithin acyltransferase preferentially esterifies cholesterol with polyunsaturated fatty acids derived from the 2-
carbon position of phospholipid glycerol (31), these findings suggest that a relatively constant amount of LDL cholesterol esters in all samples was derived from esterification of cholesterol within the plasma. The cholesterol esters with more saturated fatty acids may have been derived from esterification of cholesterol within the liver or within the small intestinal mucosa. In the rabbit, preferential esterification and incorporation of dietary cholesterol into lymph chylomicrons and very low density lipoproteins has been shown (32). Increased dietary cholesterol may lead to lymph chylomicrons and very low density lipoproteins that have an increased particle content of cholesterol ester. Catabolism of these particles may then lead to formation of cholesterol ester-enriched remnants (33) and, ultimately, to cholesterol ester-enriched LDL particles. Although no data is available in the monkey, the high content of palmitic, stearic, and oleic acids in cholesterol esters would reflect the activity of an intestinal cholesterol-ester synthetase that preferentially utilizes more saturated and monounsaturated fatty acids, as has been described in the rat (34). If the high cholesterol test diet results in chylomicrons that contained an increased number of saturated and monounsaturated cholesterol esters then the remnant would also contain these esters.

LDL molecular weight was shown to bear a strong correlation with the development of coronary atherosclerosis in M. fascicularis, independent of total plasma cholesterol concentration (9). Variables of LDL composition and structure might explain this correlation. LDL molecular weight was strongly correlated with the content of saturated fatty acids of cholesterol ester (r = 0.99), which suggests the hypothesis that atherogenicity of the high molecular weight LDL is related to the fatty-acid composition and, thus, the physical state of its cholesterol esters. In culture of monkey aortic smooth muscle cells, abnormal Rhesus monkey LDL (35, 36) or high molecular weight M. fascicularis LDL (37) stimulate cholesterol ester accumulation to a greater extent than does normal monkey LDL. The cholesterol ester accumulating in response to hyperlipemic serum is thought to be derived largely from uptake of serum cholesterol ester (36). Because the affinity for binding to LDL receptors on smooth muscle cells is similar for both the high molecular weight and normal LDL, accumulation of excess cholesterol ester may simply reflect the internalization of two to three times more cholesterol ester molecules per layer LDL particle. In addition, because the atherogenicity of the high molecular weight LDL may depend upon the physical state of its cholesterol esters, accumulation of cholesterol ester contained in these particles may reflect a relative inability of lysosomal acid lipase to degrade the more ordered cholesterol esters, perhaps as a result of the limited accessibility of the enzyme to the ester linkage in the smectic phase (25, 38). Thus, the structure of LDL core lipids may play an important role in determining a relative deficiency of acid lipase. The latter has been suggested by Peters and DeDuve (39), and Berberian and Fowler (40) to be a factor in the development of rabbit and human atherosclerosis.

ACKNOWLEDGMENTS

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ESSENTIALS OF CLINICAL PRACTICE

SECOND EDITION

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LITTLE, BROWN AND COMPANY    BOSTON
Diseases of the biliary tract and gallbladder may be divided simply into two areas: diseases involving gallstones and their sequelae, and disorders that are unrelated to gallstones. To the practicing physician, by far the most important are the diseases related to gallstones, which extend from the "silent" stone to ascending cholangitis. Among the disorders that are unrelated to gallstone disease are primary tumors, sclerosing cholangitis, and uncommon infections leading to cholecystitis or cholangitis.

The Enterohepatic Circulation and the Gallbladder
Molecules that are secreted into the bile by the liver, absorbed by the intestine, and then resecreted by the liver are said to have an enterohepatic circulation (EHC). Quantitatively the most important class of compounds undergoing EHC are bile salts, which are synthesized in the liver from cholesterol and are then conjugated with glycine or taurine to produce detergent-like molecules resistant to the acid pH of the duodenum. Conjugated bile salts are secreted by an active transport mechanism into the bile canaliculi and progress down the biliary tract to enter the duodenum and gallbladder. Studies in baboons show that during fasting about half the secreted bile salts enter the gallbladder, while the rest pass into the duodenum to be recycled; as fasting continues, the bile salts recycle repetitively, and nearly all eventually pass into the gallbladder. Since the volume of bile entering the gallbladder during a 12-hour fast may be 300 to 600 ml in man, the gallbladder must reabsorb considerable fluid and electrolytes to maintain its volume of about 25 to 50 ml. Thus during gallbladder filling, sodium bicarbonate, chloride, and water are reabsorbed to leave a bile about 10 times as concentrated as the original hepatic bile with respect to bile salts and bilirubin.

During eating, specific amino acids and fatty acids stimulate the duodenal mucosa to release cholecystokinin-pancreozymin (CCK-PZ), causing the gallbladder to contract, Oddi's sphincter to open, and concentrated gallbladder bile to enter the duodenum. Bile salts aid in the digestion of dietary fats by augmenting the action of pancreatic lipase and by removing the products of lipolysis (fatty acids and monoglycerides) from the reaction site. Bile salts solubilize fatty acids and monoglycerides in mixed micelles and bring them in contact with the mucosa of the small intestine, where they are absorbed. Conjugated bile salts, however, are not readily absorbed in the upper intestine, and so they progress to the ileum to be reabsorbed efficiently by an active transport process. The bile salts then enter the portal vein, are bound by albumin, and are transported to the liver, where they are rapidly extracted. Normally, of the total bile salts secreted into the intestine, at least 95 percent is reabsorbed and less than 5 percent is excreted in the feces. Furthermore, about 95 percent of the bile salts passing through the liver is extracted in a single passage. Thus, as a result of efficient ileal absorption and hepatic extraction, bile salts are largely contained in the EHC (liver, biliary tract, gallbladder, intestine, and portal vein), and only very small amounts are present in the rest of the body (estimated at about 2 percent of the total). This fact accounts for the very low concentration of bile salts in the systemic circulation. In the presence of liver disease, hepatic extraction and secretion may be impaired, leading to increased concentrations of serum bile salt. Thus, measurement of bile salt concentration may be considered to be a liver function test, but further study is required to determine its clinical utility.

A normal 70-kg person has a total pool of bile salts of 2 to 4 gm and a 24-hour liver-to-duodenum bile salt secretion of 15 to 35 gm. For example, assume that a typical person has a bile salt pool size of 3 gm and that he secretes 24 gm of bile salt per 24 hours. These figures imply that his 3-gm pool must circulate, on the average, about 8 times in 24 hours, or about 2 to 3 times per meal. Of the 24 gm secreted, he will reabsorb about 23.5 gm and lose 0.5 gm into the feces. During a 24-hour period the liver synthesizes an amount equal to the loss, thereby maintaining a normal pool size. Of the 24 gm of bile salts secreted, 23.5 gm (98 percent) is recycled,
and only 0.5 gm (2 percent) is newly synthesized.

If a person suddenly loses his bile salt pool (for example, by ingesting the drug cholestyramine resin, which binds bile salts in the gut and prevents their reabsorption), the rate of bile salt return to the liver will markedly decrease. The liver senses the low return rate of bile salts and responds by increasing bile salt synthesis. A normal person can augment synthesis tenfold to about 5 gm per 24 hours; this high synthetic rate will persist until the liver senses a high return rate and slows synthesis. Thus bile salt synthesis is effectively controlled by the rate of bile salt return to the liver.

The secretion of bile salts varies throughout the day; it is highest in the morning following gallbladder contraction as the ejected pool recycles to the liver. Secretion remains relatively high throughout the day, since gallbladder filling is minimal until the stomach is empty and gallbladder contraction ceases. At this point, part of the secreted bile is directed to the gallbladder, and the hepatic return diminishes. Therefore secretion decreases at night and reaches a low level early in the morning. It is obvious that dietary habits may greatly affect the temporal aspects of the secretion rate of bile salts and their EHC.

Many other substances are secreted in the bile and some, including certain drugs and hormones, have an important EHC. Other substances that are secreted into bile and that play a role in diseases of the biliary tract include cholesterol, phospholipid, and bilirubin. Normally a 70-kg person secretes about 750 mg of cholesterol, 8 gm of phospholipid, and 250 to 300 mg of bilirubin daily.

**Diagnostic Assessment of Biliary Tract and Gallbladder Disease**

**CLINICAL FEATURES**
The history and physical examination of the patient together with standard laboratory tests aid in establishing the correct diagnosis and help in assessing the severity of the disorder. For example, the appearance of *biliary colic* (a persistent, severe pain in the right upper quadrant, often radiating to the tip of the scapula, sometimes increasing in severity for a period of minutes or hours and then gradually declining), accompanied by an enlarged and tender gallbladder, is a good indication of a stone impacted in the cystic duct. If the stone dislodges or is passed into the duodenum, the pain abates and the patient recovers. Persistent pain accompanied by chills, fever, and leukocytosis suggests that *cholecystitis* has developed in an obstructed gallbladder. The appearance of jaundice and bilirubin in the urine, with similar bouts of pain, suggests *choledocholithiasis*, that is, a stone lodged in the common bile duct. Continuing chills, fever, leukocytosis, increasing jaundice, and impaired liver function tests indicate the presence of *ascending cholangitis*, a bacterial infection and inflammation of the biliary tract extending into the liver; this complication has a more serious prognosis than does cholecystitis. Intermittent partial biliary tract obstruction is often accompanied by right upper quadrant pain with little or no jaundice, but the presence of a high serum alkaline phosphatase level should lead the physician to suspect a stone partly blocking the common duct. Chronic intermittent biliary obstruction may lead eventually to *secondary biliary cirrhosis*. Complete biliary obstruction accompanied by steady pain located deep in the epigastrium or in the back suggests a tumor of the head of the pancreas or of the biliary passages. Finally, intermittent small bowel obstruction in an elderly patient whose abdominal x-ray shows air in the biliary tract should lead to the diagnosis of a *fistula* between the gallbladder or common duct and the small bowel and the presence of *gallstone ileus*.

**SPECIAL DIAGNOSTIC PROCEDURES**
To identify patients at high risk for gallstones that are not clinically apparent, to demonstrate "silent" or asymptomatic stones, or to pinpoint the specific position of an obstructing lesion, more sophisticated diagnostic procedures are necessary.

**Flat Plate of the Abdomen**
The flat plate may reveal calcified gallstones, calcified wall of the gallbladder, or "milk of calcium bile," in which the gallbladder fluid appears to be more radiopaque than the surrounding tissues. Air in the biliary tree may be the result of a fistula between the biliary tract and the intestines, or it may be produced by
gas-forming bacteria such as *Clostridium* or *Escherichia coli* that are infecting the gallbladder or biliary passages. Occasionally, during barium studies of the upper or lower intestinal tract, a distended gallbladder may be identified as a mass pressing on the duodenum or the colon, or a distended common duct may be seen impressing the duodenum. Finally, in a case of intestinal obstruction, one may be able to identify a calcified gallstone as the cause.

**Oral Cholecystogram (Gallbladder Series)**

In an oral cholecystogram, an oral dose of 2 to 4 gm of an iodinated compound (iopanoic acid) is given in tablet form the night before the study. This compound is absorbed by the intestine, extracted by the liver, secreted into the bile, and concentrated in the gallbladder. The high electron density of the iodine scatters the x-ray beam and allows the gallbladder lumen to be visualized as an underexposed (radiopaque) area on the film. Radiolucent stones are seen as filling defects within the opacified gallbladder lumen; they may be differentiated from polyps and other tumors because stones usually change position when the patient is turned.

Nonvisualization of the gallbladder in cholecystography may be caused by a number of conditions that are not related to gallbladder pathology, such as failure to swallow the pills, inability to absorb the ingested pills (for example, in a patient with gastric retention), poor liver function due to intrinsic liver disease and, rarely, selectively impaired biliary secretion of iopanoic acid (the Dubin-Johnson syndrome). Blockage of the cystic duct by a stone, acute pyogenic cholecystitis or cholangitis, acute and chronic cholecystitis with reabsorption of the contrast material from the gallbladder, and inability of the gallbladder to concentrate the contrast medium may all result in nonvisualization and indicate disease of the gallbladder or biliary tract. As a general rule, a patient with normal liver function tests who absorbs the contrast material, but whose gallbladder does not fill on two successive tests in a 48-hour period, has a 95 percent chance of having gallbladder disease and at least a 90 percent chance of having gallstone disease. It is helpful to do an upright film of the gallbladder, since stones occasionally layer evenly in a single-density band and may be obscured in the ordinary supine views of the gallbladder. Finally, it may be helpful to give a fatty meal or CCK-PZ and then observe contraction of the gallbladder. Occasionally symptoms are produced during the contraction that may aid in the diagnosis of intermittent obstruction of the cystic duct, or of biliary dyskinesia if stones are absent (see later in the chapter).

**Cholangiography**

**Intravenous Cholangiography.** In patients with serum bilirubin levels of 3 mg per 100 ml or less in whom partial obstruction of the biliary tract is suspected, an iodine-labeled contrast material may be given either as an intravenous bolus injection or as an infusion during a 30- to 60-minute period. This material is taken up by the liver and secreted into the ducts, allowing the biliary tree, but usually not the gallbladder, to be opacified. Stones, tumors, and strictures may be identified as filling defects or narrowings. Since occasional idiosyncratic reactions to iodinated compounds have been observed, these tests should be performed with care.

**Percutaneous Transhepatic Cholangiography.** In patients whose serum bilirubin level is higher than 4 mg per 100 ml, visualization of the biliary tract by oral cholecystography or intravenous cholangiography usually fails. In such instances it is possible to pass a thin needle through the skin to the liver substance, locate a bile duct by exploration with fluoroscopy, and then inject contrast material directly into a hepatic bile duct. The entire biliary tract may be visualized, and the site (and in some cases the cause) of a complete or partial obstruction may be identified. The success rate in demonstrating the site of the obstruction is close to 90 percent. This procedure is usually followed by definitive surgery. Transjugular passage of a catheter that traverses the superior vena cava and a hepatic vein before puncturing through the liver into a bile duct has been employed in patients with impaired hemostasis; however, experience with this technique is limited.

**Transduodenal Cholangiography.** In transduodenal cholangiography, a side-viewing endoscope is first
passed into the duodenum; the ampulla of Vater may then be identified and a catheter inserted into the common bile duct. Retrograde injection of contrast material then permits visualization of the biliary tree. Although this test is both difficult to perform and time-consuming, experienced endoscopists perform successful cannulations in 70 to 80 percent of the cases attempted. Transduodenal cholangiography is the procedure of choice when transhepatic percutaneous cholangiography cannot be performed.

Intraoperative Cholangiography. Intraoperative cholangiography may be performed just prior to removal of the gallbladder by injecting the contrast material directly into the common duct, or after cholecystectomy and T tube insertion, by injecting it into the T tube. This procedure identifies tumors, strictures, or retained stones in the common and hepatic bile ducts.

Duodenal Intubation and Bile Collection
In the duodenal intubation procedure, a tube is placed in the duodenum and a secretagogue such as CCK-PZ is given to produce gallbladder contraction. Bile is collected before giving the secretagogue, during the ejection of gallbladder bile, and after completion of gallbladder contractions. The finding of cholesterol crystals and bilirubin granules in the aspirate correlates well with the presence of gallstones. Gallbladder bile may be collected for chemical determinations of cholesterol saturation (see the following section).

Epidemiology of Gallstone Disease
For many years it was taught that one should suspect cholelithiasis in females who are “fair, fat, forty, and fertile”; or in other words, in light-skinned, obese, middle-aged, and multiparous women. Recently, the true prevalence of cholelithiasis (as determined by cholecystography) has been established in a random population of Pima Indians living in Arizona. Gallstones are extraordinarily prevalent among these dark-skinned people: 70 percent of the females have gallstones by the age of 30, and males have the same prevalence in later decades. In nearly all the females, the stones appear between the ages of 20 and 30; in males, they develop gradually from ages 30 to 60.

Comparison of the true prevalence of gallstone disease in the Pima Indians to the clinical prevalence shows that symptoms do not appear until several years after the development of the stones, and that about half the women and two-thirds of the men with gallstones never have any clinical symptoms. The true prevalence of gallstone disease in the general population is unknown; however, on the basis of data reported in the Framingham study, an estimated 15 million women and 5 million men in the United States have gallstones. In about half of the approximately 800,000 new cases of cholelithiasis each year, surgery is ultimately performed. About 6,000 deaths per year are attributed to gallstone disease, and the total cost of morbidity approaches $1 billion annually.

In the United States and other high-prevalence countries, gallstones are mostly of the cholesterol type. Japan and India, on the other hand, have a lower prevalence, and gallstones in these countries are mainly the pigment variety. Certain populations, such as the Masai tribe of East Africa, do not have gallstones at all.

Pathogenesis of Gallstone Disease
Human gallstones fall into two chemically different categories: those composed of bile pigments or their derivatives, and those composed primarily of cholesterol. In the former case, the pathogenesis involves bile pigment metabolism and in the latter, cholesterol and bile salt metabolism. Although each category will be considered separately, the fact that cholesterol stones often contain a small pigment center and that mixed pigment-cholesterol stones occur suggest that, in a given patient, both disorders may be present at some time.

PIGMENT STONES
Pigment stones probably form only when excess unconjugated bile pigments or their derivatives are present in bile and precipitate to form insoluble, polymer-like complexes with calcium and copper (see Fig. 40-1). These stones are dark brown or black, do not contain cholesterol, and as far as is known, cannot be dissolved in vivo or in solutions of artificial bile. Since pigment stones contain calcium, they are usually seen on x-ray films of the abdomen. The source of excessive free pigment may involve increased hepatic secre-
Figure 40-1. Mechanisms of pigment stone formation. True calcium bilirubinate stones result from excess secretion of free bilirubin or deconjugation of conjugated bilirubin in bile. Other pigment stones of unknown chemical structure (not bilirubin) may also result from secretion of these pigments from the liver or their production from known pigments such as bilirubin in the bile. Substances such as calcium and copper may be important in the precipitation process of both true calcium bilirubin stones and nonbilirubin pigment stones. Pigment stones may also form a nidus for precipitation of cholesterol from cholesterol-supersaturated bile. (Adapted from D. M. Small. Gallstones: Current concepts. N. Engl. J. Med. 279:388, 1968.)

Distribution of bilirubin in disorders associated with rapid hemoglobin breakdown, or chemical alteration of conjugated bilirubin followed by the precipitation of calcium bilirubinate or related compounds in the biliary tract or gallbladder by the action of bacterial deconjugating enzymes.

Since little is known of the chemical and physical makeup of pigment stones, and nothing of the solubility of the precipitating molecules, one cannot discuss the early stages of this disorder, nor can one predict from bile composition whether or not a patient will develop such stones. One can, on epidemiologic grounds, suggest that patients with increased pigment production resulting from severe chronic hemolytic anemia (e.g., sickle cell anemia, thalassemia, spherocytosis) and infants with severe erythroblastosis fetalis will tend to get stones. Pigment stones are more common than cholesterol gallstones in alcoholic cirrhosis. Pure pigment stones comprise about 10 percent of all gallstones in Western countries. The true prevalence of pigment stone disease may be much higher than is indicated by the prevalence of pure pigment stones, since predominantly cholesterol stones often have a small pigment stone at their center. Strictly speaking, such patients first have pigment stone disease, which acts as a nucleus for the precipitation of cholesterol during a subsequent bout of cholesterol stone disease.

CHOLESTEROL GALLSTONES
Cholesterol stones (that is, stones of pure cholesterol or those having cholesterol as the major chemical component) account for most of the gallstone disease in the Americas, Europe, and South Africa. Gallstones usually present clinically as a medical or surgical emer-
Bile Composition and Cholesterol Solubility
Normal bile is a liquid, whereas bile from patients with cholesterol cholelithiasis also contains cholesterol in crystalline form that has precipitated from solution. The solubility of cholesterol in bile is limited and depends on the three major lipid components of bile: conjugated bile salts, phospholipids, and cholesterol. These components make up about 90 percent of the dry weight of gallbladder bile.

Bile salts are water-soluble, detergent-like molecules which, in aqueous solution, form small aggregates called micelles. At least 90 percent of the phospholipid in bile is phosphatidylcholine (lecithin), which is insoluble in aqueous systems but can be dissolved by bile salts in micelles. Cholesterol is also insoluble in water, but it becomes soluble when incorporated into the lecithin–bile salt mixed micelle, whose capacity to dissolve cholesterol is related to their relative contents of lecithin and bile salts.

Triangular coordinates were used by Admirand and Small to define the maximum solubility of cholesterol in lecithin–bile salt mixtures in a system containing 90% water and 10% bile salts together with phospholipids and cholesterol (Fig. 40-2). The line ABC represents the effective maximum solubility of cholesterol in varying mixtures of bile salt and lecithin.

A mixture in the zone below line ABC forms a single homogeneous liquid from which cholesterol does not readily precipitate. A mixture that has a composition falling above line ABC contains readily precipitable excess cholesterol. In physical terms, bile with excess cholesterol may present either as a single, liquid phase that is supersaturated with cholesterol or as a two-phase system of liquid bile and solid crystalline cholesterol. The crystals may be either very small or large enough to be called stones. As a general rule, patients with cholesterol gallstones have a bile that contains excess cholesterol, whereas subjects without stones have a bile composition lying below line ABC.

Figure 40-2. Three major components of bile plotted on triangular coordinates. The percent of total moles of bile salt, lecithin, and cholesterol constituted by each of the separate components is shown on the scales along the sides of the triangle. Since the sum of the three components equals 100 mol, the composition of any bile can be represented as a single point within the triangle. For instance, point P represents a bile consisting of 80% bile salt, 5% cholesterol, and 15% lecithin. Line ABC represents the maximal effective solubility of cholesterol in varying mixtures of bile salt and lecithin as determined by Admirand and Small. P falls below line ABC and within the zone of a single phase of micellar liquid, and is less than saturated with cholesterol. To calculate the percentage saturation of a bile having composition P, draw a line through to cholesterol apex. The intersection of this line with line ABC (point X) gives relative cholesterol concentration at 100 percent effective saturation, in this case, 8% cholesterol. Percent saturation of point P is percent saturation = 5/8 (100) = 62.5 percent saturated.

Stages of Cholesterol Gallstone Disease
The first diagnosable abnormality of cholesterol gallstone disease becomes apparent when the patient begins to secrete bile containing an excessive proportion of cholesterol relative to bile salts and phospholipids; that is, a bile composition falling above line ABC in Figure 40-2. Whereas all normal persons may secrete such a bile occasionally during fasting, the potential gallstone patient secretes a bile in which the
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<th>Stage</th>
<th>Stones Absent</th>
<th>Stones Present</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abnormality</td>
<td>Bile becomes supersaturated with cholesterol</td>
<td>Growth to macroscopic stones</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>Duodenal drainage shows that gallbladder bile has excess cholesterol (estimated from triangular plot as in Fig. 40-2) but no crystals by microscope</td>
<td>Blockage of cystic duct, cholecystitis, and/or jaundice</td>
</tr>
<tr>
<td></td>
<td>Duodenal drainage shows cholesterol crystals by microscope. No stones by cholecystography</td>
<td>Cholecystography reveals stones or nonfunctioning gallbladder. Usually cholesterol crystals and/or abnormal bile are found by duodenal drainage. Patient is asymptomatic</td>
</tr>
</tbody>
</table>

mean 24-hour bile composition contains excess cholesterol. The gallbladder bile from these patients thus becomes supersaturated with cholesterol. If the level of supersaturation is very marked, precipitation of cholesterol may occur rapidly and spontaneously and result in a large number of small stones. If only a moderate degree of supersaturation is present, nucleation may be necessary to initiate precipitation. Many small cholesterol crystals may flocculate to produce aggregates, or a single crystal may grow to form an individual stone. The rate of growth depends on the degree of supersaturation: The greater the supersaturation, the more rapid is the growth.

The pathogenesis of cholesterol gallstones can be subdivided into five stages (Table 40-1). Stage I involves the genetic, biochemical, or metabolic defect that leads to the production of gallbladder bile supersaturated with cholesterol. Stage II, the chemical stage, involves the production of an abnormal, supersaturated bile. Stage III, the physical stage, involves a change in the physical state of bile from a single, liquid phase that is supersaturated with cholesterol to a system containing both a liquid bile phase and cholesterol crystals. The key processes in this stage are nucleation, flocculation, and precipitation of cholesterol from the supersaturated bile. Stage IV involves the growth of the small crystals into macroscopic stones; and the final stage (stage V) involves the appearance of clinical symptoms.

The diagnosis of cholesterol gallstone disease may currently be made as early as stage II, using duodenal intubation and collection of bile discharged from the gallbladder after administration of CCK-PZ. If the bile is supersaturated (by the criteria shown in Fig. 40-2) but contains no crystals, the patient can be considered to be in stage II. Stage III may be diagnosed by finding cholesterol crystals in a patient who has a normal cholecystogram; stage IV by finding, in addition, stones on cholecystography; and stage V by the presence of signs and symptoms.

Pathophysiologic Types of Cholesterol Gallstone Disease

Cholesterol gallstone disease is not a single entity; there are at least six different categories of pathophysiologic abnormalities that may lead to abnormal bile and ultimately to cholesterol gallstones (see the following section). All these abnormalities result in an excess quantity of cholesterol relative to bile salt and phospholipid so that gallbladder bile becomes supersaturated. An understanding of these different mechanisms requires knowledge of the normal relationship between the bile salt secretion rate and bile composition. The relationship between the saturation percentage of cholesterol in bile and the bile salt secretion rate is illustrated schematically in Figure 40-3. At high bile salt secretion rates, a bile is less...
saturated with cholesterol, but as the secretion rate decreases, the percentage of cholesterol saturation increases. Since the bile salt secretion rate decreases during fasting, some normal persons have a bile that is supersaturated with cholesterol for short periods of time; however, stones are not produced in this instance because the small amount of supersaturated bile mixes with large amounts of normal bile in the gallbladder to give a mean composition that is unsaturated.

The application of techniques for measuring bile acid pool size, synthetic rate, secretion rate, and hepatic return rate, as well as cholesterol secretion rates, permits a tentative classification of gallstone disease resulting from disorders of bile salt and cholesterol metabolism into six types (Table 40-2).

Type 1: Excessive Bile Salt Loss. Excessive bile salt loss, such as occurs in ileectomy, certain kinds of ileal disease, and perhaps in ileal bypass, results in a decrease in the bile salt pool and a very low hepatic return rate. Bile salt synthesis increases to its maximum of about 5 gm per 24 hours, but it cannot make up for the loss; thus the pool remains decreased, the bile salt secretion rate is low, and the bile becomes supersaturated. This mechanism probably accounts for the increased prevalence of gallstones in patients with ileectomy and ileal disease.

Type 2: Oversensitive Bile Salt Feedback. When hepatic return rate falls below a certain level, bile salt synthesis increases to augment the secretion rate and thus return it to normal. If the decreased hepatic return is the result of an increased loss, bile salt pool size and secretion rate can be maintained provided the loss does not exceed the ability of the liver synthesis to compensate. Minor bile salt loss, such as occurs with cholestyramine feeding, a small ileal resection, or a partial ileal bypass, is compensated by increased synthesis, while bile composition remains unsaturated. However, certain patients develop a low pool size and a low return rate without the compensation of increased synthesis; they appear to have an oversensitive feedback mechanism in which even relatively low rates of hepatic return act to depress bile salt synthesis. Assuming that type 2 patients start with a normal secretion rate and pool size, as oversensitive feedback develops the patient makes less bile salt than he loses, and a period of negative bile salt balance ensues. In time, a new steady state is reached characterized by a decreased pool size, decreased bile salt secretion rate, and a decreased hepatic return. As a result of the decreased secretion rate, bile composition becomes supersaturated. This appears to be the main cause of gallstones in Caucasians of normal weight.

Type 3: Excessive Cholesterol Secretion. Some patients whose bile salt pools and estimated bile salt secretion rates are within the normal range nevertheless have supersaturated bile, because they have a cholesterol secretion rate that is higher than normal. Obesity, which increases the synthesis of cholesterol in man, has been shown to augment cholesterol secretion into bile and lead to supersaturation. Clofibrate, estrogens, and other similarly acting drugs that appear to mobilize cholesterol pools and increase biliary cholesterol secretion probably induce cholesterol stones in some chronic users. Rapid weight loss enhances cholesterol secretion and induces supersaturation. Increased cholesterol intake may also be implicated in increased cholesterol secretion.

Type 4: Mixed Defect. Most American Indians of the Southwest and some Caucasians who are cholesterol stone formers have a double defect that is a combination of type 2 and type 3 defects. These patients have a decreased bile salt secretion rate and a high cholesterol secretion rate. It has been postulated that there may be a defect in the conversion of cholesterol to bile salt in these patients resulting in excessive cholesterol secretion and inadequate synthesis of bile salt.

Type 5: Rapid Bile Salt Circulation with Decreased Pool Size. It has been suggested that the bile salt secretion rate might be normal in some patients with gallstones and that stones could result from a primary disorder in the EHC. This disorder might develop as follows: First, an excessive stimulus for gallbladder contraction or intestinal motility arises and causes a rapid circulation of the pool. Bile salt secretion would increase and result in excessive return of bile salts to
the liver. Hepatic synthesis would then decrease and
give rise to a period of negative bile salt balance until
a new steady state developed, characterized by a
decreased but rapidly circulating pool and a normal
secretion rate. Under these circumstances the mean
24-hour bile composition would not be supersaturated.
However, during fasting the small bile salt pool would
mix with a relatively large amount of potentially
supersaturated bile coming from the liver; thus, the
gallbladder bile composition following fasting might
be supersaturated, even though the mean 24-hour bile
was not. This hypothesis is intriguing but needs
further evaluation.

Type 6: Primary Disorders of the Gallbladder, Ducts,
or Sphincters. Extrahepatic mechanisms by which
the gallbladder, the sphincter of Oddi, and the bile
ducts might be implicated in the formation of gall-
stones include primary cholecystitis and the biliary
dyskinesia syndromes. First, inflammation associated
with primary cholecystitis might cause the gallbladder
to absorb bile salts abnormally or might even cause a
chemical degradation of bile salts and biliary lipids,
thus converting a normal hepatic bile into a super-
saturated bile within the gallbladder. These mecha-
nisms have not been investigated in man, but bile
salt reabsorption in the gallbladder appears to be the
cause of stones in mice that are fed cholesterol and
cholic acid. Certainly, cholecystitis that is secondary
to existing stones, by causing bile salt absorption or
by chemically altering biliary lipids, may make gall-
bladder bile composition even more supersaturated,
which could result in the formation of cholesterol
stones around existing pigment stones (see Fig. 40-1)
or accelerate the growth rate of existing stones.
Second, more subtle defects may result from abnor-
mal biliary dynamics. If, for instance, the cystic duct
failed to open when the gallbladder contracted, chole-
cystitis might be produced. Furthermore, if the
Oddi’s sphincter failed to open when the gallbladder
contracted, a condition of intermittent biliary
obstruction would result, which could lead to the
production of supersaturated bile.

Complications of Gallstone Disease

CHOLECYSTITIS
Most patients with acute cholecystitis have gallstones.
Symptoms may result either from local irritation
caused by the gallstones within the gallbladder, or
more likely from obstruction of the cystic duct by
the gallstones, causing edema and consequent inflam-
mation. An acute attack of cholecystitis is character-
ized by prolonged biliary colic, chills, fever, leuko-
cytosis, and tenderness in the right upper quadrant.
The attack either may abate spontaneously, or may
persist, leading to perforation of the gallbladder into
the peritoneum or into a contiguous organ. Early in
the attack the gallbladder contents are often sterile,
but if obstruction continues beyond a week, the gal-
bladder and its contents often become infected with
enteric bacteria. Acute cholecystitis rarely occurs in
the absence of gallstones. Chronic cholecystitis,
either as a microscopic diagnosis or as fibrosis of the
gallbladder, accompanies most cases of chronic choles-
lithiasis and probably has little clinical importance.

The diagnosis of acute cholecystitis is made pri-
marily from the clinical symptoms. The flat plate of
<table>
<thead>
<tr>
<th>Type of disorder</th>
<th>Hepatic return rate</th>
<th>Bile acid synthetic rate</th>
<th>Bile acid pool size</th>
<th>No. of circulations of pool/24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal values (estimated for 70-kg man)</td>
<td>14–34 gm/24 hr</td>
<td>350–550 gm/24 hr</td>
<td>2–4 gm</td>
<td>5–15 gm</td>
</tr>
<tr>
<td>Disorders of the EHC and liver affecting bile acid and cholesterol metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1: Excessive bile salt loss</td>
<td>Decreased (very low)</td>
<td>Increased to maximum level</td>
<td>Decreased</td>
<td></td>
</tr>
<tr>
<td>Type 2: Oversensitive bile acid feedback</td>
<td>Decreased</td>
<td>Normal to low</td>
<td>Decreased</td>
<td>Normal</td>
</tr>
<tr>
<td>Type 3: Excessive cholesterol secretion</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Type 4: Mixed (types 2, 3) Disorders primarily extrahepatic in origin</td>
<td>Decreased</td>
<td>Normal to low</td>
<td>Decreased or normal</td>
<td>As in type 2</td>
</tr>
<tr>
<td>Type 5: Rapid bile salt circulation</td>
<td>Normal</td>
<td>Normal</td>
<td>Decreased</td>
<td>High</td>
</tr>
<tr>
<td>Type 6: Disorders of gallbladder, ducts, or sphincters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bile salt secretion rate</th>
<th>Cholesterol secretion rate</th>
<th>Primary defect</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>15–35 gm/24 hr</td>
<td>0.5–1 gm/24 hr</td>
<td>.....</td>
<td>.....</td>
</tr>
</tbody>
</table>

| Decreased               | Normal to low              | Loss of mechanisms to absorb bile salts results in decreased pool and bile salt secretion rate. Synthesis cannot fully compensate loss. | Occurs in ileectomy, ileal bypass, ileal disease; congenital loss of ileal active transport system of bile salts. |
| Decreased due to small pool circulating normally | .....                     | A relative depression in bile acid synthesis. Decreased hepatic return excessively inhibits bile acid synthesis. | Occurs in many of the cholesterol stone cases in Caucasians, especially those who are not obese. |
| Normal                  | High                       | Excessive cholesterol is secreted into bile despite normal bile salt secretion rate. | Occurs in obese patients. |
| Decreased               | High                       | Mixture of types 2 and 3                                                      | American Indians, perhaps some Caucasians. |
| Normal                  | Normal                     | Small pool of normal bile collecting in gallbladder cannot compensate for abnormal bile entering gallbladder during fasting. | May occur in some patients with decreased bile acid pool but further proof is needed. |
| .....                    | .....                      | Absorption of bile salts and/or phospholipids or secretion of cholesterol by gallbladder; chemical alteration of normal hepatic bile in the gallbladder to produce supersaturated gallbladder bile. | Cholecystitis, aseptic or bacterial, may secondarily complicate other types of gallstone disease, including pigment stone disease. |
the abdomen occasionally shows either air in the gallbladder or radiopaque stones. Despite normal liver function tests, the gallbladder fails to visualize on oral cholecystography. The presence of either jaundice or abnormal liver function tests indicates partial obstruction of the common duct resulting from severe inflammatory disease, abscess formation, or stones in the common duct. Treatment consists in supportive therapy for dehydration, nasogastric suction, and antibiotic therapy if the course extends beyond 1 week. In patients with a severe attack and suspected perforation, surgery should be performed immediately; less severely ill patients should have a cholecystectomy after the acute attack has subsided.

**CHOLEDOCHOLITHIASIS**

Gallstones often pass through the cystic duct into the common duct, where they must pass through the ampulla of Vater and into the duodenum to be eliminated. Stones retained in the common duct constitute choledochothiasis. Patients with choledochothiasis may be asymptomatic with normal laboratory findings; they may have an occasional bout of colic-like pain with intermittent signs and symptoms of biliary tract obstruction; or they may develop complications—specifically, ascending cholangitis and secondary biliary cirrhosis. Choledochothiasis with partial, intermittent biliary tract obstruction is characterized by intermittent bouts of abdominal pain, intermittent jaundice, and often a strikingly elevated serum alkaline phosphatase level. Occasionally, an impacted stone causes mild, chronic, partial obstruction; in such cases the patient may be asymptomatic, with a normal or only slightly increased bilirubin. The diagnosis is made by one of the cholangiographic techniques mentioned earlier. The treatment is surgical removal of the stone and exploration of the common duct for other retained stones.

**CHOLANGITIS**

A serious complication of choledocholithiasis is cholangitis, or inflammation of the biliary tree above a partially obstructed common duct. The major cause is the presence of gallstones, but rarely tumors, polyps, or parasitic inflammation (common in the Orient) may be responsible. The symptoms of biliary colic, jaundice, fever, and chills indicate inflammation of the biliary tree. If the obstruction is removed (for example, by the passage of the stone), the symptoms may abate. However, if obstruction is not relieved, bacterial infection ascends the biliary tree, and the patient becomes more severely ill and may develop septicemia with enteric organisms. The condition may progress rapidly to severe toxic delirium with high fever. Diagnosis often must be established at some risk, by percutaneous cholangiography. Occasionally, pus rather than bile may be aspirated from the liver during this test. Suppurative cholangitis requires emergency surgical drainage of the biliary tree. Cholangitis may lead to abscesses within the liver, a condition with a very high mortality rate. Except for parasitic infestations, nearly all cholangitis is related to an obstructing lesion in the ductal system. Surgical removal of the obstruction is the ultimate therapy; however, treatment with antibiotics that are effective against enteric organisms may permit stabilization of the patient prior to surgery.

**SECONDARY BILIARY CIRRHOSIS**

A late complication of chronic choledocholithiasis, with or without cholangitis, is secondary biliary cirrhosis. This condition may follow several attacks of acute cholangitis, but it may also develop silently in a patient with chronic, asymptomatic, partial biliary tract obstruction due to a stone. Diagnosis is made by liver biopsy to establish the presence and severity of the biliary cirrhosis, and by appropriate cholangiographic tests to locate the obstructing stone. Occasionally biliary cirrhosis may lead to hepatic failure or to portal hypertension and bleeding. Once secondary biliary cirrhosis has developed, removal of the obstructing lesion may fail to stop the progression of the cirrhosis.

**POSTCHOLECYSTECTOMY COMPLICATIONS**

A serious complication of gallbladder surgery is injury to the common duct. Occasionally the common duct is unknowingly completely severed, and the bile drains into the peritoneum to form a local chemical peritonitis, requiring a second surgical intervention. In the absence of complete transection, trauma to the common duct may lead, after a period of weeks, months, or years, to stricture formation and result in
partial or complete biliary tract obstruction. The symptoms that develop are related to biliary tract obstruction, with or without infection, and include abdominal pain, jaundice, increased serum alkaline phosphatase level, and fulminant cholangitis. A rare surgical complication, ligation of the common duct, results in an identical clinical syndrome, which begins, however, within a day or two postoperatively and is characterized by progressively increasing jaundice. An important consideration in the differential diagnosis is the presence of stones that have been retained since the operation, or of stones that have formed in the biliary tree as a result of persistent cholangitis. Therefore, the diagnostic tests include an appropriate type of cholangiography. The problem of removing the retained or reformed stones is difficult; it may be helpful in this regard to place a large T tube in the duct at operation. The duct can then be cannulated and the stones removed or irrigated with stone-dissolving solutions. Some success in stone fragmentation and dissolution has been reported with the use of infusions of sodium cholate into the common duct. If a stricture is found, surgical reconstruction is necessary. Occasionally it is necessary to attach the gallbladder to the duodenum if the common duct is so badly damaged that it cannot be repaired. Such patients are often difficult to manage and develop relapsing cholangitis, often complicated by sepsisemia and subsequent biliary cirrhosis.

FISTULAS AND GALLSTONE ILEUS
During acute cholecystitis, the gallbladder may rupture not only into the peritoneum or locally to form an abscess, but also into one of the hollow organs, such as the stomach, duodenum, or colon. The most common place of perforation is the duodenum. If the patient does not undergo surgical repair, he may be left with a chronic choledochojenal fistula. Occasionally, a gallbladder containing large stones may erode, with few or no symptoms, into the duodenum or colon and result in a chronic fistula. These chronic fistulas rarely produce symptoms; occasionally, however, a large gallstone that passes from the gallbladder to the duodenum will cause intermittent intestinal obstruction as it moves down the small bowel and will lodge at the ileocelecal sphincter to produce complete small bowel obstruction (gallstone ileus). This diagnosis is strongly suspected in a patient without previous abdominal surgery who develops small bowel obstruction and has air in the biliary tree. In some instances a radiopaque stone may be seen at the site of the obstruction. Gallstone ileus represents a surgical emergency.

GALLSTONE PANCREATITIS
A major cause of acute pancreatitis in nonalcoholic persons is a gallstone lodged in the common channel that drains both the pancreas and the biliary system. The diagnosis may be made by cholangiography, and the treatment is surgical (see Chap. 42).

Other Disorders Affecting the Gallbladder and Biliary Tract

TUMORS
Benign or malignant tumors may affect the biliary tree. Polyps of the gallbladder are usually benign and are rarely associated with cholecystitis. Malignant tumors of the gallbladder are often accompanied by stones, but there is no proof that one leads to the other. Certainly a tumor may alter bile composition and precipitate stones in the gallbladder, and conversely, stones may possibly irritate the gallbladder in such a way that a tumor forms. Symptoms from cancer of the gallbladder are often minimal until either the cystic duct or the common duct becomes involved, producing signs of obstruction. Tumors of the head of the pancreas, the common duct, or the large hepatic ducts often produce progressive, unrelenting jaundice; enlargement of the gallbladder (in the absence of chronic cholecystitis); steady, deep, epigastric pain; and anorexia and weight loss. These tumors are rarely complicated by cholangitis. Metastases to the liver are common. Whereas persistent or progressive obstructive jaundice is common in carcinoma of the ampulla of Vater, this lesion may also produce intermittent or fluctuating jaundice associated with occult bleeding into the intestinal lumen. Necrosis and sloughing of the tumor mass, temporarily relieving the obstruction of the bile duct, appears to be responsible for these phenomena. Surgical intervention to relieve obstruction may reduce the clinical symptoms, but prolonged survival is uncommon.
CHOLESTEROSIS OF THE GALLBLADDER
Cholesterosis of the gallbladder is a pathologic entity in which the wall of the organ is infiltrated with small flecks of lipid, presumably cholesterol esters. This condition is often found in association with chronic cholecystitis and cholelithiasis, but it is rarely seen in patients without gallstones. Its clinical significance is uncertain.

BILIARY DYSKINESIA
Some patients who complain of colic-like biliary pain have a normal cholecystogram and at operation are found to have a normal gallbladder without gallstones. Although it is conceivable that such a patient had a stone and passed it, it is also possible that alterations in the pressure dynamics of the biliary tract might be responsible for the pain. It has been suggested that a dose of CCK-PZ given during oral cholecystography may show that the gallbladder contracts but does not discharge its contents. The development of pain at the same time would suggest the presence of spasm of the cystic duct. The clinical significance of biliary dyskinesia is not known, but it is possible that alterations in biliary dynamics may be related to the formation of certain types of gallstones (see the section on gallstone disease).

SCLEROSING CHOLANGITIS
Sclerosing cholangitis is a rare disease of unknown etiology resulting in chronic inflammation and stenosis of the common bile duct, the hepatic bile ducts, or the intrahepatic radicles, in the absence of gallstone disease or prior biliary tract surgery. It may be associated with ulcerative colitis, retroperitoneal fibrosis, thyroiditis, or other autoimmune disorders. The initial symptom is a slowly progressive jaundice, often without pain or fever. Cholangiography, either percutaneous, retrograde, or operative, is necessary to establish the diagnosis. The possibility of sclerosing bile duct carcinoma must always be considered, and because histologic diagnosis is extremely difficult, malignancy can be excluded only by the passage of time. Although surgical decompression of the biliary tract and immunosuppressive treatment with corticosteroids and azathioprine have been attempted, the prognosis is generally poor, with death resulting from secondary biliary cirrhosis.

Bibliography

Interaction of Collagen with the Lipids of Tendon Xanthomata

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ABSTRACT To determine the physical state of lipids in tendon xanthomata, six specimens surgically removed from three patients with familial hypercholesterolemia were studied by microscopy, calorimetry, and X-ray diffraction. The major constituents of the xanthomata were lipid (33% of dry weight) and collagen (24% of dry weight). The principal lipids were cholesterol ester and cholesterol. Light microscopy and thin-section electron microscopy showed occasional clusters of foam cells separated by masses of extracellular collagen. Polarized light microscopy of fresh, minced tissue showed rare droplets of free cholesterol ester. When heated, the tissue shrank abruptly at ≈70°C and, consequently, a large amount of cholesterol ester was released. Scanning calorimetry of fresh pieces of xanthoma showed a single, broad, reversible liquid crystalline transition of cholesterol ester with peak temperature from 32 to 38°C. The enthalpy (0.71 ± 0.07 cal/g) was reduced compared with the isolated cholesterol ester from each xanthoma (1.1 ± 0.01 cal/g). There was a large irreversible collagen denaturation endotherm (peak temperature = 67°C; enthalpy 9.9 cal/g collagen) that corresponded to the tissue shrinkage noted by microscopy. After the collagen denaturation, the sample displayed double-peaked reversible liquid crystalline transitions of cholesterol ester, of enthalpy 1.18 ± 0.1 cal/g, that were identical to transitions of isolated cholesterol ester.

Fibers dissected from xanthomata were examined by X-ray diffraction at temperatures below and above the cholesterol ester transition. At 20°C there was a weakly oriented equatorial reflection of Bragg spacing 36 Å, which corresponded to the smectic phase of cholesterol ester, and a series of oriented collagen reflections. At 42°C the cholesterol ester reflection disappeared. Stretched fibers examined at 10°C showed good orientation of collagen and cholesterol ester reflections and, in addition, meridional spacings which indicated oriented crystallization of cholesterol ester. These studies suggest that a major component of tendon xanthomata is extracellular cholesterol ester which displays altered melting and molecular orientation as a result of an interaction with collagen. At xanthoma temperatures, the cholesterol ester is in a smectic liquid crystalline state, probably layered between collagen fibrils, with the long axis of the cholesterol ester molecules perpendicular to the axis of the collagen fiber. Such collagen-cholesterol ester interactions may favor the extracellular deposition of cholesterol ester derived either from intracellular sources or directly from plasma lipoproteins.

INTRODUCTION

Tendon xanthomata are deposits of lipid and connective tissue commonly found in patients with familial hypercholesterolemia. They display slow growth and regression, tending to parallel the degree of hyperlipidemia. These deposits show some similarities to atherosclerotic plaques, both chemically and structurally. Thus, cholesterol and cholesterol ester are their main lipid components (1, 2) and they contain large amounts of connective tissue and clusters of cells filled with lipid droplets (3) which resemble the foam cells of fatty streaks. Xanthomata probably do not synthesize significant quantities of cholesterol but derive this lipid from the plasma (4). After intravenous injec-
tion of radioactive cholesterol, there is a gradual rise in xanthoma cholesterol specific activity which indicates slow uptake or exchange of cholesterol with the plasma compartment (5, 6).

Recently, the physical state of lipids in atherosclerotic tissue (7–9), in the spleen from a patient with Tangier disease (10), and in the plasma lipoproteins (11–13) has been investigated with techniques such as polarized light microscopy, scanning calorimetry, and X-ray diffraction. These procedures permit fresh, whole tissue to be examined, and give information about the structure and interactions of lipid under physiological conditions.

With similar techniques we have now undertaken a study of tendon xanthoma, in an attempt to define the structure of the lipid deposits and possible interactions with other tissue components.

METHODS

Patients. Three patients with severe, familial hypercholesterolemia had tendon xanthomas removed because the xanthomas were causing discomfort and had failed to respond to medical treatment.

Patient A was a 36-year-old white woman who had developed tendon xanthoma at age 13 yr and had experienced an acute myocardial infarction at age 19 yr. During the previous 10 yr her plasma cholesterol was 300–400 mg/100 ml, with low density lipoprotein (LDL) cholesterol 250–280 mg/100 ml, very low density lipoprotein (VLDL) cholesterol 20–30 mg/100 ml, and high density lipoprotein (HDL) cholesterol about 25 mg/100 ml. At different times she had been treated with nicotinic acid, cholestyramine, neomycin, and &beta;-sitosterol, and had undergone ileal bypass surgery at age 24 yr. Although partially successful, none of these treatments had reduced her plasma cholesterol to below 300 mg/100 ml. Tendon xanthoma was removed from both elbows and from the extensor tendon of her right fourth finger.

Patient B was a 30-year-old black man with a 7-yr history of tendon xanthomatosis and hyperlipidemia. During this period his total plasma cholesterol was 300–400 mg/100 ml, with LDL cholesterol 240–290 mg/100 ml, VLDL cholesterol 25–40 mg/100 ml, and HDL cholesterol 25–35 mg/100 ml. He had never followed dietary or drug treatment. A xanthoma was removed from his right elbow.

Patient C was a 56-year-old white woman with hyperlipidemia and tendon xanthomatosis of at least 15 yr duration. During the previous 6 yr her plasma cholesterol was 250–350 mg/100 ml with normal triglyceride, LDL cholesterol 190–230, VLDL cholesterol 40–60, and HDL cholesterol 35–40 mg/100 ml. She had not adhered to dietary or drug treatment. Tendon xanthomas were removed from the extensor tendon of her right second finger and from her left patellar tendon. In all cases the xanthomas were wrapped in saline-soaked gauze and examined by different techniques within 2–3 h after surgical removal. The samples were not cooled below 25°C and, except for specimens examined by standard light and electron microscopy, were analyzed without fixation.

Bovine tendon collagen was purchased from Sigma Chemical Co., St. Louis, Mo.

Chemical methods

Thin-layer chromatography. Lipids from homogenates of xanthoma were doubly extracted in 10 vol of chloroform:methanol (2:1 vol/vol), after which a Folch procedure (14) was carried out. Quantitative thin-layer chromatography was used to measure the free cholesterol, fatty acid, triglyceride, cholesterol ester, lecithin, sphingomyelin, and lecithin according to the method of Downing (15), as modified by Katz et al. (8). Cholesterol ester was isolated from the xanthoma lipids by preparative thin layer chromatography, with a hexane:ether (94:6 vol/vol) solvent system. Cholesterol ester spots were scraped and the silica removed by eluting the lipid with chloroform:methanol (2:1 vol/vol) through a sintered glass funnel.

Gas-liquid chromatography. Cholesterol esters were hydrolyzed in 2% alcoholic KOH, according to the method of Albrink (16) as modified by Smith and Slater (17).

Amino acid analysis. The delipitated xanthoma were hydrolyzed with 6 N HCl in sealed glass vials, under N2 at 110°C, for 24 hours, and amino acid composition was determined with a Technicon amino acid AutoAnalyzer (Technicon Instruments Corp., Tarrytown, N. Y.), according to the method of Hamilton (18). Hydroxyproline content of the hydrolysates was also determined by the method of Bergmann and Lowxley (19).

Light and electron microscopy. For preparation of thin section electron micrographs, fresh samples were fixed in glutaraldehyde/paraformaldehyde within 10 min of excision from the patients, washed in Na cacodylate, fixed in OsO4, washed in distilled water, dehydrated with acetone, embedded in Epon (Shell Chemical Co., Houston, Tex.), and sectioned with a glass knife (20). Sections were stained with Pb citrate and post-stained with uranyl acetate (21). Sections were examined with an AE1-68 electron microscope (AE1 Scientific Apparatus Inc., Elmsford, N. Y.) calibrated with a catalase standard. All samples were also sectioned and stained with hematxylin and eosin for conventional light microscopy.

Homogenization experiments. Xanthoma was cut into small pieces and homogenized in 0.15 M NaCl, with a VirTis homogenizer (VirTis Co., Inc., Gardiner, N. Y.) at a setting of 5,000 rpm for about 2 min. The homogenates were centrifuged for 1 h at 40,000 rpm in a Beckman 40.3 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and the top, middle, and bottom 2 ml of each tube were analyzed for lipid.

Physical methods

Polarized light microscopy. A Zeiss standard NL microscope (Carl Zeiss, Inc., New York) fitted with a heating and cooling stage was used to identify the physical states of lipids in fresh, minced xanthomata. Samples were initially examined at 25°C and then heated or cooled at 2–3°C/min. Liquid crystalline cholesterol ester was identified from its characteristic reversible liquid crystalline transitions between 30 and 40°C (22). The smectic phase was identified from its positive sign of birefringence and the cholesteric phase from its negative

Abbreviations used in this paper: CE, cholesterol ester(s); HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.
sign. Cholesterol monohydrate was identified as plate crystals with a characteristic angle of 79° (8).

**Differential scanning calorimetry.** Pieces of fresh xanthoma were minced with a scalpel on a glass slide, transferred into 75-μl stainless steel pans, and examined with a Perkin-Elmer DSC-2 differential scanning calorimeter (Perkin-Elmer Corp., Norwalk, Conn.). Samples were heated at 5°C/min and cooled at 5, 10, or 20°C/min. The area enclosed by the transition endotherm and the base line, in conjunction with the mass of material in the sample pan, the instrumental sensitivity, and a calibration factor was used to calculate the transition enthalpy. Enthalpy and temperature were calibrated as described previously (23). Each sample was scanned a number of times in the temperature range of interest and multiple samples (three to five) were examined from each specimen.

**X-ray diffraction.** 1- to 3-cm fibers were dissected with fine scissors from the cut surface of xanthoma. In some instances unstretched fibers were placed in sealed Lindeman glass tubes (Lindeman Corp., Indianapolis, Ind.) and examined immediately after removal from the patient. However, better orientation of diffracted X rays was obtained with fibers that had been aligned by being stretched with a small weight (about 50 g) for about 24 h. The aligned fibers were examined while still under tension. X-ray diffraction studies were performed with nickel-filtered CuKα radiation from an Elliot CX6 rotating anode generator (Baird and Tatlock, London, England) and Elliot toroidal (Baird and Tatlock) or Franks mirror optics as described previously (11).

Results are expressed as mean±SEM. Significance of differences between means was determined by Student's t test.

**RESULTS**

**Compositional analysis: lipid analysis.** The tissue dry weights were 28.5–29.9% of the wet weights. Lipid constituted about 33% of the dry weight of the tissue (or 9.5±0.62% of the wet weight). The six xanthomata showed variable lipid composition (Table I) with a preponderance of cholesterol ester and cholesterol, and a paucity of phospholipid and triglyceride. Samples taken from different sites within the same xanthoma also showed some variability of lipid composition (Table I). A plot of the cholesterol, cholesterol ester, and phospholipid compositions on triangular coordinates (8) predicted the existence of separate cholesterol ester and phospholipid liquid crystalline phases in patients A and B and, in addition, a cholesterol crystal phase in patient C.

**Cholesterol ester fatty acid analysis.** To obtain enough material for analysis of fatty acid composition, samples were pooled from the different xanthomata in each patient. The cholesterol ester fatty acid composition was fairly uniform, and showed a relatively high content of saturated and monounsaturated fatty acids, and a low content of cholesterol linoleate compared, for example, to plasma LDL (11) or atherosclerotic fibrous plaques (17) (Table II).

**Amino acid analysis.** The material left after extraction of lipid from the xanthomata was hydrolyzed in HCl and subjected to amino acid analysis (Table III). Amino acids constituted 58±3.4% of the chloroform:methanol insoluble residue, or 39% of the dry weight. The amino acid composition was notable for a high content of hydroxyproline (7%) and glycine (25%), which indicates that collagen was the major protein constituent of the xanthomata. Assuming an 11% content of hydroxyproline in the tendon xanthoma collagen (based on the hydroxyproline content of tendon collagen), collagen constituted 36±1.5% of the nonlipid content of the xanthomata, or 24% of the dry weight. Direct analysis for hydroxyproline, with the method of Bergmann and Loxley (19) gave a value of 38.5±1.8%.

**Table I**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site</th>
<th>Cholesterol</th>
<th>Triglyceride</th>
<th>CE</th>
<th>Lyssolecithin</th>
<th>Sphingomyelin</th>
<th>Lecithin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Right</td>
<td>14</td>
<td>1</td>
<td>70.8</td>
<td>0</td>
<td>4.3</td>
<td>9.5</td>
</tr>
<tr>
<td></td>
<td>elbow</td>
<td>11</td>
<td>0.2</td>
<td>74.8</td>
<td>1</td>
<td>5.2</td>
<td>8.1</td>
</tr>
<tr>
<td>Left</td>
<td>11</td>
<td>0.2</td>
<td>79</td>
<td>0.5</td>
<td>4.3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>elbow</td>
<td>9</td>
<td>1</td>
<td>75</td>
<td>1.5</td>
<td>4.3</td>
<td>9</td>
</tr>
<tr>
<td>Finger</td>
<td>16.1</td>
<td>—</td>
<td>71.2</td>
<td>—</td>
<td>4.8</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Finger</td>
<td>7.1</td>
<td>3.0</td>
<td>80.5</td>
<td>0.25</td>
<td>3.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.9</td>
<td>1.8</td>
<td>80.8</td>
<td>0.35</td>
<td>3.7</td>
<td>5.0</td>
</tr>
<tr>
<td>C</td>
<td>Knee</td>
<td>42</td>
<td>6.5</td>
<td>38</td>
<td>2.5</td>
<td>4.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>34</td>
<td>5.4</td>
<td>46</td>
<td>1.3</td>
<td>5.5</td>
<td>7.3</td>
</tr>
<tr>
<td>Finger</td>
<td>50</td>
<td>8</td>
<td>26</td>
<td>—</td>
<td>4.4</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>7.4</td>
<td>46</td>
<td>—</td>
<td>4.4</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

* Lipids were analysed by double Folch (14) extraction and quantitative thin-layer chromatography (15). Results of duplicate samplings from different sites in the xanthomata are shown.
TABLE II
CE Fatty Acids (%) of Xanthomata

<table>
<thead>
<tr>
<th>Patient</th>
<th>14:0</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3(ω3)</th>
<th>20:2</th>
<th>20:3(ω6)</th>
<th>20:4</th>
<th>22:5(ω6)</th>
<th>22:5(ω3)</th>
<th>22:6(ω3)</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.8</td>
<td>9.0</td>
<td>6.5</td>
<td>2.1</td>
<td>50.9</td>
<td>19.2</td>
<td>1.4</td>
<td>2.6</td>
<td>3.2</td>
<td>0.9</td>
<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>B</td>
<td>0.8</td>
<td>7.3</td>
<td>6.8</td>
<td>1.0</td>
<td>50.4</td>
<td>17.6</td>
<td>2.0</td>
<td>2.4</td>
<td>3.2</td>
<td>4.0</td>
<td>1.3</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>C</td>
<td>0.5</td>
<td>11.7</td>
<td>0.7</td>
<td>0.8</td>
<td>42.3</td>
<td>23.3</td>
<td>1.7</td>
<td>2.0</td>
<td>3.8</td>
<td>6.2</td>
<td>2.1</td>
<td>0.6</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Compared with collagen, the total amino acid content of the xanthomata was slightly enriched in acidic amino acids.

Centrifugation of homogenized xanthoma. When homogenized xanthoma was subjected to preparative ultracentrifugation, about 50% of the lipid formed a floating skin while 50% remained in the pellet. The composition of the lipid skin was cholesterol 3.9%, triglyceride 0.1%, cholesterol ester 93.8%, sphingomyelin 1.3%, and lecithin 0.9%; while the pellet contained cholesterol 13.6%, cholesterol ester 74.1%, lyssolecithin 0.4%, sphingomyelin 5.2%, and lecithin 6.8%. The amount of cholesterol ester liberated from the tissue depended upon the time and vigor of homogenization. Thus, when the pellet formed from a first centrifugation was homogenized and centrifuged a second time, it liberated an equal proportion of its cholesterol ester. These results suggest that the homogenization procedure per se liberated cholesterol ester from the tissue.

Light microscopy. Sections stained with haematoxylin and eosin showed a few clusters of cells with foamy cytoplasm which resembled foam cells (the cell membranes represent the phospholipid liquid crystals predicted from lipid composition). In most areas of the tissue the cells were interspersed with thick bands of connective tissue.

Electron microscopy. Two of the three xanthomata from patient A, and the xanthoma from patient B, were examined by thin section electron microscopy. The principal constituent of all the specimens was bundles of fibrillar material (Fig. 1), which at high magnification was identified as collagen from its characteristic 620±12 Å periodicity (n = 13 micrographs). The mean diameter of the collagen fibrils was 455±20 Å, and although the space between fibrils ranged between 0 and 2,000 Å, the average distance was about 250 Å. All sections were remarkable for a paucity of large lipid droplets or cellular elements.

Polarized light microscopy. Examination of pieces of minced tissue at 25°C showed occasional smectic liquid crystals of free cholesterol ester and, also, in patient C, crystals of free cholesterol. The amorphous bulk of the tissue showed a diffuse background birefringence, which underwent no changes when cooled from 25°C to 10°C or subsequent heating to 60°C. When heated further, there was an abrupt shrinkage of the tissue, associated with separation of an oily phase, most evident at about 70°C. When cooled to 20°C, the oily material was identified as cholesterol ester which displayed characteristic reversible liquid crystalline transitions (22). In all three xanthomata of patient A, the smectic to cholesteric transitions occurred at 34°C, and the cholesteric to isotropic liquid transition at 37–38°C, while in patient B the respective temperatures were 37 and 41°C (Table IV). In patient C, both xanthomata showed a smectic-disordered transition at about 31°C, but no cholesteric phase could be seen, as expected from the high triglyceride content (Table I), because triglyceride abolishes the optical and calorimetric properties of the cholesteric phase (11).

Differential scanning calorimetry. Examination of fresh xanthoma by differential scanning calorimetry showed a reversible thermal transition between 36 and 45°C, of peak temperature 36–37°C, for patient A, 37–38°C for patient B (Fig. 2), and 31–32°C for patient C (Table IV). The enthalpy of this transition was 0.71±0.07 cal/g cholesterol ester (13 experiments on

TABLE III
Amino Acid Composition (%) of Tendon Xanthomata*

<table>
<thead>
<tr>
<th>Hydroxylysine</th>
<th>Lysine</th>
<th>Histidine</th>
<th>Arginine</th>
<th>Cysteic acid</th>
<th>Hydroxyproline</th>
<th>Aspartic acid</th>
<th>Threonine</th>
<th>Serine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.54±0.02</td>
<td>4.3±0.26</td>
<td>1.2±0.06</td>
<td>5.5±0.09</td>
<td>0.57±0.05</td>
<td>7.12±0.4</td>
<td>6.9±0.26</td>
<td>3.1±0.71</td>
<td>4.4±0.24</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Proline</td>
<td>Glycine</td>
<td>Alanine</td>
<td>Valine</td>
<td>Isoleucine</td>
<td>Leucine</td>
<td>Tyrosine</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>9.1±0.26</td>
<td>9.9±0.21</td>
<td>25.6±0.77</td>
<td>9.6±0.27</td>
<td>4.1±0.22</td>
<td>2.14±0.2</td>
<td>4.72±0.31</td>
<td>1.48±0.1</td>
<td>2±0.16</td>
</tr>
</tbody>
</table>

* Results shown are mean±SEM obtained from individual xanthomata (n = 6).
six xanthomata). When heated further, there was a large irreversible endotherm at 67±0.6°C, of enthalpy 9.9±1.2 cal/g collagen. The mean onset and end temperatures (measured from the intersections of tangents to the transition with the base line) were 60 and 75°C. With subsequent cooling and heating, there were reversible smectic-cholesteric and cholesteric-liquid transitions of cholesterol ester at 32 and 37°C in patient

**TABLE IV**

*Temperature of CE Transitions*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Site</th>
<th>Intact xanthoma smectic-disordered</th>
<th>Xanthoma after thermal collagen denaturation</th>
<th>CE/Triglyceride</th>
<th>Is/18:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Right elbow</td>
<td>37</td>
<td>32–34</td>
<td>37</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td>Left elbow</td>
<td>36</td>
<td>32–34</td>
<td>37</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>Finger</td>
<td>37</td>
<td>33</td>
<td>37</td>
<td>∞</td>
</tr>
<tr>
<td>B</td>
<td>Finger</td>
<td>38</td>
<td>34–37</td>
<td>41</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.88</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Knee</td>
<td>31</td>
<td>-31–5</td>
<td>6.9</td>
<td>1.84†</td>
</tr>
<tr>
<td></td>
<td>Finger</td>
<td>32</td>
<td>-31–5</td>
<td>4.5</td>
<td></td>
</tr>
</tbody>
</table>

* Temperatures refer to the peak temperatures noted by differential scanning calorimetry and the median melting temperature of CE droplets observed by polarized light microscopy.
† Average value for patient.
‡ In patient C, this was a single-peaked smectic-liquid transition.
ultracentrifugation of homogenized xanthomata showed sharp transitions identical to unbound or unassociated CE (Fig. 2d). Delipidated material from the xanthoma showed a sharp, irreversible transition of peak temperature (65°C) and enthalpy = 10 cal/g collagen, resembling the high temperature denaturation endotherm observed in the native tissue (Fig. 2e). A denaturation endotherm of identical temperature and enthalpy was obtained from a sample of bovine tendon collagen (not shown).

To determine the temperature dependence of the release of CE from intact xanthoma, tissue was systematically heated, cooled, and reheated to progressively higher temperatures (Fig. 3). Heating through the large, irreversible endotherm of peak temperature 67°C clearly resulted in a double-peaked endotherm of increased enthalpy (Fig. 3). When the increase in enthalpy of the CE transition was expressed as a function of temperature, a sigmoidal curve resulted (Fig. 4). The sigmoidal curve approximates the integrated form of the endotherm of peak temperature 67°C, and has the same midpoint temperature (65°C). Thus, the release of CE paralleled the thermal denaturation of collagen.

**X-ray diffraction.** The cut surface of the fresh xan-

![Graph](image)

**Figure 2** Differential scanning calorimetric heating curves of xanthoma specimens (a) fresh, minced xanthoma, (b) same specimen after being heated to 100°C, (c) CE isolated from xanthoma lipids, (d) lipid skin formed by preparative ultracentrifugation of homogenized xanthoma, (e) chloroform: methanol insoluble residue of xanthoma. All samples were heated at 5°C/min in hermetically sealed 75-μl sample pans. Endothermic (ENDO) transitions are represented by upward deflections of the baseline, and exothermic (EXO) transitions by downward deflections. In (a), the base sensitivity between 0 and 50°C is shown at 0.4 mcal/s, and between 55 and 100°C at 2 mcal/s (×% sensitivity). Upon heating the intact xanthoma (a), there is a liquid crystalline transition of CE between 25–45°C, followed by an irreversible collagen denaturation. Upon subsequent heating (b), the CE transition displays an increased enthalpy. Results shown are for patient B.

A, 34 and 41°C in patient B (Fig. 2b), and a single transition of peak temperature 31°C in patient C. The total enthalpy of these liquid crystalline transitions of cholesterol ester of heat denatured xanthomata was 1.18±0.10 cal/g cholesterol ester which was significantly greater (P < 0.01) than that of intact xanthoma. Xanthoma kept for 1 mo at −20°C showed an initial endotherm of peak temperature ~38°C (patient B) and enthalpy 7 cal/g cholesterol ester, which indicates that the cholesterol ester had crystallized (12). When recooled, liquid crystal transitions were observed which were similar to those of the native xanthoma. When heated further, the collagen transition was observed which indicated that although storage at −20°C produced crystallization of cholesterol ester(s) (CE), this did not alter the structure of the collagen.

Pure CE isolated from the xanthoma of patient B showed liquid crystalline transitions (Fig. 2c) at identical temperatures to the heat-denatured, intact xanthoma (Fig. 2b). When examined by differential scanning calorimetry, the lipid skin obtained by preparative ultracentrifugation of homogenized xanthomata showed sharp transitions identical to unbound or unassociated CE (Fig. 2d). Delipidated material from the xanthoma showed a sharp, irreversible transition of peak temperature (65°C) and enthalpy = 10 cal/g collagen, resembling the high temperature denaturation endotherm observed in the native tissue (Fig. 2e). A denaturation endotherm of identical temperature and enthalpy was obtained from a sample of bovine tendon collagen (not shown).

To determine the temperature dependence of the release of CE from intact xanthoma, tissue was systematically heated, cooled, and reheated to progressively higher temperatures (Fig. 3). Heating through the large, irreversible endotherm of peak temperature 67°C clearly resulted in a double-peaked endotherm of increased enthalpy (Fig. 3). When the increase in enthalpy of the CE transition was expressed as a function of temperature, a sigmoidal curve resulted (Fig. 4). The sigmoidal curve approximates the integrated form of the endotherm of peak temperature 67°C, and has the same midpoint temperature (65°C). Thus, the release of CE paralleled the thermal denaturation of collagen.

**X-ray diffraction.** The cut surface of the fresh xan-

![Graph](image)

**Figure 3** Differential scanning calorimetric heating curves of xanthoma. Xanthoma minced with a scalpel, was sealed in a 75-μl pan and then heated to 50°C, cooled to 0°C, then heated to 60°C, cooled to 0°C, heated to 70°C, cooled to 0°C, heated to 100°C, cooled to 0°C, and heated to 100°C. (Figure reads top to bottom.) The sensitivity was 0.4 mcal/s, except in sections initiated by dashed lines, where the sensitivity was 2 mcal/s. With heating to higher temperatures there is a progressive increase in the enthalpy of the transition between 25 and 45°C (CE), especially after the irreversible high temperature denaturation of peak temperature 67°C (collagen). ENDO and EXO show the direction of endothermic and exothermic transitions.
The 36-Å spacing, characteristic of the smectic phase of CE, disappeared when heated to 45°C, while the diffuse fringes persisted. The 15-Å spacing is part of the diffraction pattern of collagen (24, 25). To obtain greater resolution, X-ray diffraction was performed on oriented fibers.

Partially oriented, unstretched fibers, examined below the CE transition (e.g., at 10°C) immediately after removal from the patient, showed a weakly oriented, 36-Å equatorial reflection arising from smectic liquid crystalline CE, and also a weakly oriented equatorial reflection at 15 Å arising from the collagen (Fig. 5e). To improve the alignment of collagen, fibers were stretched and examined while under tension. Under these conditions well oriented equatorial reflections at 36 and 15 Å were observed (Figs. 5b and c). In addition, a series of meridional reflections were observed, identical in spacing to those of crystalline CE isolated from the xanthomata (Fig. 5d). Thus, oriented crystallization of CE occurred during the preparation of these specimens. When heated to temperatures above the calorimetric transition of CE (e.g., 42°C) all of the CE diffractions disappeared, while the equatorial collagen spacing persisted (Fig. 5e). Examination of the low angle diffraction pattern of oriented, stretched fibers at 10°C showed a series of sharp, meridional reflections (Fig. 5f) which proved to be the 4th and 6th to 11th orders of the 620-Å periodicity of the collagen fibril (Table V). All areas of the xanthomata examined by X-ray diffraction showed collagen and CE diffractions with the relative intensities paralleling the chemical composition. Furthermore, 10 different fiber preparations showed the same orientation of CE with respect to the collagen fiber axis. The same patterns were observed for different sites in the same sample. In the xanthomata from patient C, which showed cholesterol monohydrate crystals by microscopy, there were additional reflections arising from crystalline cholesterol (e.g., note the

![Figure 4](image)

**Figure 4** The increase in enthalpy of the xanthoma CE transition as a function of temperature. The xanthoma specimens (six experiments) were heated to progressively higher temperatures and the percent increase in the enthalpy of the CE liquid crystal melt measured as a function of the temperature of the previous heating run. In each experiment, the percent increase in enthalpy was determined from the increase in area of the CE transition after heating to a certain temperature + total increase in area after heating to 100°C. The midpoint temperature of the increase in enthalpy (i.e., the temperature at which 50% of the increase occurred) corresponded to the peak or midpoint temperature of collagen denaturation (68°C), which suggests that the liberation of CE was a result of denaturation of collagen.

![Figure 5](image)

**Figure 5** X-ray diffraction of xanthoma fibers (a) unstretched fiber, 10°C (toroidal camera), (b) stretched fiber, 10°C (toroidal camera), short exposure, (c) as in (b), but longer exposure, (d) purified xanthoma CE, at 10°C, toroidal camera, (e) stretched fiber, 42°C, toroidal camera, (f) stretched oriented fiber, 10°C, Franks camera (lower angle region). All specimens are shown with the fiber axis (meridional) parallel to the length of this page. In (a), there are weakly oriented equatorial reflections, which correspond to Bragg spacings of 36 and 15 Å, arising from smectic CE and collagen, respectively. The spotty equatorial reflections at 34 and 17 Å are a result of crystalline cholesterol monohydrate. In (b), the 36-Å equatorial reflection is seen as an intense fringe close to the center of the film. In addition, there are a series of meridional reflections arising from crystalline CE. In (c), the orientation of the 36-Å fringe is not seen because of overexposure, but the equatorial reflection from collagen at 15 Å can be more clearly seen. On the original film, a 2.8 Å meridional spacing arising from collagen can also be seen. All of the fringes of crystalline CE shown in (d) can be seen on the original films of (b) and (c). CE of the xanthoma fibers (b and c) melts when heated to 42°C, shown in (e), leaving only the collagen reflections (15-Å equatorial and 2.8-Å meridional—latter is seen on the original films). In (f) is shown the low-angle meridional diffractions of collagen (orders of 630 Å) and a strong 36-Å equatorial reflection arising from CE. There is also a weak 34-Å reflection due to unesterified cholesterol. (a) is from patient C and (b-f) from patient B.
TABLE V
Low Angle Meridional Spacings (d) of Oriented Xanthoma Fiber*

<table>
<thead>
<tr>
<th>Spacing (d)</th>
<th>Order (n)</th>
<th>nX d</th>
<th>Relative Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>155</td>
<td>4</td>
<td>620</td>
<td>Very weak</td>
</tr>
<tr>
<td>106</td>
<td>6</td>
<td>636</td>
<td>Strong</td>
</tr>
<tr>
<td>91.2</td>
<td>7</td>
<td>638</td>
<td>Weak</td>
</tr>
<tr>
<td>78.9</td>
<td>8</td>
<td>631</td>
<td>Weak</td>
</tr>
<tr>
<td>69.6</td>
<td>9</td>
<td>626</td>
<td>Strong</td>
</tr>
<tr>
<td>63.5</td>
<td>10</td>
<td>634</td>
<td>Weak</td>
</tr>
<tr>
<td>57.8</td>
<td>11</td>
<td>635</td>
<td>Intermediate</td>
</tr>
</tbody>
</table>

mean = 631 Å

* These spacings were recorded with Franks' mirror optics at 10°C. Orders 1–3 could not be observed because of the backstop.

equatorial diffraction spots at 34 and 17 Å in Fig. 5a, arising from large cholesterol crystals).

DISCUSSION

The composition of the tendon xanthomata obtained from three patients with familial hypercholesterolemia resembles that reported previously (1–3), particularly the preponderance of cholesterol and CE among the lipids, the increased ratio of cholesterol oleate to cholesterol linoleate compared to plasma lipoproteins, and the relative abundance of connective tissue (3). Tuberous xanthomata, which arise in similar clinical situations to tendon xanthomata have a similar lipid composition. By contrast, eruptive xanthomata are transient, highly cellular lesions, packed with lipid-laden foam cells, rich in triglyceride.

Our studies show that under physiological conditions the CE of the xanthoma was in a smectic liquid crystalline state (see footnote 2) because the peak temperatures (37, 38, and 32°C) of the CE smectic-disordered transitions were well above that of the sites from which the xanthomata were removed (28–30°C as measured by application of a liquid crystal thermometer to the skin surface). Indeed, the cooler temperatures of extensor surfaces may favor the accumulation of the smectic liquid crystalline CE. The relatively high transition temperature of xanthoma CE, compared to human plasma LDL (11), for example, is a result of an increased ratio of CE to triglyceride and to a greater degree of saturation of CE fatty acids.

Compared to isolated CE, the smectic-disordered transition of xanthoma CE was reduced in enthalpy (from 1.1 to 0.7 cal/g CE), which indicates constraints on the melting of CE induced by interaction with another tissue component. The heating of the tissue to 100°C resulted in release of these constraints, as shown by subsequent melting typical of bulk CE. The release of CE occurred primarily between 55 and 75°C (Fig. 4), which corresponds to a high enthalpy cooperative transition, typical of tendon collagen denaturation (26). The latter was positively identified as a collagen denaturation by the presence of an identical endotherm in the chloroform:methanol insoluble xanthoma residue, and in a control sample of bovine tendon collagen. Thus, in the fresh tissue, the melting of CE was modified by an interaction with collagen.

The X-ray diffraction experiments suggest a structural relationship between xanthoma collagen and CE. At 20°C, fibers dissected from xanthomata showed smectic liquid crystalline CE, partially oriented with respect to the collagen fiber axis. Better orientation of CE was obtained in stretched, cooled fibers where CE was crystalline. Because the 36-Å reflection arising from the layering of CE molecules was perpendicular to the reflections arising from the long axis of the collagen fibrils (orders of 620 Å), the layering of the smectic CE occurs between fibrils rather than along them (shown schematically in Fig. 6). That is, the long molecular axis of individual CE molecules lies perpendicular to the collagen fiber axis.

Oriented crystallization (epitaxial) on the surface of the collagen molecule has been demonstrated for some inorganic salts and for organic urea derivatives (24, 27). A prerequisite for epitaxis is that one of the dimensions of the crystalline-unit cell should bear a simple numerical relationship to the collagen layer line at 9.4 Å. This condition is apparently fulfilled by CE which has a strong meridional reflection at 4.8 Å, and perhaps by crystalline cholesterol. In contrast to the organic ureas, which intercalate between collagen molecules within fibrils (27), interaction of CE with collagen did not change the 15-Å equatorial spacing of the collagen. Furthermore, there were no lower angle equatorial spacings to indicate hexagonal or other ordered arrangements of CE between individual collagen molecules. Thus, the CE lies between and not within collagen fibrils. This view is supported by the normal thermal denaturation of collagen and the normal thickness of collagen fibrils as seen by electron microscopy (25).

The proposed collagen/CE structure (Fig. 6) may explain the altered melting behavior of CE of xanthomata. The enthalpy of the single-peak transition of CE in the intact xanthoma is similar to that of the smectic-cholesteric transition of the isolated CE (Fig. 2), which suggests that in the native tissue, CE lacks a cholesteric to liquid transition. Thus, above the transition, CE may be held in a cholesteric-like or
nematic state by the collagen molecule, with loss of layering between CE molecules but a persistent one-dimensional alignment of their long molecular axes.

It is unlikely that CE of xanthomata exists within intact LDL because the chemical composition, the low phospholipid content, and the high cholesterol oleate:linoleate ratio are unlike that of plasma LDL. Furthermore, xanthomata show partially oriented CE X-ray diffractions whereas LDL gives only unoriented CE reflections (11). Although we have identified an interaction between collagen and CE, we cannot exclude possible interactions of collagen or CE with other unidentified tissue components, for example, cholestanol esters or mucopolysaccharides.

That cholesterol and CE probably exist between and not within collagen fibrils suggests that the lipids are deposited after secretion of collagen from cells. Pro-collagen molecules are secreted from cells and assembled into fibrils subsequent to the action of extracellular enzymes which cleave off an NH$_2$-terminus peptide (28). CE could be deposited onto collagen directly from LDL, perhaps because of an affinity of the hydrophobic surface of the collagen fibril for CE. Alternatively, LDL CE may be taken up, hydrolyzed, and reesterified by cells which eventually die and liberate CE into the extracellular space. The latter process is suggested by the altered fatty acid composition of xanthoma CE, compared to LDL (Table II).
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The phase behavior of hydrated cholesterol

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Abstract The thermotropic phase behavior of cholesterol monohydrate in water was investigated by differential scanning calorimetry, polarizing light microscopy, and x-ray diffraction. In contrast to anhydrous cholesterol which undergoes a polymorphic crystalline transition at 39°C and a crystalline to liquid transition at 151°C, the closed system of cholesterol monohydrate and water exhibited three reversible endothermic transitions at 86, 123, and 157°C. At 86°C, cholesterol monohydrate loses its water of hydration, forming the high temperature polymorph of anhydrous cholesterol. At least 24 hours were required for re-hydration of cholesterol and the rate of hydration was dependent on the polymorphic crystalline form of anhydrous cholesterol. At 123°C, anhydrous crystalline cholesterol in the presence of excess water undergoes a sharp transition to a birefringent liquid crystalline phase of smectic texture. The x-ray diffraction pattern obtained from this phase contained two sharp low-angle reflections at 37.4 and 18.7 Å and a diffuse wide-angle reflection centered at 5.7 Å, indicating a layered smectic type of liquid crystalline structure with each layer being two cholesterol molecules thick. The liquid crystalline phase is stable over the temperature range of 123 to 157°C before melting to a liquid dispersed in water. The observation of a smectic liquid crystalline phase for hydrated cholesterol correlates with its high surface activity and helps to explain its ability to exist in high concentrations in biological membranes. — Loomis, C. R., G. G. Shipley, and D. M. Small. The phase behavior of hydrated cholesterol. J. Lipid Res. 1979. 20: 525–535.

Supplementary key words liquid crystals, crystalline structure, polymorphic forms, atherosclerosis, biological membranes, gallstones, bile, differential scanning calorimetry, x-ray diffraction

Cholesterol is universally distributed in all animal tissues where it fulfills a structural and functional role in cell membranes, serum lipoproteins, and bile. Practically insoluble in water, cholesterol is readily solubilized in solutions containing amphiphilic compounds (1–6). Cholesterol concentrations up to 50 mole% with respect to phospholipid are observed in biological membranes and lipid dispersions prepared from naturally occurring phospholipids (3, 7–9). Sonicated systems of synthetic homogeneous chain lecithins have been shown to contain up to 3 moles of cholesterol per 1 mole of phospholipid (10), and liposomes prepared from certain organic solvents contain nearly 2 moles of cholesterol to 1 mole of phospholipid in a metastable state (11). The high solubility of cholesterol in phospholipid bilayers is attributed partly to its intrinsic hydrophobicity and partly to specific head group interactions between the sterol OH group and the polar moiety of the lipid (5, 12). When the cholesterol concentration exceeds the solubility limit in lipid bilayers or micelles, deposition of crystalline cholesterol monohydrate is observed (1, 3, 7). This process of saturation and deposition of crystalline cholesterol is known to occur in certain pathological states in man, most notably, gallstone disease (4) and atherosclerosis (13, 14).

The effect of cholesterol incorporation on the structural and thermodynamic properties of model lipid systems has been intensively studied in recent years; however, only minor efforts have been made toward understanding the phase behavior of pure cholesterol in water (15). We believe that the cholesterol–water binary system needs to be described before the more complex multicomponent systems can be rigorously defined. In this report we describe the phase behavior of cholesterol monohydrate in water as a function of temperature. The physical states of hydrated and anhydrous cholesterol are compared, with particular reference to the behavior of cholesterol in aqueous biological systems.

MATERIALS AND METHODS

Cholesterol undergoes autoxidation under a variety of conditions (16, 17). Since degradative products affect the thermotropic and lyotropic phase behavior of a compound, great care was taken to ensure the purity of the samples. Cholesterol (Nu Chek Prep, Elysian, MN) was recrystallized three times from an ether—

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Abbreviations: DSC, differential scanning calorimetry.
methanol solution. The final recrystallized material was analyzed for impurities by infrared spectroscopy and by thin-layer chromatography using separate solvent systems of toluene–ethyl acetate 60:40 (v/v) and hexane–diethyl ether–acetic acid 70:30:1 (v/v/v). The recrystallized material was divided into 500-mg quantities and sealed in vials by flame under nitrogen before storing in the dark at 4°C. Each vial was analyzed on opening for antioxidative products as described above. No impurities were observed for the final recrystallized material nor for samples stored up to 9 months.

Anhydrous crystalline cholesterol was prepared by dissolving pure cholesterol in hot acetic acid and allowing the solution to slowly cool. The crystals were isolated on filter paper and placed in a heated desiccator (80°C) under vacuum for 72 hr to remove all remaining acetic acid. The anhydrous crystals were stored in a desiccator over phosphorous pentoxide at 4°C in the dark. Cholesterol monohydrate was prepared by recrystallization from 95% ethanol after the method of Stauffer and Bischoff (18). Monohydrate crystals, isolated by filtration, were washed repeatedly with triply distilled water and stored in the dark at 4°C in water. Cholesterol as a dry microcrystalline powder or dispersed in water did not show antioxidative degradation when stored up to 3 months.

Anhydrous cholesterol crystals (~5 mg) with an equal weight of distilled water were hermetically sealed in small stainless steel pans under nitrogen and heated or cooled at a programmed rate of 5°C/min in a Perkin-Elmer DSC-II differential scanning calorimeter. Samples for polarizing light microscopy were examined by direct light and between crossed nicsols in order to document the number of phases and their textures. To ensure that cholesterol monohydrate remained hydrated above 100°C, samples were placed in preflattened sealed glass capillary tubes (19). The sample tubes were immersed in a silicon oil bath fitted to a Leitz heating/CO₂ cooling microscope stage which allowed control of both the heating and cooling rates of the sample. The temperature apparatus was calibrated at heating rates of 1 and 2°C/min using known standards. The accuracy in measuring a sharp first-order transition on heating was determined to be ±1.0°C.

X-ray diffraction profiles were recorded using focussing cameras with either toroidal mirror (20) or double mirror (21) optics utilizing Ni-filtered CuKα radiation from an Elliot GX-6 rotating anode generator. Anhydrous cholesterol or cholesterol monohydrate in an excess of water was placed in 1-mm quartz capillary tubes and sealed by flame. The sample tubes were located in a specially constructed brass sample holder capable of operating with either camera. The temperature of the sample was regulated by an electrically variable temperature controller over the temperature range of 20–300°C with an accuracy of ±0.5°C as determined using standards with known transition temperatures. X-ray films were analyzed with a Joyce-Loebl model III CS microdensitometer.

The rate of cholesterol hydration in different relative humidities was determined by measuring the weight change of a cholesterol sample suspended over different sulfuric acid solutions. A triturated 200-mg sample of anhydrous cholesterol was suspended from a Cahn microbalance inside a glass chamber. The microbalance was connected to a continuous recording device which allowed the weight change of the sample to be monitored throughout the entire experiment. The relative humidity inside the chamber was determined by the volume ratio of sulfuric acid to water and the atmospheric pressure. The apparatus was placed in a closed room and the room temperature was monitored by a constant recording temperature device. The temperature for all experiments was 22 ± 0.5°C.

RESULTS

Identification of cholesterol crystals

Gravimetric analysis, polarizing light microscopy, and x-ray diffraction were used to verify the crystal structures of anhydrous cholesterol and cholesterol monohydrate. Approximately 25 mg of wet cholesterol monohydrate crystals were placed on a microbalance pan and periodically weighed. When constant weight of the sample was observed over a period of 30 min, it was assumed that all excess water had evaporated from the crystal surfaces. The crystals were immediately heated until melted, allowed to cool, and reweighed for the weight of cholesterol alone. From the weight of cholesterol, the mole ratio of water to cholesterol was calculated for several samples. The mean of 0.98 ± 0.17 (SD) mol of H₂O/mol of cholesterol indicated that the samples were the monohydrate form of cholesterol. Anhydrous cholesterol crystals treated in a similar manner showed no change in weight after heating.

Identification of anhydrous cholesterol and cholesterol monohydrate was carried out using polarizing light microscopy and x-ray diffraction as shown in Fig. 1. At 20°C anhydrous cholesterol appears as needles and cholesterol monohydrate as plates when viewed under a polarizing light microscope. The angles formed by the edges of the monohydrate plates measured on a large number of crystals were 79.15
ANHYDROUS CHOLESTEROL

CHOLESTEROL MONOHYDRATE

Fig. 1. Photomicrographs and x-ray diffraction patterns obtained from crystalline anhydrous cholesterol and cholesterol monohydrate. Photomicrographs were taken with the samples between crossed nicols. Magnification 70×.

and 100.8°. The larger angle agreed well with the angle γ (100.8°) of the unit cell of cholesterol monohydrate (22). The x-ray powder diffraction patterns obtained from the triturated samples at 20°C are shown on the right side of Fig. 1. The reflections in each powder pattern could be indexed according to the unit cell data in the crystal structure of anhydrous cholesterol (23) and cholesterol monohydrate (22).

Thermotropic phase behavior of the two crystalline forms of cholesterol

Fig. 2 shows a typical heating scan of anhydrous cholesterol on first heating from 0 to 170°C. A small, relatively broad endotherm was observed centered at 38.9 ± 1.4°C and having a calculated heat change of 0.91 ± 0.5 Kcal/mol cholesterol. No further changes were observed on heating until 150.7 ± 0.8°C when a large, sharp endotherm occurred with a calculated heat change of 6.59 ± 0.25 Kcal/mol. Both transitions were reversible on cooling, with the large transition at approximately 115°C and the small transition undercooling to approximately 20°C. On the second heating of the sample, the small transition sharpened and moved down to 36°C. The larger transition remained at 150°C. At the bottom of Fig. 2, x-ray diffraction patterns show that there are structural changes associated with the two transitions of cholesterol. The x-ray powder diffraction pattern obtained at 20°C is typical of anhydrous cholesterol as shown previously in Fig. 1. On heating the sample to 45°C, the dif-
In contrast to anhydrous cholesterol, cholesterol monohydrate in excess water produced a different DSC profile on first heating the sample from 10°C as shown in Fig. 4. Three endotherms were observed at 86.4 ± 0.5, 123.4 ± 0.8, and 156.8 ± 0.5°C. The cal-

Fig. 2. Differential scanning calorimetry and x-ray diffraction results obtained from an anhydrous cholesterol sample as a function of temperature.

fraction pattern has changed from that observed at 20°C in accordance with the small transition observed by DSC at 39°C. Heating the sample to 135°C did not change the powder diffraction pattern from that observed at 45°C. At 160°C, above the large 150°C DSC endotherm, the diffraction pattern dramatically changed. Two diffuse scattering maxima centered at equivalent Bragg spacings of 6 and 21 Å indicated that the sample had melted to a liquid.

X-ray diffraction patterns obtained above and below the 39°C transition and the microdensitometer traces from these patterns are shown in Fig. 3. The traces clearly show that several crystal reflections have changed intensity during the transition (dashed lines indicate reflections that have changed intensity). Except for the appearance of one new reflection at a Bragg spacing of 5.4 Å (arrow), no change in the diffraction spacing was observed. These results are consistent with the existence of a crystalline polymorphic transition of cholesterol first described by Spier and van Senden (24) and later confirmed by van Putte et al. (25), and suggest that the crystal structures of the two polymorphic forms are similar.

Fig. 3. X-ray diffraction patterns of anhydrous cholesterol above and below the 39°C polymorphic transition. The microdensitometer traces of the two x-ray diffraction patterns are shown at the top of the figure. The dashed lines indicate the reflections that undergo intensity changes during the transition. These reflections have been indexed according to the unit cell parameters of anhydrous cholesterol (29). The arrow designates a new reflection not seen in the low temperature polymorph.
culated heat change for each transition was 2.35 ± 0.22, 3.42 ± 0.20, and 2.29 ± 0.14 Kcal/mol cholesterol, respectively. If the sample was cooled at 5°C/min., three exotherms were observed at 149, 85, and 10°C. The latter two transitions showed marked undercooling. The calculated heat change of the 149 and 85°C exotherms compared well with those of the 156 and 123°C transitions observed on heating. The 10°C exotherm gave an average heat change of only 0.8 Kcal/mol cholesterol, well below that calculated for the 86°C endotherm of the heating run. The second heating run, shown in Fig. 4, no longer exhibited an 86°C transition but instead showed a small endotherm at 36.4 ± 0.5°C. The two high-temperature endotherms remained unchanged. The calculated heat change for the 36°C transition was 0.81 ± 0.21 Kcal/mol cholesterol, which is similar in temperature and enthalpy to the low-temperature polymorphic transition of anhydrous cholesterol described in Fig. 2. To verify that the cholesterol monohydrate sample exhibited a polymorphic crystalline transition on the second heating run, anhydrous cholesterol crystals were placed in a DSC pan, an equal weight of water was added, and the pan was immediately sealed and heated from 10°C at 5°C/min in the differential

Fig. 4. Comparison of differential scanning calorimetry heating scans of cholesterol monohydrate and crystalline anhydrous cholesterol in water.

Fig. 5. Photomicrographs illustrating the changes in cholesterol monohydrate in water as a function of temperature. All pictures are of same field, magnification 100×. Top left, cholesterol monohydrate crystals, crossed nicols; bottom left, melted cholesterol droplets, direct light; top right, cholesterol smectic liquid crystals, crossed nicols; bottom right, same field, crossed nicols with full wave compensators inserted. Sign of birefringence +.
scanning calorimeter. At the bottom of Fig. 4 is shown the resulting heating scan which remained unchanged on consecutive heating runs. The scan was identical to the second heating scan of cholesterol monohydrate. This identity indicated that after cholesterol monohydrate was heated to high temperatures and cooled in water, the cholesterol crystallized to the anhydrous form.

Cholesterol monohydrate samples sealed in capillary tubes with water were studied by polarizing light microscopy and x-ray diffraction in an attempt to identify the phases undergoing the transitions observed by DSC. The types of phases observed by polarizing light microscopy during heating are shown in the photomicrographs of Fig. 5. At 20°C, cholesterol monohydrate displays birefringent plate crystals. No consistent changes could be discerned on heating the sample from 20°C to 125°C. At 124°C, there was a sudden change from crystalline cholesterol to a birefringent phase exhibiting flow characteristics. The two photomicrographs on the right of Fig. 5 show the same field with crossed nicols (top) and with crossed nicols plus the full wave compensator inserted (bottom). Notice the myelin figure (upper right at 125°C) and the large number of focal conics dispersed in a continuous water phase. The focal conics gave a positive sign of birefringence, indicating a smectic type of liquid crystalline phase. This is the first observation that pure cholesterol will form a liquid crystalline phase in water. On further heating, the liquid crystalline phase melted at 157°C to an isotropic oil dispersed in water (Fig. 5, bottom, left).

Fig. 6 shows the results from the x-ray diffraction experiment. The powder diffraction pattern of cholesterol monohydrate, identical with that shown in Fig. 1, was observed at 20°C. Between 20 and 85°C, the diffraction pattern remained unchanged, but at 90°C a different powder pattern was recorded. The new diffraction pattern was identical to one obtained from the anhydrous cholesterol sample at 90°C. Thus the transition observed by DSC at 86°C represents the transformation of cholesterol monohydrate to the high-temperature anhydrous cholesterol polymorph with the loss of the water of hydration. At 124°C, a new diffraction pattern was obtained which contained two sharp low-angle diffraction maxima at the equivalent Bragg spacings of 37.4 and 18.7 Å and a diffuse wide angle maximum at 5.7 Å. Although only two low-angle diffraction maxima were observed, their ratio of 1:½ was compatible with a smectic liquid crystalline
phase. The diffuse wide-angle maximum indicated a semi-melted or fluid state in the short-range packing of the molecules.

The effect of temperature on the liquid crystal structure was studied by obtaining x-ray diffraction patterns at 2°C intervals between 124 and 156°C. The experimental results are shown in Fig. 7. Over the total temperature range of the liquid crystal, only two sharp low-angle diffraction maxima could be obtained. This graph shows that the three liquid crystal reflections remain invariant with temperature. When the sample was heated above 156°C, two diffuse maxima were observed at approximately 25 and 5.9 Å, indicating that the cholesterol liquid crystal had melted to a liquid with no long-range order.

**Hydration rate of cholesterol**

Anhydrous cholesterol suspended in water will form cholesterol monohydrate with time (26). Since anhydrous cholesterol undergoes a polymorphic transition near physiologic temperature (36–39°C), the dependence of the hydration rate on the crystal form of cholesterol was investigated. These studies were carried out in bulk water at 20 and 45°C and in different relative humidities at 22°C.

For the bulk water experiments at 20 and 45°C, the degree of hydration in a sample was measured by differential scanning calorimetry using the areas of the 36 and 86°C endotherms of anhydrous cholesterol and cholesterol monohydrate, respectively. The ratio of the areas under the peaks, standardized to the mean area per mole of cholesterol, gave the ratio of anhydrous cholesterol to cholesterol monohydrate in the sample. A series of DSC pans were prepared, each containing 7 mg of anhydrous cholesterol. Since the crystal surface area will greatly affect the hydration rate, the cholesterol in each pan was heated above the melt and cooled to form a thin crystalline film on the bottom of the pans of approximately equal surface area from sample to sample. Half of the pans were placed in a 20°C constant temperature room and half were placed in an oven at 45°C. At time = 0, each pan received 7 μl of triply distilled water and was sealed under nitrogen. Equilibration was carried out at the appropriate temperature and each pan was examined by DSC after a specified period of time ranging up to 48 hr.

Several of the DSC heating scans from samples equilibrated at 20 and 45°C are shown in Fig. 8. On the left, the samples equilibrated at 20°C have formed the monohydrate in the first 24 hr. In contrast, the samples equilibrated at 45°C showed no evidence of monohydrate formation until after 36 hours. The rate of cholesterol monohydrate formation may be determined from a plot of percent cholesterol monohydrate vs. time. This is shown in Fig. 9, where the rate of formation is given by the slope of the line. Assuming all the samples equilibrated at 20°C have reached maximum hydration in 24 hr, the data points were fitted to a straight line by least-squares linear regression analysis ($m = 4.17, b = -6.5$). A regression coefficient of $r = ±0.927$ indicates the assumption is valid. The rate, determined from the slope and standardized to 1 g of cholesterol, was found to be $1.94 \times 10^{-3} \text{g H}_2\text{O/g cholesterol per hr}$. If the sample of anhydrous cholesterol was introduced to water as a powder, the rate was increased.

In contrast, samples equilibrated at 45°C did not readily convert to cholesterol monohydrate in 48 hr.
water of hydration into the surrounding water. The water molecules in the crystal lattice of cholesterol monohydrate form three hydrogen bonds per oxygen atom (22). In addition, every other cholesterol hydroxyl group is hydrogen bonded to its neighbor. Therefore, three hydrogen bonds must be broken to release one water molecule. In anhydrous cholesterol at 22°C and presumably in its higher melting polymorph, every hydroxyl group contains two hydrogen bonds (23). Thus, when cholesterol monohydrate loses its water of hydration, one new hydrogen bond must form for every cholesterol hydroxyl group. The net energy absorbed during the release of water from the cholesterol monohydrate structure is equivalent to the breaking of two hydrogen bonds or approximately the absorption of 9 Kcal/mole of cholesterol.

In ice, a water molecule tends to hydrogen bond with four neighboring water molecules in a tetrahedral arrangement (28). When ice melts, the energy required to break all the hydrogen bonds would be 9 Kcal/mol of water (one hydrogen bond is formed between two water molecules; therefore, two moles of hydrogen bonds are broken per mole of water). However, the heat of fusion of water is much smaller (1.43 Kcal/mol) and indicates that only 16% of the hydrogen bonds are actually broken in the ice-liquid transition. The water released from the monohydrate structure will presumably form the same number of hydrogen bonds as the surrounding water. Thus, approximately 16% fewer hydrogen bonds are formed per mole of released water than are broken in the transition to anhydrous cholesterol. In other words, a small amount of energy, equivalent to the heat of fusion of water, must be added to the system to bring about the transition to anhydrous cholesterol.

A similar approach may be applied to the behavior of hydrated cholesterol at high temperatures (>100°C). At 90°C, cholesterol is in the same crystalline state regardless of the presence of water and the final state of cholesterol at high temperatures is a liquid for both systems. If we assume that the change in enthalpy is not influenced by the path taken to get from initial to final state and that variables which specify the state of the system, such as temperature and pressure, have a small effect on the enthalpy, then the total enthalpy change should be the same for the anhydrous and hydrated systems. A total enthalpy change of 6.59 Kcal/mol cholesterol was obtained from the anhydrous system and 5.71 Kcal/mol for the hydrated system. The difference between the two values of 0.88 Kcal/mol cholesterol was highly significant. A possible explanation for this difference may be that the liquid phases in the dry and hydrated systems are not exactly the same. X-ray diffraction patterns obtained on the liquids at 160°C show differences in the low-angle maxima of 25 and 21 Å for hydrated cholesterol and anhydrous cholesterol, respectively, which could indicate that water of hydration is present in the melted hydrated system.

Two separate experiments were designed to measure the hydration rate of crystalline anhydrous cholesterol. The first experiment determined the rate of hydration in bulk water above and below the 39°C polymorphic transition. The results conclusively showed that the low-temperature polymorph hydrated faster than the polymorph present at 45°C, the difference in the hydration rates being $1.9 \times 10^{-3}$ g H$_2$O/g cholesterol per hr and $1.56 \times 10^{-4}$ g H$_2$O/g cholesterol per hr for 20 and 45°C, respectively.

The second experiment was designed to measure the rate of hydration of anhydrous cholesterol at 22°C in different relative humidities. Relative humidities less than 95% failed to hydrate cholesterol. Stauffer and Bischoff (18) reported that cholesterol monohydrate remained stable at 80% relative humidity and at 38°C. It may be that after formation of the monohydrate, it will remain stable at lower relative humidities. At 95% relative humidity, the hydration rate of cholesterol held a constant value of $7.3 \times 10^{-5}$ g H$_2$O/g cholesterol per hr over a period of 200 hr. Crystalline cholesterol at 100% relative humidity showed a sharp increase in the amount of hydration for the first 40 hr before the rate slowed and reached a constant value of $7.7 \times 10^{-5}$ g H$_2$O/g cholesterol per hr. This value is very close to the rate obtained at 95% relative humidity. However, the maximum rate in 100% relative humidity of approximately $4.6 \times 10^{-4}$ g H$_2$O/g cholesterol per hr is an order of magnitude below the rate obtained in bulk water at 20°C by DSC. Although the discrepancy cannot be adequately explained at this time, the hydration experiments have shown that formation of cholesterol monohydrate in water is a slow process and is dependent on the polymorphic crystalline form of anhydrous cholesterol.

In biological systems, cholesterol is usually found in cellular membranes. Such membranes have a general structure of a bilamellar leaflet of phospholipid into which proteins and other molecules are imbedded (29). Cholesterol is readily incorporated into pure phospholipid lamellar liquid crystalline phase (2, 3). In fact, cholesterol can form lamellar liquid phases with a large number of aliphatic amphiphiles (1, 30). Therefore, it would appear that high temperatures or the presence of fatty acyl chains is required to disrupt the steroid ring packing. In systems containing only cholesterol and water the interaction between the sterol hydroxyl group and water is apparently not strong enough to overcome the packing forces at low temperatures. The maximum amount of cholesterol that can be incorporated into membranes might also
depend on the sterol hydroxyl–phospholipid head group interactions (5, 12). Cholesterol in excess of a 1:1 mole ratio with phospholipid would experience both side-to-side packing and competition for phospholipid head groups with other cholesterol molecules. The excess cholesterol would eventually precipitate as crystalline cholesterol monohydrate (3), a process which also occurs in atherosclerosis (13, 14).

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Quantitation of the Transfer of Surface Phospholipid of Chylomicrons to the High Density Lipoprotein Fraction during the Catabolism of Chylomicrons in the Rat

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ABSTRACT  Small chylomicrons (CM) labeled with cholesterol, cholesteryl ester, phospholipid, and, in some cases, protein, were used to study the fate of these constituents as the CM are catabolized in the circulations of the heptatectomized and intact rat. In the heptatectomized animal after 1/2 h, CM are greatly reduced in volume, surface area, and diameter. During this period, the CM lost >92% of the mass of their triacylglycerol, >77% of the mass of their phospholipid, and >39% of their protein. Compared to the injected CM, the chemically altered particles, called CM "remnants," have a reduction in volume of 96% and in surface area of 88%. The labeled cholesterol esters remain with the CM remnants but, strikingly, a major fraction of the labeled phospholipids and labeled soluble apoproteins leave the CM and are found in the high density lipoprotein (HDL) fraction.

The chemical composition of this HDL fraction contains relatively more phospholipid and less cholesterol ester than normal rat HDL. Because of the difference in composition of HDL between normal rats and those given CM, we estimate that the HDL phospholipid pool increased by 25% by the infusion of 4–5 mg of CM phospholipid. Approximately 5 mg of phospholipid is secreted on CM by a fed rat in 1 h. The findings in heptatectomized rats indicate that a major fraction of the phospholipid and a minor fraction of the protein (soluble non-B apoproteins) of newly secreted CM are transferred from the CM to the HDL fraction during remnant formation. The same process probably occurs in intact rats except that the remnant particles are rapidly removed from the plasma by the liver and a smaller fraction of the surface of the CM enters the HDL fraction.

INTRODUCTION

The increase in human plasma high density lipoproteins (HDL)1 phospholipids that accompanies absorption of a fatty meal was described as early as 1957 (2), but its metabolic basis remains unknown. In rats, as chylomicron (CM) or very low density lipoprotein (VLDL) triglycerides are hydrolyzed, cholesterol ester-rich "remnant" particles are formed (3, 4). During remnant formation in heptatectomized rats, it was shown that CM diameter decreased from ≈200 to ≈90 nm (3). This corresponds to a reduction in surface area of ≈80%. Mjos et al. (4) also showed that triglyceride-rich particles (CM and VLDL) lose surface components. In intact rats 63% of 32P-labeled CM phospholipids stayed in plasma after the CM remnants were taken up by the liver indicating loss of CM surface during CM catabolism (4). In the present experiments, CM, double labeled in vivo with [3H]cholesterol and either [14C]phosphatidylcholine (PC) or 32P-apoproteins were injected into rats to trace the fate of CM components during CM remnant formation. From mass and radiolabeled studies, we conclude that during CM catabolism a major fraction of the surface components of the CM especially phospholipids enter the HDL fraction.

METHODS

To obtain CM, male Wistar rats weighing 180–250 g (Charles River Breeding Laboratories, North Wilmington, Mass.) were

1 Abbreviations used in this paper: CM, chylomicron(s); HDL, high density lipoprotein(s); LCAT, lecinthin-cholesterol-acyltransferase; PC, phosphatidylcholine; VLDL, very low density lipoprotein(s).
prepared with thoracic duct (5) and intestinal cannulae and maintained postoperatively as previously described (6). I d after surgery, a sonicated lipid emulsion was injected through the intestinal cannula at 1–2 ml/h. The emulsions contained pure egg PC (Lipid Products, South Nutfield, Surrey, England) 16 mg/ml; triacylglycerol, 20 mg/ml; cholesterol, 0.2 mg/ml; [1,2-3H]cholesterol, 1.5 μCi/ml (New England Nuclear, Boston, Mass.); and [N-methyl-14C]egg PC, 2 μCi/ml. Radioactive PC was synthesized from enzymatically prepared phosphatidic acid and [methyl-14C]choline chloride (New England Nuclear); sp act was 6.8 Ci/mmol. In some experiments, L-5-5S-methionine (New England Nuclear; sp act 573 Ci/mmol) was added to the emulsion at a concentration of 10–20 μCi/ml. Milkly lymph was collected in the presence of EDTA and ethyl mercaptobasalicate (6).

CM were isolated by centrifugation in a discontinuous salt gradient in the SW25.2 rotor of the Beckman L2-65 ultracentrifuge (Beckman Instruments, Inc., Spino Div., Palo Alto, Calif.) (8). The gradient was prepared from 12 ml NaCl solutions of d 1,006, 1,020, 1,041, and 1,065 g/ml. After removing cells by low-speed centrifugation, the lymph was adjusted to a d = 1.1 g/ml with solid KBr, degassed, and then 12 ml as carefully injected under the gradient. The gradient was centrifuged first for 21.5 min at 15,000 rpm (38,460 g-max). The rotor was stopped with the brake on and the top 0.5 cm was removed (fraction 1). The centrifuge was then started and run for 72.5 min at 22,500 rpm (84,290 g-max). The rotor was again stopped with the brake and the top 0.5 cm of the gradient was removed (fraction 2). The two fractions were resuspended in 0.15 M NaCl containing EDTA 0.1 mg/ml. Fraction 1 contained large particles (>220 nm Dian) but <25% of the CM mass, whereas fraction 2 contained the smaller particles (75–220 nm Dian). The smaller CM were used within 3 d to produce remnants.

Remnant particles were prepared in functionally hepatocereattomed rats anesthetized lightly with ether (4). 10 mg of heparin and 1 ml of a milky suspension of 75–220 nm Dian CM containing 47±18 mg (SD) of total lipid was injected into a tail vein. This dose was equivalent to a 1 h production of lymph. After 30 min, blood was collected by cardiac puncture into EDTA and 5,5-dithiothreitol solution (9) (final concentrations 5 and 2 mM, respectively) and the plasma was harvested by centrifugation at 1.5 g for 15–30 min. The plasma always appeared clear, the turbidity of the injected CM having disappeared. The clearing indicated that the size of the CM was reduced. The plasma was then adjusted to d 1.1 g/ml with the addition of solid KBr and 12 ml as loaded under the gradient described earlier and centrifuged for 20 h at 20,000 rpm (66,000 g-max). Eight separate fractions were removed and their density measured in an Anton Paar density meter (Graz, Austria). To obtain an HDL fraction, the bottom three fractions (d > 1.063) were pooled, the density readjusted to 1.21 g/ml, and then recentrifuged (10). The HDL fraction was examined for chemical composition, radioactivity, and for size in four or a smear by Na phosphotungstate-negative electron microscopy. The HDL fraction of a normal rat and two hepatocereattomed rats given heparin but not CM, isolated using the same procedures as CM-infused animals, were examined by negative-stain electron microscopy for comparisons to CM-infused animals.

The fate of CM constituents was tracked in intact nonfasted, nonheparin-treated 250-g male rats given (by tain vein) 25 mg of a CM suspension labeled with [3H]cholesterol and cholesterol ester and [14C]PC. After 5, 15, or 30 min, rats were exsanguinated by cardiac puncture and the liver was removed. The livers were extracted for lipid analysis and radioactivity. Plasma was fractionated by sequential ultracentrifugation at d 1.006, 1.063, and 1.21 g/ml in the Spineco 40.3 rotor (Beckman Instruments, Inc., Spino Div.) (10) and the lipoprotein fractions were analyzed for lipid and radiactivity. Rat plasma volume was calculated from the relationship 0.175 W0.70, where W = body weight (11).

Lipids were extracted in 25 vol of chloroform-methanol (2:1 vol/vol), and lipid composition was analyzed by quantitative thin-layer chromatography and densitometry (12, 13).

Total lipoprotein protein was estimated by the method of Lowry et al. (14) using bovine serum albumin as a standard. Lipid turbidity was extracted with CHCl3 after color development. B and non-B peptides were estimated by the method of Kane (15) except that B peptides were precipitated by 2-propanol. The conditions for ultracentrifugation were calculated by applying the nomogram of Dole and Hamlin (16), assuming that the gradient retained its discontinuities of density throughout centrifugation. The mean diameter of lipoprotein particles was calculated from their chemical composition assuming spherical particles with all apolar lipids in the core and all polar lipids and protein in a 2.15-nm thick surface layer (17).

RESULTS

Recovery of labeled CM constituents. When CM were injected into hepatocereattomed rats, and allowed to circulate for 30 min, 91.6±2.37% (mean±SEM, n = 24) of total cholesterol, 91.9±1.96% (n = 24) of PC, and 100±4.53% (n = 4) of protein radioligactivity were recovered in the plasma compartment. These recoveries indicate that the cholesterol, PC, and protein moieties of the injected CM remain largely in the plasma of the hepatocereattomed rat. Of the total PC label recovered, 96±0.3% was as diacylglycophosphatidylcholine and <4% was recovered as lysophosphatidylcholine. This indicates that very little of the injected PC was hydrolyzed.

The changes in CM composition, mass, and surface area during 30-min circulation. The chemical composition of the injected CM and the resultant remnant-containing fraction from the plasma are given in Table I. Compared with their parent particles, the particles in the remnant fraction were depleted in triacylglycerol and enriched in cholesterol, cholesterol esters, phospholipid, and protein. Knowing the injected dose and composition of the CM and amount of each constituent recovered in the remnant fraction, we have estimated the percent of the total CM-injected mass recovered in the remnant fraction (Table I). Because of lipolysis, only 8% of the triacylglycerol mass is recovered. Furthermore, only 23% of the phospholipid and 59% of the protein is recovered. However, the recovery of free and especially esterified cholesterol is greater than that injected with the CM. These results require some explanation. It was shown that dog CM incubated for 30 min with dog serum increase their free cholesterol by ≥10% but cholesterol esters were neither gained nor lost (8). The gain in cholesterol esters in our remnant fraction is probably explained by the fact that VLDL, which have a similar lipid composition and density compared to the CM,

Transfer of Chylomicron Phospholipid to High Density Lipoprotein 163
TABLE I
Composition and Recovery of Injected CM

<table>
<thead>
<tr>
<th></th>
<th>Injected CM (n = 8)</th>
<th>Remnant fraction (n = 8)</th>
<th>Percent of total CM injected mass recovered in remnant fraction* (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall composition</td>
<td></td>
<td></td>
<td>% total mass</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>86.6±1.13†</td>
<td>56.9±3.51</td>
<td>8±2</td>
</tr>
<tr>
<td>Cholesterol ester</td>
<td>1.3±0.18</td>
<td>13.4±2.30</td>
<td>139±30</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.9±0.17</td>
<td>7.8±0.92</td>
<td>121±30</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>9.5±0.95</td>
<td>16.2±2.21</td>
<td>23±7</td>
</tr>
<tr>
<td>Protein</td>
<td>0.9±0.14</td>
<td>5.2±0.74</td>
<td>59±10</td>
</tr>
</tbody>
</table>

* This column overestimates the mass recovery of CM components in remnants as the remnant fraction also contains some other plasma lipoproteins of similar density, for instance hepatic VLDL.
† Mean±SEM.

remnant, are isolated with the CM remnant and thus contaminate the remnant composition. Because VLDL also contain triacylglycerol, phospholipids, and protein, not only is the “mass recovery” of CM cholesterol ester too high, but the mass recovery of the other constituents is also too high. Therefore, the actual recovery of CM triacylglycerol is <8%, of CM phospholipid <23%, and of CM protein <50%. This indicates that there is a marked loss of both CM triacylglycerol and of surface components during remnant formation.

The size and density of the injected CM are compared to the remnant fraction particles in Table II. The mean diameter of the CM is decreased to 35%, the surface area to 12%, and the mass to 4% of the original. If each CM produced one remnant particle, then not only has the mass of the particle been decreased greatly by the removal of triacylglycerol but the surface of the particle has also been diminished. Thus, both mass recovery and size changes indicate that during circulation of the CM in the hepatectomized rat not only is triacylglyceride removed by lipolysis but also a major portion of the surface is lost from the CM.

The distribution of radiolabeled CM components 30 min after CM injection. 30 min after injection of CM labeled with [3H]cholesterol, and either [14C]PC or [32S] apoproteins, the plasma was isolated and centrifuged on the density gradient. Fig. 1 shows the percentage of recovered label found in fractions of different density. The remnants are contained in fractions 1 and 2 (d < 1.020). Most of the plasma cholesterol radioactivity floated with the remnant-containing fractions, but, strikingly, PC and non-B-protein radioactivities were found in the more dense fractions. The majority of the cholesterol label in the remnant fraction was found as cholesterol ester, but free cholesterol accounted for most of the label in the more dense fractions.

The data for all experiments are given in Table III. The data are expressed as three density cuts: a remnant fraction (d < 1.020); pooled middle fraction (d 1.025–1.050); and the bottom fractions (d > 1.063). About 80% of the recovered [3H]cholesterol floated with the remnant fraction (Table III). Because the total recovery of injected CM [3H]cholesterol in plasma was 91.6% we calculate that ≈73% of the total injected [3H]cholesterol

TABLE II
Calculated Density and Size of CM and the Particles Recovered in the Remnant Fraction

<table>
<thead>
<tr>
<th></th>
<th>Injected CM</th>
<th>Remnant fraction particles</th>
<th>CM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density, g/ml†</td>
<td>0.93</td>
<td>0.96</td>
<td></td>
</tr>
<tr>
<td>Diameter, nm‡</td>
<td>131±12.8</td>
<td>46±3.62</td>
<td>35</td>
</tr>
<tr>
<td>Surface area, nm²¶</td>
<td>53,900±10,500</td>
<td>6,650±1,050</td>
<td>12.3</td>
</tr>
<tr>
<td>Molecular weight, ×10⁶¶</td>
<td>8.2±2.2</td>
<td>0.34±0.08</td>
<td>4.1</td>
</tr>
</tbody>
</table>

* The density is estimated from the composition given in Table I and the partial specific volumes of the components as given in Sata et al. (17).
† The diameter, surface area, and molecular weight are calculated by the method of Sata et al. (17) with the assumptions: (a) that the particles are spherical and not widely heterogeneous in size; (b) that all the protein, phospholipid, and free cholesterol form a 2.15-mm thick surface shell around a core of triacylglycerol and cholesterol ester; and (c) that the apparent partial specific volumes of the individual components are similar to their partial specific volume in the intact lipoproteins.

164  T. G. Redgrave and D. M. Small
remained with the CM remnant. Most of the \(^{3}H\)cholesterol in the remnant fraction was esterified (79±2.2\%). The rest of the \(^{3}H\)cholesterol was divided between the middle and bottom fractions. The bottom fraction contained 10\% of the recovered label or 9.2\% of the injected label and it was mostly unesterified (17±3.8\% esterified). Only 8.6\% of the recovered \(^{14}C\)PC and 15.9\% of the recovered \(^{35}S\)-soluble non-B apoprotein was recovered in the remnant fraction. Most of these labels were found in the bottom fractions. We estimate, based on the total recovery of injected radioactivity, that the bottom fraction contained 71.4\% of the injected CM \(^{14}C\)PC and 68\% of the injected CM \(^{35}S\)-soluble apoproteins.

Recovery of HDL from bottom fractions. By raising the density of the bottom fractions of the density gradient \((d > 1.063)\) to 1.21 g/ml and recentrifuging, an HDL fraction was obtained, composed of 25.8\% protein (±2.87), 1.6\% triacylglycerol (±1.64), 40.5\% phospholipid (±3.48), 24.4\% cholesterol ester (±2.89) and 6.8\% free cholesterol (±0.87, \(n = 5\)). Most of the radioactivity present in the pooled bottom fractions of the density gradient was recovered with this HDL fraction. The recoveries given as a percent of counts in the bottom fraction were: \(^{3}H\)cholesterol 83.1±2.57\% (mean±SEM, \(n = 5\)); \(^{14}C\)PC 71.1±2.20\%; \(^{35}S\)-soluble apoprotein 71.0\%. The small amount of total cholesterol radioactivity in the HDL fraction was 83\% unesterified. Phospholipid radioactivity remained as diacylphosphatide in the HDL fraction with <4% lysophosphatide present. The HDL fraction of four separate animals was negatively stained with Na phosphotungstate and examined by electron microscopy. Fig. 2 shows that even in a fairly dense preparation no rouleaux are seen. Most of the particles appear round. A rare collapsed vesicle and a very rare flattened particle resembling a 5.0-nm thick disk on edge was seen in some preparations. These particles represented <1 in 200 particles. Occasional large particles were also seen. The distribution of particle size for each animal are given in Fig. 3. The mean in each animal varied from 17.7 to 24.1 nm. The distributions are rather wide but with the exception of a very few large particles, the distributions are not bimodal. With the same electron microscopy techniques a normal rat HDL had a mean diameter of 13.3±2.3 nm (\(n = 245\)) and two control hepatectomized animals given heparin but not CM had a mean diameter of 13.3±3.8 nm (\(n = 146\)) and 13.7±4.8 nm (\(n = 117\)). These values compare reasonably to diameters of rat HDL estimated by negatively stained electron microscopy by others (18, 19). Thus rats given CM have larger particles in their HDL fraction.

The lack of an appreciable discoidal or vesicle fraction and the increased size of the HDL suggests that surface components of the CM might fuse with normal HDL to produce the larger chemically altered particle.

Injection of labeled CM into intact rats. Because the preceding observations were made in hepatectomized rats, it was necessary to show that the catabolic events described also occurred in the intact animal. Table IV shows the disappearance of labeled cholesterol and PC from the plasma and the appearance of label in the liver after intravenous injection of CM. In accordance with previous reports (3, 4), CM cholesterol disappeared quickly from the plasma. At 5 min, 54–71\% of the injected \(^{3}H\)cholesterol was recovered in plasma but only 5–6\% remained 30 min after injection. Cholesterol label removed from the plasma was quantitatively recovered in the liver. Removal of \(^{14}C\)PC from the plasma was slower, so that 40–44\% remained 30 min after injection. Recovery of cleared
PC in the liver was much less than for cholesterol, but 27–30% of the injected dose was found in this organ after 30 min.

Fractionation of plasma radioactivity after CM injection into the major lipoprotein classes by ultracentrifugation (Table V) showed that [3H]cholesterol ester radioactivity was associated with the lightest fraction (d < 1.006 g/ml). However, after 30 min very little cholesterol ester radioactivity was present in any fraction. Cholesterol radioactivity in the HDL fraction (d 1.063–1.21 g/ml) was due mainly to free cholesterol, cholesterol ester was never >2% of the injected dose. In marked contrast, 23–32% of the injected CM [3H]PC radioactivity appeared in the HDL fraction as early as 5 min after injection of CM. Only 14–22% of the injected [3H]PC radioactivity remained in the simultaneously recovered fraction containing the remnants (d < 1.006 g/ml). Radioactivity in HDL as a result of [3H]PC remained fairly constant during the 30 min whereas at this time <2% of the injected dose was found at 30 min in d > 1.006 g/ml. Because of some loss of some lipoproteins during the process of ultracentrifugal separation of the plasma into lipoprotein fractions, we recovered only 61 and 70% of the counts originally found in plasma. Therefore, the percent of injected CM radioactivity reported in Table V is low by 30–39%. Assuming that the loss is evenly distributed over the three lipoprotein fractions we estimate that the true percent of the injected CM label appearing in each fraction should be ≥30–39% higher. Thus, we estimate that 38–48% of the injected [3H]PC goes to HDL in 5 min, and at 30 min 32–53% is still in the HDL fraction. In contrast, we estimate that 21–36% of the injected [3H]PC remained with the remnant at 5 min but no >3% was present at 30 min. Up to 8% of radioactivity of both isotopes was recovered with the 1.006– to 1.063-g/ml fractions; like the d < 1.006-g/ml fraction, this activity declined rapidly for [3H]cholesterol, but, like HDL, it persisted for [3H]PC. This suggests that the 1.006–to 1.063-g/ml fraction may contain at least two populations, one of CM remnants rich in cholesterol ester which are rapidly taken up by the liver and one of PC-rich particles of low-protein content allowing flotation at 1.063 g/ml.

Exchange and net loss of phospholipids has been observed when CM are incubated with serum. Up to 20% of dog CM phospholipid mass and somewhat more CM phospholipid radioactivity could be lost in 30-min incubation with dog serum (8). To test for net loss of lipid and loss of radioactive CM phospholipid we incubated 25 mg of fresh rat CM labeled with PC with 10 ml of saline or rat plasma. (A 275-g rat has ≈10 ml of plasma.) The CM were incubated for 0, 5, 15, and 30 min and then isolated on a density gradient. There was no net loss of rat CM triacylglycerol or phospholipid but radioactive phospholipid was lost. In saline, the percent of original phospholipid radioactivity remaining on the CM was 84, 87, and 87% after 5, 15, and 30 min, respectively. In plasma more radioactive phospholipid was lost. 66, 60, and 55% of the original radioactivity remained with the CM after 5, 15, and 30 min, respectively. Thus, since there is no net loss of

<p>| Table III |</p>
<table>
<thead>
<tr>
<th>Distribution of CM Radioactivity in Plasma Fractions of Different Density after 30-min Circulation in Hepatectomized Rats*</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]cholesterol</td>
</tr>
<tr>
<td>% Total</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Top remnant fractions</td>
</tr>
<tr>
<td>(d &lt; 1.020)</td>
</tr>
<tr>
<td>Middle fractions</td>
</tr>
<tr>
<td>(d 1.025–1.050)</td>
</tr>
<tr>
<td>Bottom fractions</td>
</tr>
<tr>
<td>(d &gt; 1.063)</td>
</tr>
</tbody>
</table>

* The values are given as percent of total recovered counts. The values in parentheses give calculated recovery as percent of injected dose. This value was obtained by multiplying the total plasma recovery of each CM label injected ([3H]cholesterol = 91.6%; [3H]PC = 91.9%; [3S]-Soluble non-B apoprotein = 100%) by the percent total recovered counts in each fraction. The individual values were used to calculate the SEM. 
† The percent esterified is the percent of label of [3H]cholesterol which was esterified. The [3H]cholesterol in the injected CM was 64±2.6% esterified. Thus, the remnant fractions are enriched in labeled cholesterol ester and the bottom fractions which contain HDL are very poor in labeled cholesterol esters. 
§ The value for [3H]cholesterol and [3H]PC are mean±SEM of 24 experiments. The values for [3S]-Soluble apoprotein represent the mean of four separate experiments.
FIGURE 2  Morphology of HDL fraction isolated from CM-injected hepatectomized rat. After the HDL were isolated by ultracentrifugation, they were placed on a Formvar-coated grid and negatively stained with Na phosphotungstate. ×318,000.
phospholipid, the decrease in CM radioactivity with time indicates that phospholipid exchange occurs.

When CM labeled with [3H]lysine were incubated with rat plasma ≈30% of the labeled protein was lost in 30 min. However, the total mass of CM protein increased by about fourfold. Thus, CM exposure to

**DISCUSSION**

The changes in CM mass and composition during 30-min circulation in the hepatectomized rat show that as the mass of the CM triacylglycerol is reduced to <8% of the original CM, phospholipid mass is decreased to <23%, and protein to <59%. Triacylglycerol is the major constituent of the core of the CM (20), so its loss results in a shrunken remnant particle, which we estimate contains only ≈4% of the original mass of the CM.

<table>
<thead>
<tr>
<th>TABLE IV</th>
<th>Percentages of Injected CM Radioactivities Found in Liver and Whole Plasma 5, 15, and 30 min after CM Injection in Two Groups of Intact Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
</tr>
<tr>
<td>Rats (a)</td>
<td></td>
</tr>
<tr>
<td>Time, min</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>5</td>
</tr>
<tr>
<td>Plasma</td>
<td>84</td>
</tr>
<tr>
<td>Total recovery</td>
<td>95</td>
</tr>
<tr>
<td>Rats (b)</td>
<td></td>
</tr>
<tr>
<td>Time, min</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>5</td>
</tr>
<tr>
<td>Plasma</td>
<td>76</td>
</tr>
<tr>
<td>Total recovery</td>
<td>98</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE V</th>
<th>Percentages of Injected CM Radioactivities Found in Isolated Lipoprotein Fractions 5, 15, and 30 min after CM Injection in Two Groups of Intact Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipoprotein fraction</td>
<td>PC</td>
</tr>
<tr>
<td>Rats (a)</td>
<td></td>
</tr>
<tr>
<td>Time, min</td>
<td></td>
</tr>
<tr>
<td>d &lt; 1.006 g/ml</td>
<td>5</td>
</tr>
<tr>
<td>d = 1.006–1.063 g/ml</td>
<td>6</td>
</tr>
<tr>
<td>d = 1.063–1.21 g/ml</td>
<td>23</td>
</tr>
<tr>
<td>Rats (b)</td>
<td></td>
</tr>
<tr>
<td>Time, min</td>
<td></td>
</tr>
<tr>
<td>d &lt; 1.006 g/ml</td>
<td>14</td>
</tr>
<tr>
<td>d = 1.006–1.063 g/ml</td>
<td>5</td>
</tr>
<tr>
<td>d = 1.063–1.21 g/ml</td>
<td>32</td>
</tr>
</tbody>
</table>
When the core of a spherical particle shrinks, surface must also be lost if the particle is to remain spherical. With the assumptions (17, 20) that all the protein, phospholipid, and most of the free cholesterol form a 2.15-nm thick surface around a core of triacylglycerol and cholesterol esters, the mean composition of both the CM and the remnant fractions (Table I) are consistent with spherical emulsion particles (Table II). We calculate that the remnant particle has lost \( \approx 85\% \) of its original surface, which compares well with the 77% net loss of phospholipid, the major surface component. During in vitro incubation of CM with plasma triacylglycerol and phospholipids are not lost and proteins are actually increased so the loss of phospholipid and protein occurring during formation of the CM remnant must be linked to that process.

The composition of the HDL fraction isolated 30 min after CM injection in the hepatectomized rat is quite different from normal rat HDL (18, 19). For instance, the relative weight proportions of the three major lipids of normal rat HDL (phospholipids, cholesterol esters, and cholesterol), expressed as weight percent of the sum of the weight of three lipids are: 48% phospholipids, 43% cholesterol esters, and 9% cholesterol. Expressed in the same way the HDL fraction isolated from our rats has 56% phospholipid, 33% cholesterol ester, and 9% cholesterol. Thus, our HDL contain relatively more phospholipid and less cholesterol ester. These changes are illustrated on triangular coordinates (21) in Fig. 4, which shows that the direct addition of about one part CM surface lipids to four parts normal HDL can account for the lipid composition of our CM-injected rat HDL. In absolute terms, we have given these animals \( \approx 47 \) mg of CM containing \( \approx 4.5 \) mg of phospholipid. During CM catabolism to remnants \( \approx 77\% \) or \( \approx 3.5 \) mg of the CM phospholipid was lost. The HDL phospholipid pool in normal rats is between \( \approx 3.4 \) and 7.3 mg (10, 23, 24). If all the phospholipid lost from the CM were transferred to HDL the preexisting HDL phospholipid pool would be appreciably expanded.

Exchange of PC between CM and HDL (8) is insufficient to account for our observations. About 45% of the PC radioactivity exchanged when 25 mg of CM was incubated with rat plasma for 30 min. However, when a larger amount of similar CM was injected into hepatectomized rats, \( \approx 92\% \) of the injected radioactive PC left the CM by 30 min, and most of it was found in HDL. Because this enormous loss of labeled PC in vivo cannot be accounted for solely by exchange, net loss of both labeled and unlabeled PC must have occurred, consistent with the loss of mass already described.

The CM also loses some of its protein during CM remnant formation (4, 25). Mjös et al. (4) and Schaefer et al. (25) showed that the proteins lost are almost entirely soluble non-B apoproteins. In our experiments the CM proteins labeled during \(^{35}\)S-methionine infusion were both insoluble B apoproteins and soluble non-B apoproteins. We presume that most of the soluble apoproteins labeled on our CM were of the A species, which are synthesized in the gut and secreted on CM (26, 27). After infusion the CM loses \( \approx 84\% \) of its labeled soluble apoproteins and most of these are found in the HDL fraction probably because of a net movement of A peptides to HDL during CM remnant forma-

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Lipid composition of normal HDL and the HDL fraction isolated from CM-injected hepatectomized rats plotted on triangular coordinates. Triangle above represents simplified phase diagram (21) of the major lipid classes of HDL. Zone I contains a single phospholipid lamellar liquid-crystal phase, into which 33% cholesterol (C) and 2% cholesterol ester (CE) can be incorporated. Zone II also contains a single phase, composed of liquid or liquid-crystalline cholesterol ester. Zone III contains both the cholesterol ester and phospholipid (PL) phases. In zone IV, a third phase, cholesterol-monohydrate crystals, is also present. Below, the CM-injected HDL fraction from hepatectomized rats (B) contained more phospholipid and less cholesterol ester than normal rat HDL (A). A line connecting the compositions A and B extended to the cholesterol-phospholipid axis gives the composition of the material which must be added to normal HDL (A) to produce the composition at point B. The composition is given by the point X which is precisely the composition of the CM surface in respect to phospholipid and cholesterol. (From Table I, the CM phospholipid:cholesterol ratio is 90:10.) This indicates that CM surface lipids were added to circulating HDL (A) to produce the HDL fraction of composition B. The ratio of the mass of X to A is given by the law of levers (22). Thus, the ratio of X to A to produce B is \( (X/A) = (AB)/(BX) = 0.25 \). Thus, about one part of surface composition X added to four parts of normal HDL will result in composition B.
tion. The same mechanism probably operates in man (28). Normal human lymph CM contain a large amount of A-1 peptide (29) and CM isolated from chylous plural effusion (25) or from urine of humans having fistulae between intestinal lymphatics and the urinary tract contain an abundance of A peptides especially A-1 and an A-4-like peptide (30). CM obtained from plasma lack A-1 (31). Thus, once the CM has circulated in plasma, the A-1 is lost. Further, when CM labeled with 125I-A-1 are injected into normal humans, >90% of the labeled peptide appears in HDL in 1 h (32). Finally, both HDL phospholipid (2) and total protein (33) increase during fat feeding.

In the intact rat a similar transfer of phospholipid to HDL appears to occur. 5 min after CM injection only 14–22% of the injected CM [14C]PC remains within the CM remnant formation, whereas in vitro incubation of similar quantities of CM with 10 ml of plasma for 5 min show that much more of the radioactive PC (≈66%) is retained by the CM. This suggests that there is also a net loss of phospholipid from CM to HDL in the intact animal. Most of the CM [14C]PC lost can be recovered in the HDL fraction and at this time only a very small percent of the injected dose (≈11%) is found in the liver. However, because remnants are rapidly taken up by the liver, some of the [14C]PC which otherwise goes to HDL in hepatectomized animals enters the liver at 15 and 30 min. In the intact animal, CM remnants larger than those produced by 30 min catalysis in the hepatectomized rat will be taken up by the liver.

Both the perfused rat liver (18) and rat intestine (19) secrete discoidal “nascent” HDL particles which contain mainly surface components (phospholipids, free cholesterol, apoproteins) but very little cholesterol ester. The importance of lecithin-cholesterol-acyltransferase (LCAT) in the conversion of discoidal HDL particles to spherical HDL has been shown in vitro (18) and implicated in LCAT deficiencies. Familial LCAT deficiency (34) and the LCAT deficiency in alcoholic hepatitis (35) are both associated with the presence of discoidal HDL in the plasma. Compared to these discoidal nascent particles, HDL isolated from normal plasma are spherical (19, 34) and contain relatively more cholesterol ester. Discoidal particles are not readily found in the normal HDL fraction and it is likely that after their formation, they are acted on by LCAT which leads to increased cholesterol esters, decreased PC, and the subsequent structural change from disk to sphere (18, 36).

In our experiments the composition and size of the HDL fraction isolated from CM-injected, hepatectomized animals are different from normal HDL. It contains more phospholipid, less cholesterol ester, and the particles are larger than normal rat HDL. The LCAT reaction would form new cholesterol esters and simultaneously decrease phospholipid. Thus, the particle would subsequently increase core components (cholesterol esters) and decrease surface components (phospholipids). However, it would gain an absolute amount of cholesterol ester so its mass and diameter would be bigger than the original circulating HDL. Such a mechanism might explain the rather specific increase in the larger HDL (HDL2) found after fat feeding (33).

Significant formation of lysocephatidate was shown by Eisenberg and Schurr (37) when rat plasma VLDL were degraded in vitro by postheparin plasma, leading to the conclusion that hydrolysis of PC was a major mechanism for the removal of phospholipids from VLDL during their degradation. Our data in vivo suggest that very little PC is converted to lysocephatidate during CM catalysis, so we must conclude either that the surface phospholipids of CM and plasma VLDL are metabolized by different mechanisms or that studies in vitro must be applied with caution to physiological events in vivo.

Fresh CM produced by the intestine have a very low cholesterol-phospholipid ratio (0.18), whereas remnant has a very high ratio (0.95) (Table I). Therefore, the surface lipids of the CM can rapidly pick up cholesterol. The HDL isolated after CM injection also has a low cholesterol-phospholipid ratio and may accept cholesterol from other tissues (38). Thus, the addition of large amounts of phospholipid to the HDL fraction not only increases one of the substrates for LCAT but also permits the other to enter the HDL fraction from other lipoproteins and cell membranes. Such a mechanism might protect against tissue cholesterol accumulation and atherosclerosis. Man has a fasting pool of HDL phospholipid of ≈1.5 g (39). After a fatty meal, man can absorb 50 g of triacylglycerol which would be secreted into lymph as CM containing ≈3 g phospholipid. If only 25% of the CM phospholipid entered the HDL fraction it would account for half the pool of HDL phospholipid, and would be important in the formation of HDL and cholesterol homeostasis (38).

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Lectin-Induced Agglutination of Phospholipid/Glycolipid Vesicles†

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ABSTRACT: A model membrane system is described which exhibits characteristics similar to those observed for ligand binding to cell surfaces, in the absence of cellular energetic machinery or a cytoskeleton. *Ricinus communis* agglutinin (RCA), a plant lectin, reversibly agglutinates model membrane vesicles composed of egg yolk lecithin (EYL) and the glycolipid lactosylceramide (LC). The initial velocity of this agglutination depends upon pH, temperature, and the EYL/LC mole ratio. Vesicles containing less than 5 mol % LC are not agglutinated by RCA. At higher LC contents, agglutination increases with increased LC content. The temperature profile of RCA-induced agglutination exhibits a maximum at 25 °C. Decreased agglutination at higher temperatures may be due to a temperature-dependent reversible transition of the lectin. Decreased agglutination at low temperatures is correlated with differential scanning calorimetry studies of the EYL/LC vesicles, which suggest that a glycolipid lateral phase separation occurs at low temperatures. RCA-agglutinated EYL/LC vesicles can be deagglutinated by addition of sugars which compete with the vesicles for the lectin. Electron microscopy of deagglutinated vesicles indicates that extensive vesicle fusion has not taken place. Vesicles composed of EYL and bovine spinal cord cerebroside (galactocerebroside), a glycolipid which possesses the requisite terminal galactose for RCA binding, are not agglutinated by RCA, indicating that the glycosyl receptor must extend out from the bilayer surface to support lectin-induced agglutination.

The ability of mammalian cell-surface receptors to move in the membrane plane is affected by transformation (Rosenblith et al., 1973; Nicolson, 1973) and differentiation (Moscona, 1971). Various lines of evidence have suggested that receptor topography is controlled both by cytoskeletal components (Edelman et al., 1973; Ji and Nicolson, 1974; Pasté et al., 1975; Bales et al., 1977; Aubin et al., 1975) and by membrane fluidity (Hirwitz et al., 1974; Ben-Bassat et al., 1977; Maccecchini and Burger, 1977; Hatten et al., 1978). The determination of the extent to which each of these factors influences receptor mobility will depend upon the development of model systems in which these effects can be separated.

The role of glycolipids as mobile cell-surface receptors has been suggested by their ability to interact with lectins and hormones in liposomes prepared from total lipid extracts (Rendi et al., 1976) or from pure glycolipids and lecithin (Suroliya et al., 1975; Redwood and Polefka, 1976; Alo et al., 1977; Boldt et al., 1977; Curatolo et al., 1977). In this paper, we characterize the interactions of a lectin from castor beans (*Ricinus communis*) with sonicated vesicles composed of egg yolk lecithin (EYL) and lactosylceramide (LC), a neutral glycolipid whose structure is shown as structure I.

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Increased agglutination at low temperatures is correlated with differential scanning calorimetry studies of the EYL/LC vesicles, which suggest that a glycolipid lateral phase separation occurs at low temperatures. RCA-agglutinated EYL/LC vesicles can be deagglutinated by addition of sugars which compete with the vesicles for the lectin. Electron microscopy of deagglutinated vesicles indicates that extensive vesicle fusion has not taken place. Vesicles composed of EYL and bovine spinal cord cerebroside (galactocerebroside), a glycolipid which possesses the requisite terminal galactose for RCA binding, are not agglutinated by RCA, indicating that the glycosyl receptor must extend out from the bilayer surface to support lectin-induced agglutination.

Materials and Methods

Materials. *Ricinus communis* agglutinin (RCA) was purified according to Nicolson and Blaustein (1972), as modified by Podder et al. (1974). RCA was stored in 0.01 M Tris-HCl-0.2 M NaCl (pH 7.2). EYL was extracted and purified according to Litman (1973). Lactosylceramide ([N-palmitoylhydrolactocerebroside, lot no. 3]) was purchased from Miles Laboratories, Elkhart, Ind., and was repurified by silicic acid chromatography. After repurification, LC exhibited one spot on TLC in CHCl₃/CH₃OH/H₂O (65:25:4). Bovine spinal cord cerebroside (galactocerebroside) was from Supelco, Inc., Bellefonte, Pa. Galectose (Ga) and lactose (Lac) were from Sigma Chemical Co., St. Louis, Mo. All other reagents were reagent grade. Water was doubly distilled. [N-methyl-³H]-Choline-labeled EYL ([³H]EYL) was synthesized using egg phosphatidic acid and [N-methyl-³H]-choline (New England Nuclear, Boston, Mass.) as previously described (Sears et al., 1976).

EYL/LC Vesicles. Solutions of EYL (2:1 chloroform/methanol, v/v) and LC (in chloroform) were mixed in appropriate proportions in a round-bottomed flask, and the solvent was removed in a rotary evaporator. After vacuum desiccation overnight, 8 mL of a buffer composed of 0.01 M Tris-HCl (pH 7.2) and 0.2 M NaCl was added to the flask, and the lipid was suspended by shaking for ~15 min at room temperature. This dispersion was sonicated for 20 min at 4 °C under a N₂ atmosphere, followed by centrifugation at 12 000g for 20 min at 4 °C to remove titanium fragments and multilamellar liposomes.

Lectin-Induced Agglutination. *Ricinus communis* agglutinin, in 0.01 M Tris-HCl (pH 7.2). 0.2 M NaCl, was added rapidly to an EYL/LC vesicle suspension and vortexed. The
AGGLUTINATION OF LIPID VESICLES

FIGURE 1: Turbidity increase observed upon the addition of RCA to a suspension of sonicated EYL vesicles containing 10 mol % LC. Incubation mixtures contained vesicles at an EYL concentration of 0.24 μmol/mL and various amounts of RCA, in a total volume of 2.1 mL.

FIGURE 2: (a) Initial velocity of agglutination of EYL vesicles containing 10 mol % LC as a function of RCA concentration. Incubation conditions are identical to those in Figure 1. (b) Initial velocity of agglutination as a function of vesicle concentration. 100 μL of RCA (1.5 mg/mL) was added to various amounts of EYL/LC vesicles (10 mol % LC), in a total volume of 2.1 mL.

FIGURE 3: Relationship between the turbidity (A450) of RCA-agglutinated EYL/LC vesicle suspensions after a 1 h incubation and the mass of agglutinated material which can be sedimented at 23000g (cpm). Incubation mixtures contained EYL/LC vesicles (10 mol % LC) at an EYL concentration of 0.24 μmol/mL and various amounts of RCA, in a total volume of 2.1 mL.

FIGURE 4: Deagglutination of RCA-agglutinated EYL/LC vesicles (10 mol % LC) by lactose (● -●) and galactose (● -●). Sugar concentrations are: (a) 46, (b) 46, (c) 150, and (d) 460 μM lactose and (e) 460 μM galactose.

mixture was immediately transferred to a spectrophotometer, and agglutination was followed by the turbidity (A450) increase with time. Lectin-induced agglutination was studied systematically as a function of lectin concentration, vesicle concentration, phospholipid/glycolipid ratio, pH, and temperature. Throughout this study, the initial velocity of agglutination is reported because prolonged incubation (>30 min) of EYL/LC vesicles with RCA results in the formation of very large aggregates (~0.5-mm diameter) which sediment to the bottom of the tube.

Electron Microscopy. Sonicated vesicles were negatively stained with 2% sodium phosphotungstate (pH 7.4) on Formvar-coated copper grids. Electron micrographs were obtained with an AEI-GB electron microscope, calibrated with a catalase standard.

Differential Scanning Calorimetry (DSC). EYL/LC vesicles for DSC were prepared as above, concentrated in an A-75 minicon dialyzing apparatus (Amicon Corp., Lexington, Mass.), and hermetically sealed in stainless-steel pans (50 μL capacity). Scanning calorimetry traces were obtained on a Perkin-Elmer DSC-2 differential scanning calorimeter (Perkin-Elmer Corp., Norwalk, Conn.) at a heating/cooling rate of 5 °C/min at 0.2 mcal/s sensitivity. Peak areas were determined by planimetry, and enthalpies were calculated on the basis of phosphorus assays of the contents of the DSC pans (Gomori, 1942).

Results

Addition of Ricinus communis agglutinin to a clear sonicated suspension of EYL containing 10 mol % LC results in an increase in the turbidity of the suspension which can be monitored by absorbance at 450 nm. The time course of the lectin-induced agglutination at various RCA concentrations (at 24 °C) is shown in Figure 1. Both the rate and the extent of the agglutination depend upon RCA concentration. The initial velocity of agglutination (A450/min) increases linearly with RCA concentration over the range 50–150 μg of RCA/mL, while very little agglutination is observed below 50 μg/mL (Figure 2a). The dependence of the initial velocity of agglutination upon vesicle concentration shows a similar lag at low vesicle concentrations, as shown in Figure 2b. To validate the use of turbidity as a quantitative measure of agglutination, [3H]EYL vesicles containing 10 mol % LC were agglutinated with various amounts of RCA, and the turbidity (A450) was measured following a 1-h incubation at 24 °C and vigorous vortexing to break up large aggregates. The suspensions were centrifuged at 23000g for 20 min (24 °C), and the radioactivity in the clear supernatants was measured. Figure 3 is a plot of counts per minutes in the precipitate (obtained by subtraction) as a function of turbidity after a 1-h incubation. The plot is linear (r = 0.987) and extrapolates to the origin, indicating that turbidity at 450 nm is directly proportional to the mass of material agglutinated.

The agglutination of EYL/LC vesicles can be reversed by the addition of sugars which compete with the glycolipid in the vesicles for sugar-binding sites on the lectin. In Figure 4 is shown the effect of the addition of lactose and galactose on the turbidity of lectin-agglutinated vesicle suspensions. The rate and extent of deagglutination are dependent upon sugar concentration, as shown for lactose (the more effective of the two
sugars). Electron microscopy of lactose-deagglutinated vesicle suspensions shows only small particles, verifying that lectin-induced agglutination does not result in extensive vesicle fusion (Figure 5).

The pH dependence of the initial velocity of agglutination of EYL vesicles containing 10 mol % LC is presented in Figure 6. The agglutination exhibits a broad pH maximum, ranging from pH 5.4 to 8.7. The decrease in agglutination at pHs below and above this range is presumably due to conformational changes in the protein, since neither EYL nor LC exhibit a pKa in this range. The initial velocity of agglutination undergoes a significant variation with the phospholipid/glycolipid ratio, as illustrated in Figure 7. No agglutination is observed at LC contents less than 5 mol %, with minimal agglutination observed at 5 mol %. At higher LC contents, the initial velocity of agglutination increases with increasing LC content.

The temperature dependence of the initial velocity of agglutination of EYL vesicles containing 10 mol % LC was determined and is shown in Figure 8. Agglutination velocity increases with increasing temperature, reaching a maximum at 25 °C, and decreases at higher temperatures. When a sample containing EYL and RCA is incubated at 37 °C (a temperature at which minimal agglutination is observed) and then decreased in temperature to 25 °C, the turbidity increases.

If this sample is returned to 37 °C, a decrease in turbidity is observed, indicating deagglutination. However, if vesicles are agglutinated at 25 °C, they do not deagglutinate when the temperature is lowered to 4 °C and remain as a stable aggregate at this temperature for several days. In order to determine whether the temperature dependence of agglutination is related to some property of the EYL/LC vesicles, the vesicles were studied by differential scanning calorimetry. DSC thermograms of sonicated EYL vesicles containing 10 mol % LC are presented in Figure 9. The temperature dependence of agglutination is superimposed on the calorimetric heating run. A broad transition is observed, which spans the temperature range 0–32 °C, with a single peak at 17 °C on heating. The calorimetric transition coincides with the ascending portion.
AGGLUTINATION OF LIPID VESICLES

(5-25 °C) of the temperature profile of agglutination.

RCA was unable to agglutinate EYL vesicles containing 20 mol % bovine spinal cord cerebroside (galactocerebroside) at 25 °C, even though this glycolipid possesses the requisite terminal galactose for RCA binding. It appears that the terminal galactose must be located at least two sugar residues out from the ceramide backbone to support vesicle agglutination. EYL vesicles which lack glycolipid are not agglutinated or fused by RCA.

Discussion

The development of model systems for the study of cell-surface phenomena is a necessary prerequisite for separation of the various effects involved in ligand binding to cell surfaces and in cell-cell interaction. The phospholipid/glycolipid vesicle system described in this paper allows the study of the interactions of a lectin with a simple model membrane in the absence of the cellular energetic machinery and cytoskeleton.

When Ricinus communis agglutinin is added to a suspension of sonicated phospholipid/glycolipid vesicles, agglutination of the vesicles into large aggregates occurs, indicated by an increase in the turbidity of the suspension. The possibility that this turbidity increase represents irreversible fusion of vesicles to form larger particles is eliminated by the observation that the process can be completely reversed by the addition of saturating amounts of lactose. This deagglutination is evidenced by a decrease in the turbidity to the level observed before addition of lectin and is corroborated by the observation that no large fused vesicles are seen by electron microscopy. The agglutination presumably occurs via RCA cross-bridges between glycolipid molecules on different vesicles. Since RCA is divalent (Olsnes et al., 1974; Poder et al., 1974), one RCA molecule may be able to bridge two vesicles, or, alternatively, self-aggregated multimers of RCA may form the cross-bridges.

The agglutination of EYL/LC vesicles by RCA exhibits specificity: RCA can agglutinate EYL/LC vesicles but not EYL/cerebroside vesicles or vesicles composed of EYL alone. Space-filling models of galactocerebroside and lectin indicate that when in a bilayer the monogalactosyl moiety of galactocerebroside is positioned at the level of the lectin chain head groups and so may be inaccessible to RCA (Figure 10). The terminal galactose residue of lactosylceramide, on the other hand, projects out above the phospholipid chain head groups and is more accessible to binding by RCA.

The surface concentration and distribution of lactosylceramide in the EYL/LC vesicles would be expected to be important determinants of the extent of RCA-induced agglutination. No agglutination of vesicles containing less than 5 mol % LC occurs, as was also observed by Redwood and Polefka (1976) for wheat germ agglutinin-induced fusion of EYL/ganglioside vesicles. A similar requirement for a threshold concentration of surface receptors has also been reported for antibody-induced agglutination of red blood cells (Hoyer and Trabold, 1970; Tsai et al., 1978). Above 5 mol % LC, the velocity of agglutination of EYL/LC vesicles increases with increasing LC content. This suggests that the formation of stable lectin bridges between vesicles is a function of the surface density of glycolipid receptors. This dependence can be explained by the hypothesis that a stable vesicle aggregate requires more than one attachment point between two vesicles, as has been suggested for lectin-induced agglutination of transformed cells (Nicolson, 1971).

The temperature dependence of agglutination described in Figure 8 indicates that factors other than gross receptor surface concentration are involved in lectin-induced agglutination.

Sonomicated EYL vesicles containing 10 mol % LC undergo a broad thermal transition which spans the temperature range 0-32 °C. EYL alone exhibits a transition at ca. -5 °C. If all the lipid in the vesicles (EYL + LC) is undergoing an acyl chain order disorder transition, the calculated enthalpy (ΔH) for this transition is 1.02 ± 0.12 cal/g. This is an unusually small ΔH for an acyl chain order–disorder transition (Laddbrooke and Chapman, 1969). If, on the other hand, the transition involves only the lactosylceramide, a ΔH of 11.9 ± 2.77 cal/g is calculated, which is a more reasonable enthalpy for an acyl chain order–disorder transition. We infer that, over the temperature range 0-32 °C, the LC in these EYL/LC sonicated vesicles undergoes an acyl chain transition. By analogy with studies of mixtures of phospholipids (Shimshick and McConnell, 1973; Grant et al., 1974), this transition may be accompanied by a lateral phase separation into LC-rich regions at low temperatures (below the transition). Above the transition, a relatively homogeneous distribution of LC in the EYL bilayer would exist. Since DSC cannot provide direct evidence for lateral phase segregation, we are currently studying phospholipid/glycolipid phase behavior by 2H NMR, using deuterated lipids.

The maximum initial velocity of agglutination is observed at a temperature (25 °C) at which the calorimetric transition is almost complete (on heating), while at lower temperatures less agglutination takes place. The failure of RCA to agglutinate EYL/LC vesicles at low temperatures may be due to either (1) the inability of LC to diffuse laterally at temperatures below its acyl chain order–disorder transition or (2) the inability of RCA to bind to the lactosyl head group of LC when the glycolipid is in a laterally segregated surface. In such a state lateral interactions between the glycosyl head groups could serve to prevent binding.

The decrease in agglutination with increasing temperature above 25 °C may not be related to the thermal behavior of the EYL/LC vesicles. RCA is also able to agglutinate human serum low-density lipoprotein (LDL) (Yau A., Curatolo, W., Small, D. M., and Sears, B., manuscript in preparation) and the temperature dependence of LDL agglutination shows a similar decrease at temperatures greater than 25 °C (and a plateau below 25 °C). This decreased agglutination observed
in both systems may be due to a reversible temperature sensitivity of the lectin. Preliminary quasiliastic laser light-scattering studies of RCA indicate that the lectin may undergo a small conformational transformation at about 25°C (Young, C. Y., personal communication), while UV difference spectroscopy reveals no large temperature-dependent changes (Curatolo, W., unpublished observation).

In summary, we have described a model membrane system which exhibits characteristics similar to those observed for lectin-induced cell-cell agglutination: specificity and crypticity. We have shown that such characteristics do not necessarily rely on cellular energetic machinery or the presence of a cytoskeleton, although we would certainly not understare the importance of these components in cell-surface phenomena such as capping. A recent fluorescence photobleaching recovery study of bilayer lipid membranes has reached similar conclusions (Wolf et al., 1977). Furthermore, we have shown that the physical state of the glycolipid receptor in the EYL/LC model system plays an important role in the lectin-induced agglutination. The complexity of factors involved in agglutination in this relatively simple model system suggests that the molecular basis of agglutination in natural systems is quite complicated. Therefore, a systematic study of model systems should aid in assigning the relative roles of various membrane components and membrane-associated components in cell-ligand and cell-cell interactions.

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Interactions of cholesterol esters with phospholipids: cholesteryl myristate and dimyristoyl lecithin

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Abstract The ternary phase diagram of cholesteryl myristate–dimyristoyl lecithin–water has been determined by polarizing light microscopy, scanning calorimetry, and x-ray diffraction. Hydrated dimyristoyl lecithin forms a lamellar liquid–crystalline phase \(L_n\) at temperatures above 23°C into which limited amounts of cholesteryl myristate (<5 wt.%) can be incorporated. The amount of cholesterol ester incorporated is dependent upon the degree ofhydration of the \(L_n\) phase. Below 23°C dimyristoyl lecithin forms ordered hydrocarbon chain structures \(L_\alpha\) and \(P_\beta\) which do not incorporate cholesterol ester. Comparison with other phospholipid–cholesterol ester–water phase diagrams suggests the following general principles: i) the incorporation of cholesterol ester occurs only into liquid crystalline phospholipid bilayers, ii) the extent of incorporation is temperature-dependent, with increasing amounts of cholesterol ester being incorporated at higher temperatures, and iii) unsaturated cholesterol esters induce increased disordering of the phospholipid bilayers.—Janiak, M. J., D. M. Small, and G. G. Shipley. Interactions of cholesterol esters with phospholipids: cholesteryl myristate and dimyristoyl lecithin. J. Lipid Res. 1979. 20: 183–199.

Supplementary key words atherosclerosis · cell membranes · differential scanning calorimetry · liquid crystalline mesophases · order–disorder phenomena · phase equilibria · polarizing light microscopy · x-ray diffraction

The structural and metabolic interrelationships of systems involved in lipid transport and storage processes are complex. For example, different amounts of phospholipids, cholesterol, cholesterol esters, and triacylglycerides, in association with protein, are transported in specific plasma lipoproteins through the bloodstream (1) and high concentrations of these lipids are found in tissues such as the adrenal cortex (2) and ovaries (3). In normal arterial tissue there is an age-related increase in lipid content (4). Of particular importance is the abnormal, localized accumulation of cholesterol esters in cholesterol esters and phospholipids, which results in "fatty streak" intimal lesions. These early lesions may eventually develop into more severe lesions or plaques associated with arterial blockage and atherosclerosis (5–7). Recently, we characterized the interrelationships of the major lipids accumulating in atherosclerotic lesions (8) and have shown that the development of these lesions may be explained in physicochemical terms, at least for their lipid components (8, 9).

A critical part of these studies was the demonstration that unsaturated cholesterol esters have limited solubility in hydrated phospholipid bilayers (10). We have now examined the interaction between cholesteryl myristate and dimyristoyl phospholipid, a system in which the fatty acid component of the two lipids is constant. This, together with our earlier studies, enables us to discuss a general theory for the interaction of these two biologically important lipid classes.

MATERIALS AND METHODS

Materials

Cholesteryl myristate (CM) was obtained from Nu Chek Prep (Elysian, MN) and was determined to be greater than 99% pure by chromatographic methods. Thin-layer chromatography (TLC) showed only a single spot corresponding to cholesteryl ester and gas–liquid chromatography (GLC) revealed only minor contamination (<1%) by other fatty acids. Dimyristoyl lecithin (DML) was synthesized from glycerophosphorylcholine prepared from a choline phosphoglyceride isolated from egg yolk (11) and myristic acid (Nu Chek Prep) by the method of Cubero Robles and Van Den Berg (12). DML was isolated by silicic acid chromatography and determined to be only phosphatidylcholine by TLC, with a fatty acyl

Abbreviations: CM, cholesteryl myristate; DML, dimyristoyl lecithin; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; DSC, differential scanning calorimetry; CE, cholesteryl ester; PL, phospholipid.

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composition consisting of 99.9% myristate as judged by GLC.

**Preparation of mixtures**

Cholesteryl myristate and dimyristoyl lecithin were weighed into a flask and dissolved in chloroform-methanol 2:1 (v/v) to give a bulk solution containing a specific ratio of CM:DML (X% CM:100 – X% DML). Aliquots from each bulk solution were transferred to glass tubes with a central constriction. The solvent was evaporated in vacuo and a predetermined amount of water was added to each dry mixture. Thus, each specific weight ratio of CM:DML was prepared as a series of increasing water concentrations, the composition of each mixture being referred to as a% water: (100 – a%) total lipid, where the ratio of CM:DML of total lipid is given by (X% CM:100 – X% DML).

Rapid equilibration of this mixed lipid system occurs when both components exist in a disordered liquid-crystalline or liquid state. However, equilibration of mixtures of cholesteryl myristate and dimyristoyl lecithin is complicated due to the high temperature of the liquid crystal to isotropic liquid transition of cholesterol ester (85.5°C). In the absence of cholesterol esters, even at moderate temperatures (~50°C), hydrolysis of DML occurs after about 6 hr with detectable traces of lysolecithin being formed (0.2–0.5%). For this reason a modified equilibration procedure was used. After sealing the glass tube, the sample was centrifuged (1000 g) through the central constriction for 5 min at 90°C during which time the CM melted and the sample appeared homogeneous. The sample was then immediately transferred to a second centrifuge operating at 50°C and equilibrated by centrifugation (1000 g) through the constriction for 4–5 hr at this temperature. All samples studied contained 0.5–1.0% lysolecithin. However, of several equilibration methods investigated, this procedure resulted in minimum sample degradation and ensured sample homogeneity and reproducible observations by polarizing light microscopy, scanning calorimetry, and x-ray diffraction. Further, the presence of this small amount of lysolecithin did not affect the phase behavior of hydrated DML as determined by parallel control studies.

Immediately on opening the constricted tube, a well-mixed sample of the mixture was weighed, dried in vacuo, and reweighed to determine the water content gravimetrically. Each mixture was examined by polarizing light microscopy, differential scanning calorimetry, and x-ray diffraction.

**Polarizing light microscopy**

Each sample was placed between a slide and coverslip and immediately examined by direct light and between crossed nicols in a Zeiss standard NL microscope. A gross estimate of the viscosity of the sample was obtained by pressing on the coverslip and deforming the sample. The sample was initially examined at a temperature 2–5°C above the chain-melting transition of hydrated DML and the textural appearance and number of phases were recorded. The sample was then heated to 90°C (heating rate 1–2°C/min) to identify any changes in appearance of the sample, noting in particular the presence of any CM thermal transitions. The sample was then cooled to the starting temperature and repeatedly heated and cooled, noting the reproducibility of these changes. Samples that exhibited only one phase were cooled to -10°C, held at this temperature for 1 hr, and then re-examined in the same fashion, noting any textural difference. A second sample was examined between 5 and 90°C in a similar fashion. To a third sample, water was added at 5°C and heated to determine the temperature at which growth of myelin figures occurred.

**Differential scanning calorimetry (DSC)**

Samples taken for calorimetry (5–10 mg) were hermetically sealed in aluminum pans and placed in a Perkin–Elmer (DSC-2) differential scanning calorimeter. Samples were studied at variable heating/cooling rates from 1.25 to 10°C/min. Each sample was heated from the equilibration temperature to 90°C, cooled to 0°C, and then repeatedly heated and cooled between 0°C and 90°C.

**X-ray diffraction**

The x-ray source was an Elliott GX-6 rotating anode generator using nickel-filtered Cu-Kα radiation, a 200-μm spot, and operating at 40 kV, 40 mA. The x-ray beam was optically focused using modified double mirror (13) and toroidal (14) cameras (Baird and Tatlock, London). Unoriented specimens were contained in a sample holder adapted to either an electrical (range, ambient to 300°C; accuracy ± 2°C) or a circulating solvent (range -15 to 80°C, accuracy ± 0.1°C) variable temperature apparatus capable of operating with either camera. Specimens were contained either between two 50 μm Mylar windows (specimen thickness 2 mm) or in thin-walled capillary tubes (internal diameter 0.7 mm). The diffraction patterns were recorded using flat film cassettes with Ilford Industrial G photographic film.

**RESULTS**

**Microscopic examination**

*Cholesteryl myristate.* At 25°C, cholesteryl myristate forms a rigid birefringent texture containing sphero-
lites (Fig. 1a). The sample remains unchanged in appearance until 70°C when homeotropic droplets form out of the birefringent continuum. On depressing the coverslip at 72°C, the sample flows and transforms to the smectic mesophase textures (Fig. 1b). At 79.5°C, the sample transforms to the fine birefringent textures of the cholesteric phase (Fig. 1c). At 85°C, there is a rapid loss of birefringence and the sample is completely isotropic by 86.0°C. If the sample is cooled from 90°C, these same liquid crystalline transitions and textures are observed with slight supercooling (~1°C). On further cooling of the sample, spherulite growth is observed at ~50°C. This temperature lies at the upper limit of the temperature range where nucleation is homogeneous (15). The textures observed here are similar to those reported by Barrall, Porter, and Johnson (16). The melting behavior of cholesteryl myristate was not influenced by the presence of water and the two components were immiscible in all proportions.

Dimyristoyl lecithin–water. We have established those structural changes associated with the thermal transitions of hydrated DML (17). Hydrated DML undergoes two thermal transitions, a broad low enthalpy "pre-transition" prior to the sharp first order "chain-melting" transition. Below the pretransition, a one-dimensional lamellar \(L_{\beta}^d\) phase is observed with the hydrocarbon chains extended and tilted with respect to the normal to the plane of the lipid bilayer. The pretransition is associated with a structural transformation to a two-dimensional \(P_{\beta}\) phase consisting of lipid lamellae distorted by a periodic ripple with the hydrocarbon chains remaining tilted. At the chain-melting transition, the hydrocarbon chains assume a liquid-like conformation and the one-dimensional lamellar \(L_{\alpha}\) phase is formed.

All mixtures of hydrated DML exhibit the same textural features at water contents >10%. At 5°C, DML appears as a waxy, highly birefringent "viscous neat" texture (18) as shown in Fig. 1d. On heating the sample, no change in appearance or birefringence occurs at the pretransition temperature (11°C). At the chain-melting transition (23°C), a marked decrease in the viscosity of the sample occurs, accompanied by a loss of birefringence and the appearance of homeotropic areas and focal conic spots of positive sign of birefringence, i.e., the "neat" textured phase. On continued heating, the neat texture assumes a well-defined network of "oily streaks" (ref. 18, see Fig. 1e). On heating to 90°C, the sample becomes more homeotropic with a further loss in birefringence.

In mixtures containing between 12 and 25% water, a significant decrease in the viscosity occurs at temperatures decreasing with increasing water content, from 35°C at 15% water to a limiting value of 25°C at 25% water. If water is added to the sample at 5°C, no water penetration or myelin figure growth occurs. If the sample is then heated, water penetrates at the sample edge at ~2°C below the softening temperature (25°C) and myelin figure growth occurs within 2°C of the softening temperature (see Fig. 1f). In mixtures containing >40% water, no penetration or growth of myelin figures was observed on addition of water, indicating that water was already present as a separate phase. Thus, the neat textured phase is associated with the structure \(L_{\alpha}\) and viscous neat with \(P_{\beta}\) or \(L_{\beta}\).

(2.79% CM:97.21% DML) 20% water. At 35°C, the sample appears as a single phase (Fig. 2a) and exhibits a birefringent neat texture (Fig. 2b). As the sample is heated to 90°C, a progressive loss in birefringence occurs. If the sample is cooled to ~10°C and maintained at this temperature for 1 hr and then examined at 5°C, the sample exhibits a viscous-neat texture as observed for DML, but a second phase of small, highly birefringent angular grains is now present. On heating the sample from 5°C, a marked decrease in the viscosity occurs at 30°C and the sample assumes a neat texture. At 70–72°C, the birefringent angular grains melt to form droplets and are thus associated with cholesteryl myristate. No other changes were observed on heating to 90°C. If the sample is cooled from 90°C to between 40 and 50°C, and maintained at this temperature for several hours, the droplets slowly disappear and the sample eventually appears as one phase. If water is added to a sample at 5°C and then heated, water penetration occurs at 30°C and myelin figure growth at 32°C.

(2.79% CM:97.21% DML) 50% water. At 30°C, the sample contains three phases, a birefringent neat textured phase, cholesteryl myristate crystals, and water. As the sample is heated to 90°C, a progressive loss in birefringence occurs and large homeotropic areas are present. Between 70 and 72°C, the birefringent crystals melt to form homeotropic droplets which become birefringent between 78 and 80°C and exhibit a negative sign of birefringence. At 85°C, the droplets lose birefringence and become isotropic. At 87°C, the sample assumes a weakly birefringent neat texture (Fig. 2c). On cooling, birefringent droplets reappear abruptly at 84°C (Fig. 2d). This birefringence is lost at 78°C (Fig. 2e). Crystals of CM do not form spontaneously from the homeotropic droplets, but after about 1 hr at 25°C crystals are present. The transitions observed are reproduced on subsequent heating and cooling of the sample. If the sample is held at ~65°C on cooling from 90°C, the droplets appear to decrease in size after several hours but two phases remain. At 5°C, a birefringent viscous-neat textured
Fig. 1. Microscopically observed textures of CM (a,c) and DML containing 25% water (d,e,f); crossed polarizers, magnification 112×. For CM, (a) crystalline phase at 25°C, (b) smectic phase at 74°C, (c) cholesteric phase at 82°C. For DML, (d) "viscous neat" texture at 5°C, (e) "neat" texture at 60°C. In (f), addition of water produced the growth of myelin figures into the water phase at 28°C; sample photographed at 32°C. CM was recrystallized in situ. The heating rate was 1–2°C/min.

phase is present together with crystals of CM. On heating the sample, a marked decrease in the viscosity is observed at 25°C and the sample transforms to a neat textured phase. No myelin figure growth is observed at any temperature on addition of water since, at 50% water, the mixtures are always fully hydrated.

In general, mixtures of CM–DML–water exhibited the phase behavior of the separate components, i.e.,
hydrated dimyristoyl lecithin and cholesteryl myristate. The transition from viscous-neat to neat texture for mixtures containing CM was observed within a degree of that found for DML at comparable water contents. Growth of myelin figures occurred at comparable temperatures providing water was not present as an excess phase. When a cholesterol ester phase was present, transitions from crystal to smectic and, except at very low ester contents, smectic to cholesteric and cholesteric to isotropic were observed. The liquid-
crystalline transitions temperatures (smectic to cholesteric and cholesteric to isotropic) were lowered by \( \sim 1^\circ C \) in mixtures containing CM in comparison to CM alone. In addition, the domain size of the excess cholesterol ester phase appeared to diminish at higher temperature. The crystallization of ester from the liquid–crystalline phase was similarly dependent on domain size, crystallization of ester occurring more rapidly at higher ester content.

At 37°C, a number of changes were observed as the water content was increased in mixtures containing 2.79% CM:97.21% DML (Fig. 3). At low water contents (<16.4%), two phases were observed, a neat textured phase together with crystals of CM. Upon further increasing the water content (19.7% water), crystals of CM were no longer present. At higher water content (31.2%), two phases were again observed. All mixtures containing greater than 39.7% water contained three phases, the third phase being excess water. Similar behavior was observed in mixtures containing 1.71% CM:98.29% DML (Fig. 3).

In mixtures containing 5.5% CM:94.5% DML, no mixture produced only a single phase. Similarly, all mixtures containing greater than 5.5% CM:94.5% DML (10.7:89.3, 16.0:84.0, and 30.9% CM:69.1% DML) contained either two phases, neat and CM crystals, or three phases, the additional phase being water. At 10°C and 20°C, no mixtures showed only one phase. All mixtures contained either two phases, viscous-neat and CM crystals, or three phases, the additional phase being water.

**Differential scanning calorimetry**

Typical DSC thermograms obtained for mixtures of CM:DML containing 30% water are shown in Fig. 4b, c, and d. Mixtures containing 1.7% CM:98.3% DML (Fig. 4b) exhibit the same thermal behavior as hydrated DML alone (Fig. 4a). No thermal transitions arising from cholesteryl myristate could be detected (Fig. 4e). It is important to note that, at maximum sensitivity of the instrument where the crystal to
Smectic transition of CM can be detected with 0.01 mg of sample, no detectable transition was observed (sample contained 0.072 mg of CM). In mixtures containing larger amounts of ester (16.0:84.0 and 30.9% CM:69.1% DML), the thermal transitions of DML were similarly unaffected. However, in these mixtures, thermal transitions arising from cholesteryl ester were clearly observed (Fig. 4c and d).

For a series of mixtures containing a constant lipid ratio (e.g., 16.0% CM:84.0% DML) but varying in water content, the observed thermal transitions can be compared to those observed for hydrated DML and cholesteryl myristate. As shown in Fig. 5, this series of mixtures exhibits similar hydration dependence as observed for hydrated DML. Both the thermal pretransition and chain-melting transition are observed for mixtures containing CM in the same temperature and hydration range described for DML. The enthalpies of these transitions are unaffected. Similar observations have also been made for mixtures containing 2.8:97.2, 5.5:94.5, 10.7:89.3, and 30.9% CM:69.1% DML. These observations together with the microscopy data indicate that little or no cholesterol ester is associated with the phases of hydrated DML present at temperatures below the chain-melting transition, leaving these thermal transitions unaffected by the presence of cholesterol ester.

In addition to hydrated DML, the thermal transitions arising from cholesteryl myristate were observed and could be correlated with the microscopy results (Fig. 3). No mixtures containing one phase by microscopic observation exhibited thermal transitions associated with cholesteryl myristate, i.e., thermal transitions in the temperature range 70–90°C. For mixtures that exhibited thermal transitions in this temperature range, the transitions were similar to those of CM. In general, for all mixtures examined the crystal to smectic transition temperature, as identified microscopically and observed by DSC, was unchanged. The smectic to cholesteric and cholesteric

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**Fig. 4.** DSC thermograms of mixtures containing DML and CM. (a) Hydrated DML containing 35% water; (b,c,d) mixtures of CM:DML containing 30% water; (e) cholesteryl myristate. Mixtures contain 1.7% CM:98.3% DML (b); 10.7% CM:89.3% DML (c); 30.9% CM:69.1% DML (d).

**Fig. 5.** Comparison of thermal transition temperatures observed for mixtures containing 16.0% CM:84.0% DML varying in water content with those found for hydrated DML and cholesteryl myristate. The transition temperatures for hydrated DML are taken from references 17 and 24; the dashed line represents the onset temperature of the transition, the solid line represents endotherm maxima. The hollow symbols represent the onset temperature of the transition observed in the mixtures containing CM and the solid symbols, the endotherm maximum.
to isotropic transition temperatures were ~1°C lower in the ternary mixtures in comparison to CM. However, even in pure cholesterol esters, these liquid crystalline transitions were somewhat variable, suggesting that these minor differences may not be significant.

In contrast to the transition enthalpies associated with DML in the ternary mixtures, the transition enthalpies associated with cholesterol myristate are not identical to those of CM. In particular, the crystal to smectic transition enthalpy is 30–40% lower than predicted. Since mixtures that are only one phase by microscopy did not exhibit thermal transitions associated with cholesterol ester, it is reasonable to conclude that portions of the cholesterol ester incorporated into the liquid crystalline phase of hydrated DML, \( L_n \), do not contribute to the enthalpy of the thermal transitions of CM. Thus, the transition enthalpy is a measure of the “free” or unincorporated cholesterol myristate that forms an excess phase.

Assuming that the transition enthalpy of the crystal to smectic transition is unchanged (\( \Delta H = 18.68 \text{ cal/g} \)), the fractional part of the total mass of CM present in the sample giving rise to the transition can be determined from the area of the transition endotherm. The remaining amount of CM present in the sample is a measure of the incorporation of cholesterol myristate into the liquid crystalline phase of DML. From this value, the composition of the mixture in terms of the maximum amount of CM incorporated into the lamellar liquid crystalline phase at ~70°C can be determined. For mixtures containing 10.7:89.3, 16.0:84.0, and 30.9% CM:69.1% DML, the reetermined compositions indicating the extent of incorporation of CM into hydrated DML are plotted in Fig. 6. Maximum incorporation of CM occurs between the constant lipid ratios of 2.5:97.5 and 5.0% CM: 94.5% DML. As the water content increases, the amount of cholesterol ester decreases, but even at high water contents, where water is present as an excess phase, some cholesterol ester remains incorporated. These results are consistent with the microscopy observations that suggest increased incorporation of cholesterol ester with increasing temperature.

These conclusions are derived from measurements obtained from the excess phase of cholesterol myristate at a temperature corresponding to the crystal to smectic transition (70.5°C). However, the microscopy data suggest that the values shown in Fig. 6 are indicative of CM incorporation at lower temperatures, e.g., the equilibration temperature (50–60°C).

X-ray diffraction

X-ray diffraction patterns were obtained for ternary mixtures containing CM in the same temperature range where the structures \( L_\beta, P_\nu, \text{ and } L_n \) have been observed for hydrated DML (17). At temperatures below the thermal pretransition and above the chainmelting transition, hydrated DML exhibits several low-angle reflections arising from a one-dimensional lamellar lattice. At temperatures intermediate between the two transitions, several reflections in addition to these lamellar reflections occur and arise from a two-dimensional oblique lattice, \( P_\nu \) (Table 1). X-ray diffraction patterns obtained for ternary mixtures containing cholesterol myristate exhibit the same characteristic reflections at a given temperature (see Table 1). In the presence of low amounts of ester, only diffraction patterns associated with the structures of hydrated DML are observed. At higher concentrations of ester, additional reflections associated with CM are also observed.

Similarly the wide-angle diffraction data from mixtures containing cholesterol myristate can be compared to hydrated DML. The ternary mixtures exhibit the same temperature dependence of the wide-angle x-ray diffraction as hydrated DML in the absence of ester, i.e., at 37°C, a broad diffuse band of intensity centered at \((4.6 \text{ Å})^{-1}\); at 20°C, a broad intensity maximum centered at \((4.2 \text{ Å})^{-1}\); and at 10°C, a sharp maximum at \((4.2 \text{ Å})^{-1}\) together with a broad band at higher values of \( s = (2 \sin \theta / \lambda) \). These x-ray intensity profiles have previously been interpreted (17, 19) as arising from packing variations of the hydrocarbon chains in the \( \beta \) conformation at 10°C and 20°C, or the structure \( L_n \) at 37°C.

At 85°C, a diffraction pattern consistent with the
structure \( L_\alpha \) is observed (Fig. 7a). At 60°C, a similar diffraction pattern is observed with an additional diffraction maximum present at \( s = (33.6 \, \text{Å})^{-1} \) (Fig. 7b), corresponding to that observed for the smectic phase of CM (Fig. 7c). Since homogeneous nucleation of pure CM does not occur above 50°C (15), the presence of the smectic phase at this temperature is not surprising. At 37°C, the crystalline powder diffraction pattern of CM is superimposed on the diffraction observed for the \( L_\alpha \) structure (Fig. 7d). By comparison with the diffraction pattern for CM (Fig. 7f), the contributions to the diffraction pattern from the two phases can be distinguished. Finally at 10°C, the diffraction pattern from \( L_{\beta'} \) together with CM is observed (Fig. 7e).

In general, mixtures containing \( \geq 5.5\% \) CM exhibited diffraction from the liquid crystalline phase associated with hydrated DML and crystalline cholesterol myristate. In mixtures containing <5.5\% CM, diffraction associated with cholesterol myristate could not be observed. Thus, the presence of an excess phase of cholesterol myristate observed by microscopy could not be directly verified by x-ray diffraction at these low ratios.

In the ternary mixtures containing cholesterol myristate, the structures \( L_{\beta'} \), \( P_{\beta'} \), and \( L_\alpha \) of hydrated DML are observed at the appropriate temperatures. From the microscopy and DSC results, we concluded that below the DML chain-melting transition little or no ester was incorporated into the structures \( L_{\beta'} \) and \( P_{\beta'} \). Thus, the composition of the diffraction phase in the ternary mixtures is determined only by DML and water and the cholesterol myristate content can be subtracted from the total composition of the ternary mixture. The corrected composition of the mixtures containing cholesterol myristate is that of hydrated DML and, for a given water content, the value of the interlamellar repeat, \( d \), and cell parameters \( a \) and \( b \) in the case \( P_{\beta'} \) should be equal to the value obtained for the binary system in the absence of CM. For comparison we will refer to the values obtained from the ternary system as \( d(\text{DML} + \text{CM}) \) and from the binary system as \( d(\text{DML}) \). In Table 2, values of \( d(\text{DML} + \text{CM}) \) for mixtures containing variable amounts of cholesterol myristate (1–36%) are compared with the values of \( d(\text{DML}) \).

Table 1. Comparison of indexed reflections for mixtures containing (0% CM:100% DML) 28.8% water and (50.8% CM:69.13% DML) 18.7% water at 20°C

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\( ^a \) Subtracting the cholesterol myristate content from this mixture and correcting the composition of (0% CM:100% DML) 26.2% water.
\( ^b \) The observed reflection at 50.8 Å corresponds to the 002 reflection of CM; the 004 reflection overlaps with the 2-3 reflection from \( P_{\beta'} \).

The microscopy and DSC results indicate that incorporation of CM occurs at temperatures above the chain-melting transition. From the microscopy data at 37°C, an approximate phase boundary was defined (see Fig. 3). Using this phase boundary, the molar ratio of DML:CM at maximum incorporation can be determined as a function of water content and, for a given ternary mixture, the amount of incorporated cholesterol ester can be obtained. Again, the composition can be corrected and the composition of the diffracting phase determined (see Table 2). The limited incorporation of cholesterol myristate into the phase \( L_\alpha \) does not significantly alter the interlamellar
Fig. 7. X-ray diffraction patterns obtained for a mixture containing 31.8% water (5.5% CM:94.5% DML) at 85°C (a), 60°C (b), 37°C (c), 10°C (d), and cholesteryl myristate at 74°C (e) and 37°C (f).

repeat, \( d(DML + CM) = d(DML) \). This observation is discussed in detail below.

**Structural effects of incorporation**

Model electron density distributions for the CM–DML bilayer structure were determined for different orientations of the ester and the calculated continuous Fourier transforms were compared with the DML bilayer structure model. The bilayer structure was assumed to contain 5 wt% cholesterol ester (molar ratio DML:CM = 15.1), an upper limit of incorporation. Three orientations of cholesterol ester are considered.

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\( a \) At 10 and 20°C, the composition of the ternary mixtures is corrected assuming no ester is incorporated.

\( b \) At 37°C, the composition of the ternary mixtures is corrected assuming incorporation of CM occurs, the degree of incorporation (molar ratio DML:CM) determined from the microscopy results at 37°C.

\( c \) Mixtures that exhibited the phase \( L_p \) at 20°C.

\( d \) Does not represent maximum incorporation.

\( e \) No cholesteryl myristate incorporated.
The recent crystal structure determination of cholesteryl myristate by Craven and DeTitta (20) has provided structural data for the calculation of these model distributions. In model I, the cholesterol ester is in a “kinked” molecular geometry with both the myristate chain and the steroid ring located perpendicular to the bilayer plane and roughly parallel to the phospholipid molecule. Only the ester linkage protrudes into the polar region. In model II, cholesteryl myristate is “extended perpendicular” to the bilayer plane, again roughly parallel to the phospholipid molecules. In model III, CM is oriented “extended perpendicular” to the long axis of the phospholipid and located in the center of the bilayer. The transforms obtained for the three models and variations on each model are superimposable on that observed for hydrated DML (the details of these calculations and the Fourier transforms are given in the Appendix). Thus, with this limited incorporation of CM, analysis of the x-ray diffraction data does not yield any direct information on the molecular geometry of cholesterol ester in the phospholipid bilayer. However, some general features of cholesterol ester–phospholipid interactions are apparent. In Table 2, we have shown that the interlamellar repeat of the mixed lipid system at maximum incorporation is nearly identical to that observed for hydrated DML at the same water content. The same is true for the bilayer thickness, $d_1$, and the surface area per lipid molecule $S_1$ (see Table 3). However, the number of molecules per unit surface area, $N_1$, is on the average ~1% higher in the mixed lipid system compared to DML. This is in contrast to that observed for cholesteryl linoleate–egg lecithin–water (10) and suggests that, if cholesteryl myristate is located at the lipid–water interface, a molecular geometry occupying a surface area less than that of DML might be favored.

We can further examine the three models (Fig. 8) by calculating the surface area of DML in the mixed lipid system. From the molecular volumes and lengths calculated from CM (see Appendix), the calculated surface area for the cholesterol moiety is 40.8 Å² and that of the myristate chain is 28.2 Å². Assuming all the cholesteryl myristate is at the surface for model I (kinked), the surface area of cholesterol ester is $S(CM) = 79$ Å² and for model II (extended perpendicular), $S(CM) = 40.8$ Å². In model III (extended parallel) $S(CM) = 0$, since no cholesterol ester is assumed to be at the surface. As shown in Table 3, the calculated values of $S(DML)$ for the three models are similar, with the largest deviation from the value of hydrated DML occurring for model III. Furthermore, the small differences in the values obtained are insufficient to distinguish between models I and II.

Fig. 8. Three generalized molecular orientations of cholesteryl myristate incorporated in the DML bilayer structure. In model I, CM assumes a “kinked” molecular geometry; in model II, “extended perpendicular” to the bilayer plane; model III “extended parallel” to the bilayer plane. The details of the calculation and assigned values of $x$ and $\rho(x)$ are given in the Appendix.

Thus, in contrast to the cholesteryl linoleate–egg lecithin–water system, the differences in the structural parameters found for cholesteryl myristate–dimyristoyl lecithin–water are very small, perhaps due to similarities in the molecular lengths of the two lipid components.
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For hydrated DML, d(DML) is the interlamellar repeat distance, d(DML + CM) is the bilayer thickness, and S₁(DML) is the mean molecular area. For the mixed lipid system, d(DML + CM) is the interlamellar repeat distance, d(DML + CM) is the bilayer thickness, and S₃(DML + CM) is the mean molecular area. S₁(DML) is calculated assuming all the cholesteryl myristate is at the interface and occupies an area of 79 Å². S₄(DML) is calculated assuming all the cholesterol ester is at the interface and occupies an area of 40.8 Å². S₃(DML) is calculated assuming no cholesterol ester is at the interface.

*No cholesterol ester incorporated.

**DISCUSSION**

**Cholesteryl myristate–dimyristoyl lecithin–water**

Hydrated dimyristoyl lecithin undergoes two thermal transitions in the temperature range 0–90°C, a thermal pretransition at 11°C (at water concentrations ≥20%), and the chain-melting transitions at 23°C (at all water contents ≥20%; this transition occurs at higher temperatures at <20% water). At temperatures below the thermal pretransition, hydrated dimyristoyl lecithin forms the structure Lα. The pretransition is associated with the formation of the structure Pγ. At the chain-melting transition a lamellar liquid crystalline structure, Lα, forms.

Cholesteryl myristate does not interact with dimyristoyl lecithin below the chain-melting transition in either the Lα or Pγ phase. In the Lα phase only limited but variable incorporation of cholesterol ester occurs depending on the temperature, higher amounts of ester being incorporated at higher temperatures. Maximum incorporation of ester into the phase Lα also varies with the degree of hydration. Polarizing light microscopy results at 37°C indicate that the approximate limiting molar ratio of CM:DML increases from 1:36 to 1:20 upon increasing the water concentration from 16 to 22% (w/w). Between 22 and 25% water, the CM:DML ratio remains at ~1:20. At higher water concentrations, a progressive decrease in the ester content occurs until essentially no cholesteryl myristate remains incorporated at maximum hydration of dimyristoyl lecithin (40% water). The DSC results indicate that increased amounts of cholesterol ester are incorporated into the Lα phase at higher temperatures, e.g., 70.5°C. Although it is not clear how the incorporation of CM changes between 25 and 70.5°C, we will assume that, at the sample equilibration temperature (50–60°C), the sample composition is similar to that at 70.5°C. In addition, DSC shows that at water contents between 15 and 25% (w/w) the limiting molar ratio of CM:DML in the phase Lα is 1:17. Increasing the water content results in a progressive decrease in the limiting molar ratio to 1:26 at 40% water. The lamellar phase is the only phase (except at low water contents, <5%) in which all three components mutually interact. All mixtures of the three components having compositions outside the one-phase zone produce additional phases of cholesteryl myristate or water, or both.

Using model electron density distributions, x-ray diffraction analysis could not differentiate between the mixed lipid system and DML–water in the absence of ester, due to the limited incorporation of cholesteryl myristate (see Appendix). Calculated structural parameters for this mixed lipid system are nearly identical to DML–water in the absence of ester and are consistent with strong similarities in the molecular parameters of cholesteryl myristate and dimyristoyl lecithin.

**Interrelationships in cholesterol ester–phospholipid systems**

This study, together with the cholesteryl linoleate–egg lecithin–water (10) and cholesteryl linoleate–egg lecithin–water (21) systems, allows for comparison of three different cholesterol ester–phospholipid–water systems, the cholesterol esters varying in chain length or degree of unsaturation. Plotted in Fig. 9 is a comparison of maximum incorporation of cholesterol ester into the Lα phase of DML as a function of hydration. For both cholesteryl lin-
olenate–egg lecithin–water (C<sub>18:3</sub> system) and cholesterol linoleate–egg lecithin–water (C<sub>18:2</sub> system) the data shown are at 23°C. In these mixed lipid systems, the chain-melting transition of hydrated egg lecithin (~7°C) is unaffected by the presence of cholesterol ester. Relative to this transition these studies were carried out at a temperature 30°C higher (T + 30). For cholesterol myristate–dimyristoyl lecithin–water (C<sub>14:0</sub> system), the results are presented at 37°C (T + 14) as determined from the microscopy data and at ~T + 35°C as determined from DSC. In order to compare these systems directly, the data in Fig. 9 are plotted as the molar ratio of cholesterol ester to phospholipid (CE:PL) on the ordinate and the molar ratio of water to total lipid (cholesterol ester + phospholipid) on the abscissa.

The C<sub>14:0</sub> system (at T + 14) and the C<sub>18:2</sub> (T + 30) system exhibit very similar behavior over all hydration values, both systems incorporating no cholesterol ester at a water:lipid ratio of 28:1. The somewhat higher values of CE:PL for the C<sub>18:2</sub> system may be a temperature effect; the results for this system were obtained at a higher temperature relative to the chain-melting temperature of each lecithin (T + 30 for the C<sub>18:2</sub> system and T + 14 for the C<sub>14:0</sub> system). This observation would indicate that the temperature-induced disorder allows for increased incorporation of cholesterol ester.

The increased incorporation of cholesterol myristate in the C<sub>14:0</sub> system at higher temperatures supports this observation, as does the increased incorporation of cholesterol linolenate in the high-temperature phases of anhydrous egg lecithin (22).

Incorporation of cholesterol ester in the C<sub>18:3</sub> system exhibits a different hydration dependence. Although the molar ratios of CE:PL are quantitatively different, the C<sub>18:3</sub> system exhibits similar hydration dependence to that of the C<sub>14:0</sub> system at higher temperature (T + 35), above a water:lipid ratio of 8:1. At a water:lipid ratio of ~28:1, both systems incorporate comparable amounts of cholesterol ester (CE:PL 1:30). This observation suggests that the degree of unsaturation may be equivalent to that of thermal disordering.

To probe further the significance of chain unsaturation of cholesterol ester, the structural parameters for the C<sub>18:2</sub> and C<sub>18:3</sub> systems at the same temperature are compared (23°C, i.e., T + 30). Although the molecular orientation of cholesterol ester in the phospholipid bilayer could not be determined, the data were more consistent with the molecular axis of cholesterol ester being oriented perpendicular to the plane of the bilayer. Regardless of the orientation, incorporation of cholesterol ester could affect the mean molecular surface area calculated from the x-ray diffraction data either indirectly by being located entirely in the apolar region of the phospholipid bilayer, or directly by being located at the lipid–water interface. These effects will be reflected in the average number of lipid molecules per unit surface area, N<sub>i</sub>. By comparison of this value for the mixed lipid system, N<sub>i</sub>(CE + PL), with the values obtained for lecithin alone, N<sub>i</sub>(PL), the net difference ΔN<sub>i</sub> can be calculated for the C<sub>18:2</sub> system and C<sub>18:3</sub> system at the same temperature. A negative difference indicates that fewer molecules per unit surface area are present as compared with lecithin–water in the absence of cholesterol ester. This net difference in molecules per unit surface area is shown in Figure 10 for the C<sub>18:2</sub> system and C<sub>18:3</sub> system. Both systems show a negative deviation, the C<sub>18:2</sub> system by a maximum of only ~3%. This further suggests a disordering effect related to the degree of unsaturation of the cholesterol ester.

The significance of these deviations can be examined utilizing the calculated structural parameters d<sub>i</sub> and S<sub>i</sub>. Assuming the values of these parameters were obtained for lecithin–water in the absence of

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<sup>3</sup> The degree of incorporation of CM at the equilibration temperature of 50–60°C (i.e., ~T + 35°C) is assumed to be similar to that recorded at 70.5°C by DSC (see Results).
cholesterol ester, the temperature at which these parameters would be observed for lecithin–water alone may also be calculated. The surface areas obtained for the \( C_{18:2} \) system are essentially the same as would be obtained for egg lecithin–water at 23°C (T + 10°C). For the \( C_{18:3} \) system, the calculated values of surface areas for this mixed lipid system at T + 50°C are the same as would be obtained for egg lecithin alone at T + 34°C. These calculations suggest that for the \( C_{18:3} \) system the small differences in \( \Delta N_i \) are probably not as significant as those found for the \( C_{18:2} \) system.

These observations suggest the following regarding the interactions of cholesterol ester with phospholipids: 1) the incorporation of cholesterol ester is a temperature-dependent phenomenon requiring the existence of a thermally disordered liquid crystalline structure; 2) the extent of incorporation is also temperature dependent; increasing amounts of cholesterol ester can be incorporated at higher temperatures relative to the chain-melting transition; 3) the structural effect of thermal disordering of phospholipid bilayers (bilayer thickness, surface area, etc.) may also be induced by the presence of highly unsaturated cholesterol esters. Although this effect is not readily apparent for the \( cis \) di-unsaturated ester, cholesteryl linolate, it is observed for cholesteryl linolate \( (cis,cis,cis) \) \( 9,12,15 \)-octadecatrienoate. Similar behavior may also occur for other highly unsaturated cholesterol esters.

### Biological relevance

Biological membranes appear to contain little or no cholesterol ester. Recently, Zambrano, Fleischer, and Fleischer (23) measured the lipid content of specific rat liver subcellular fractions. Expressed in terms of weight percent cholesterol ester (based on CE + PL only), values in the range of 1.3 to 3.7% were found. The amount of cholesterol ester based on either total lipid or only CE + PL content is comparable to that determined in the model cholesterol ester–phospholipid systems described here, especially when the marked heterogeneity of lipids present in these subcellular fractions is considered. These observations strongly suggest that the limited presence of cholesterol esters in membrane systems is explained in terms of simple lipid–lipid interactions.

The authors wish to acknowledge helpful discussions with Dr. C. R. Loomis. This research was supported by U. S. Public Health Service grants HL18623, GM00176, and HL07291.

*Manuscript received 30 January 1978; accepted 24 July, 1978.*

### REFERENCES


**APPENDIX I**

**CALCULATION OF MODEL ELECTRON DENSITY DISTRIBUTION FOR CHOLESTEROL MYRISTATE–DIMYRISTOYL LECITHIN BILAYERS**

Model electron density distributions were calculated assuming 3- and 4-level square well models (including solvent).

The electron content and molecular volume of dimyristoyl lecithin was determined from the partial specific volume ($\bar{\rho}_v = 0.983$ at 37°C), the molecular weight (M = 678), and the number of electrons (n = 374). Using the values obtained by Reiss-Illusson and Luzzati (Al), the average volume of the hydrocarbon chains in the liquid-like conformation was determined and, by difference, the volume of the polar head group. From these values the electron densities of the hydrocarbon chain and polar head group region were calculated (Table A-1). The electron density of the water layer was assumed to be 0.334. The width of each level was calculated from the interlamellar repeat value obtained for hydrated DML (~30% water).

To obtain electron density values for cholesteryl myristate...
assumed to be the same as that for the phospholipid. Thus, the cholesteryl myristate molecule was divided into four segments: the side chain, steroid nucleus, ester linkage, and hydrocarbon chain. The length of the steroid nucleus and ester linkage was determined from the crystal structure by projection onto the molecular axis. The values of molecular length and electron density are summarized in Fig. A-1 and Table A-1, respectively.

Three generalized models of molecular orientation of cholesteryl myristate incorporated into the bilayer structure of hydrated DML were considered (see Fig. 8 above). Model I assumes that 100% of the CM side chain, steroid nucleus, and hydrocarbon chain are located in the hydrocarbon region of DML and 100% of the ester linkage is in the polar region. A variation on Model I also tested assumes that 100% of the CM side chain and hydrocarbon chain and 65% of the steroid nucleus are located in the hydrocarbon region of DML, and that 35% of the steroid nucleus and 100% of the ester linkage are located in the polar region.

Model II assumes that 100% of the steroid side chain and 14% of the steroid nucleus reside in the polar region and 86% of the steroid nucleus and 77% of the hydrocarbon chain of CM reside in the hydrocarbon region of DML. An extra level located near the center of the bilayer exists and

<p>| Table A-2. Contributions of molecular components to electron density of various electron density levels of hydrated dimyristoyl lecithin bilayer structure |</p>
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<td>20.7</td>
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<td>581.8</td>
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<td>150.9</td>
<td>543.0</td>
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<td>498.6</td>
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<td>CM Ester Linkage</td>
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<td>8.1</td>
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<td>310</td>
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<td>755.5</td>
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<td>100</td>
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<td>CM Steroid Side Chain</td>
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<td>100</td>
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<td>51.2</td>
<td>0.067</td>
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<td>498.6</td>
<td>1.0</td>
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<td>48.3</td>
<td>173.8</td>
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<td>51.2</td>
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<td>51.2</td>
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<td>CM Hydrocarbon Chains</td>
<td>23</td>
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<td>173.8</td>
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<td>45.1</td>
<td>162.2</td>
<td>23</td>
<td>48.3</td>
<td>173.8</td>
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</tr>
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198  Journal of Lipid Research  Volume 20, 1979
**TABLE A-3.** Model electron density distributions of CM in DML bilayers

<table>
<thead>
<tr>
<th></th>
<th>DML</th>
<th>Model I</th>
<th>Model II</th>
<th>Model III</th>
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<tr>
<td>$x_1$ (Å)</td>
<td>0</td>
<td>0</td>
<td>2.34</td>
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<tr>
<td>$\bar{n}$</td>
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<td>88.9</td>
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<tr>
<td>$\bar{\nu}$</td>
<td>171.4</td>
<td>311.4</td>
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</tr>
<tr>
<td>$(x_2)e^{-x}/Å^3$</td>
<td>0.281</td>
<td>0.285</td>
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<tr>
<td>$x_3$</td>
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<td>9.9</td>
<td>9.9</td>
<td>9.9</td>
</tr>
<tr>
<td>$\bar{n}$</td>
<td>210</td>
<td>216.8</td>
<td>164.4</td>
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<tr>
<td>$\bar{\nu}$</td>
<td>775.5</td>
<td>775.5</td>
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<td>$(x_3)e^{-x}/Å^3$</td>
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<td>17.9</td>
<td>17.9</td>
<td>17.9</td>
</tr>
<tr>
<td>$\bar{n}$</td>
<td>164</td>
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<td>$(x_4)e^{-x}/Å^3$</td>
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<td>0.468</td>
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<td>$x_5$</td>
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<td>26.25</td>
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<tr>
<td>$(x_5)e^{-x}/Å^3$</td>
<td>0.334</td>
<td>0.334</td>
<td>0.334</td>
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</table>

* See Fig. 8, above.

The percent occupancy (%T) of cholesteryl myristate in each level was determined from the molecular lengths given in Table A-1. The mole ratio of DML:CM used in the calculation was 15:1 (5 wt%), from which a multiplicity factor, $\kappa$, was determined to calculate the partial electron content ($\bar{\eta}_e$) and partial volume ($\bar{\nu}_e$) for each component occupying a given level. The calculated parameters for the three models are summarized in Table A-2 and the values of $x$ and $\rho(x)$ for each model are summarized in Table A-3.

The continuous transforms were computed after direct integration of the generalized transform

$$F(s) = \frac{2}{d} \int_0^{d/2} \cos(2\pi s x) dx$$

integrating from 0, $x_1$, $x_2$, ..., $d/2$ and are shown in Fig. A-2.

**REFERENCES**


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**Fig. A-2.** Calculated Fourier transform $F(s)$ for the models of the organization of cholesteryl myristate in a dimyristoyl lecithin bilayer. a) Dimyristoyl lecithin without cholesterol esters; b) Model I; c) Model II; d) Model III. The models and calculations are as described in the text. The transforms have been displaced vertically by one amplitude unit for clarity.
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Key words. Total bile fistula, simulated enterohepatic circulation, taurocholate infusion, bile salt dependent flow, bile salt independent flow.

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108
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Key words. Total bile fistula, simulated enterohepatic circulation, taurocholate infusion, bile salt dependent flow, bile salt independent flow.

Introduction

Clinical cholestasis associated with the administration of phenothiazine tranquillizers is of particular importance in view of the widespread use of these drugs. Chlorpromazine hydrochloride (CPZ.HCl)* (10-3-dimethylaminopropyl)2-chloro-phenothiazine hydrochloride) induces cholestatic jaundice in 1-2% of treated patients [1-4]. In prospective studies, abnormal liver function tests, increased bromosulphalein retention [2, 5] and morphological abnormalities in liver biopsies [2] have been observed in nearly 50% of patients taking CPZ.HCl for prolonged periods of time, all of which suggest a direct hepatotoxic effect of the drug. Furthermore there is experimental evidence indicating a direct impairment of the biliary secretory apparatus of the liver; thus, altered bile canalicul and reduction of bromosulphalein excretion have been observed in CPZ.HCl treated rats [6, 7], and the drug inhibits bile flow both in dogs [8] and in isolated perfused rat liver preparations [9]. Others, however, have found no effect of CPZ.HCl on the plasma clearance of bromosulphalein in mice [10], or on the rate of bile flow in rats [11, 12].

Nonetheless, based on circumstantial evidence, hypersensitivity to the drug has generally been implicated as a mechanism for CPZ.HCl associated cholestatic jaundice [1, 13, 14]. Others have suggested that CPZ.HCl cholestasis could be attributed to spasm of the sphincter of Oddi: [15] alteration of bile viscosity [16], precipitation of biliary glycoproteins [17], and inhibition of...
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PHYSICAL CHEMISTRY OF BILE

A Biophysical Approach to Cholesterol Transport from Tissues to Bile

Physical Chemistry of Bile: Lipid Solubility
M. C. Carey

Quasielastic Light Scattering Studies of Micelle Formation and Cholesterol Precipitation in Model Bile Solutions
N. A. Mazer, M. C. Carey, R. F. Kwasnick, and G. B. Benedek

Biliary Cholesterol Supersaturation, Metastability and Micronucleation
R. T. Holzbach and K. R. Holan

The Nucleation of Cholesterol Monohydrate Crystals in Model Bile Solutions
E. W. Toor, D. F. Evans, and E. L. Cussler

Discussion
D. F. Evans, Chairman

PATHOGENESIS OF CHOLESTEROL GALLSTONE FORMATION

The Role of Supersaturated Bile and Other Factors in the Genesis of Cholesterol Gallstones in Man
R. H. Dowling

Gallstone Formation in Animals
W. van der Linden

The Role of the Gallbladder in Gallstone Formation
E. A. Shaffer

Studies on the Pathogenesis of Cholesterol Gallstone Formation: Alterations of Bile Acid Transport and Liver Surface Membrane Lipid Structure by Estrogens
F. R. Simon, M. Gonzalez, and R. Davis

Discussion
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A BIOPHYSICAL APPROACH TO CHOLESTEROL TRANSPORT
FROM TISSUES TO BILE

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INTRODUCTION

Cholesterol is not only absorbed from the intestinal tract
but also synthesized in many tissues of the body. Further, many
tissues utilize plasma cholesterol under certain conditions as a
source of membrane cholesterol. Thus, since tissues take up and
synthesize cholesterol mechanisms must exist to remove cholesterol
from tissues when they have accumulated an excess. Necessarily
tissue cholesterol must first move into the intracellular space,
then into the plasma and finally be delivered to the liver where
at least some of it can be secreted into bile as biliary cholesterol
or bile acid (Figure 1). In the past we have concentrated on the
physical chemistry of the biliary lipid micelle (1-5) and have
realized the importance of phospholipids in the process of carrying
free cholesterol into bile. In this paper, we will deal with the
physical chemistry of complexes which probably allow cholesterol
to be removed from tissues and be returned to the liver.

There are a large number of studies indicating that "high
density lipoprotein" (HDL) are the important aggregates involved
in the removal of cholesterol from tissues. First, a number of
tissue culture experiments show that cholesterol can be removed
from tissue culture cells by HDL or by recombinations of HDL with
phospholipids (6-8), or by phospholipids themselves (9). Second,
there are many epidemiologic experiments suggesting that high
levels of HDL protect against cholesterol accumulation in athero-

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Fig. 1 Removal of cholesterol from tissues. Cholesterol removal must involve the movement of cholesterol from tissues into the extracellular space and then into the plasma compartment. Here the interactions of different lipoproteins become important. Finally, some of the cholesterol must be taken up by the liver where it is in turn excreted into bile as biliary cholesterol or bile acid.

sclerosis (e.g. 10). In the past, we have suggested that the structure of the lecithin bile salt micelle which carries cholesterol out in the bile has some interesting similarities to discoidal HDL (11), a particle which may well be the primitive HDL structure in plasma responsible for cholesterol removal from tissues.

What is HDL? As isolated from the plasma, it is a small particle containing 50% protein and about 50% lipid. Considerations of the physical-chemical properties of the lipids and proteins involved as well as x-ray scattering and other studies, strongly suggest that plasma HDL is a quasi spherical particle in which the phospholipid, protein and some of the free cholesterol form the surface and the less polar lipids (cholesterol ester and triglyceride) form the core. The most important lipids in HDL are the phospholipids, free cholesterol and cholesterol esters. The plot of the physical-chemical relationships of these is shown in Figure 2 (12,13). The composition of HDL as found in the plasma has been plotted on Figure 3. The composition shows that the HDL falls in a zone where one would expect two phases to exist - a phase made up of cholesterol ester (and triglyceride), and a phase made up of phospholipids. Free cholesterol would be expected to partition between these phases. X-ray scattering data of Atkinson and Shipley (14,15) confirmed the presence of these two micro-domains within the particle (Figure 3).
**Fig. 2** Phase diagram of a three-component system showing interactions of cholesterol (C), phospholipid (PL), and cholesterol ester (CE) in excess water at 37°C and 1 atmosphere pressure. (Adapted from Katz S.S., Shipley G.G., Small D.M. J Clin Invest 58: 200-211, 1976).

Zone I contains a single phospholipid lamellar liquid crystal phase into which 33 per cent cholesterol and 2 per cent cholesterol ester can be incorporated. Zone II also contains a single phase, composed of liquid or liquid-crystalline cholesterol ester. Zone III contains both the cholesterol-ester and phospholipid phases. In Zone IV, a third phase, cholesterol-monohydrate crystals, is also present. Schematic molecular representations of the phases are shown near each apex of the triangle. The irregular lines denote the phospholipid molecule, the solid symbols the cholesterol molecule, and the solid, tailed symbols cholesterol ester.
Fig. 3 Mean compositions (±S.E.M.) of human plasma HDL plotted on the phase diagram. The composition of HDL as plotted indicates that two lipid phases should be present. The schematic model for HDL showing the phospholipids and protein on the outside and the core having cholesterol ester. The two separates phases are: 1) the core and 2) the surface phospholipid free cholesterol.

Leu-Glu-Asp-Lys-Ala-Arg-Glu-Leu-Ile-Ser-Arg-Ile Lys-Gln-Ser 20 25
Leu-Glu-Ser-Ala-Lys-Met-Glu-Ile-Pro-Phe-Ser-Glu-Thr-Phe-Gln-Lys-Val-Lys-Glu-Lys-Leu 35 40 45 50
Leu-Glu-Ser-Ala-Lys-Met-Glu-Ile-Pro-Phe-Ser-Glu-Thr-Phe-Gln-Lys-Val-Lys-Glu-Lys-Leu 35 40 45 50

Fig. 4 The sequence of apolipoprotein C-I. (Adapted from Herbert et al[20]). Amino acid sequence of apolipoprotein C-I (apo C-I). The enclosed regions are those that may fold to produce amphipathic helices.
Fig. 5 αHelix of residues 33-53 from apo C-I showing amphiphilic nature of helix. (Adapted from Herbert et al.). Space-filling (CPK) models of amphipathic helical regions of apo C-I. The helical regions are shown with their axes oriented parallel to the plan of the page and the amino-terminal end toward the top of the page. Below are schematic representations used in following figures.
Structure of Apoproteins

Since HDL is about 50% protein we need to consider the role of the protein in the structure of HDL. Important concepts of the structure of the proteins came from the sequences of several different apoproteins. Of the major apoproteins so far sequenced (A peptides and C peptides), most contain sequences which can form amphiphilic helices, that is, alpha helical segments, one side of which is hydrophobic, the other side of which is hydrophilic (16,22). (See Figures 4 and 5). The major apoprotein on HDL is apo A-I. It has a molecular weight of about 28,000 and 12 sequences about 20 - 35Å long, which are amphiphilic alpha helices (20-21) (Figure 6). In simplified form, the structure is rather like a series of amphiphilic rods connected with structure breaking or beta turn forming amino acids. While the definite tertiary structure of A-I, or any of the other soluble apoproteins for that matter, are not known, it is clear that they have rather unique structures among proteins in general. In dilute solutions, these amphiphilic helices presumably attempt to hide the hydrophobic parts of the helices from the water producing a pseudo globular state.

Fig. 6 (Adapted from Small D.M.11). Schematic sketch of A-I apoprotein from HDL. The spiral arrangements consist of alpha-helical regions separated by helix-breaking amino acids. One side or portion of the helix is made up predominantly of hydrophobic amino acids, while the other side is made up of hydrophilic amino acids. In the native state, A-I in solution is globular protein in which hydrophobic areas try to minimize their interaction with water by associating hydrophobically. Thus, this native structure of the apoprotein is like a string of amphiphilic molecules bound together predominantly by hydrophobic association into the thermodynamically most suitably structured globule.
protein. However, from thermodynamic studies they are not very successful at hiding hydrophobic groups and the stability of the protein in aqueous systems is very low compared to other globular proteins (23-24). Furthermore, one might expect that these proteins would self-associate in water to form aggregates (25-27) which are somewhat analogous to micelles. Recently, it has been shown that the A-1 peptides form aggregates of up to 8 monomers depending upon the concentration (27).

Fig. 7 (Adapted from Tall et al27). Electron micrographs, negatively stained with sodium phosphotungstate, pH 7.3. ApoHDL was incubated with multilamellar liposomes of DML, under N₂ at 38° for 16 h. Mixtures contained a, 57% DML; b, 37% DML; c, 24% DML. Samples contained about 1 mg of lipid/ml. Magnification of originals was about x 100,000; bars indicate 1000 Å.
Interaction with Phospholipids such as Lecithin

Lecithin forms myelin figures which, on a molecular basis, are biomolecular leaflets of lecithin interspaced with layers of water (28). If apo A-I or a mixture of HDL apoproteins (apo HDL) is added to a cloudy suspension of myelin figures of lecithin, it acts as a detergent and within a period of hours this solution becomes clear (23, 29). This is analogous to the interaction of bile salts on lecithin (1-4). When these suspensions are looked at in the electron microscope by negative staining they are found to be a series of small discoid-like particles (Figure 7). As the lipid to protein ratio increases the disks become larger up to a limit (Figure 8). From calorimetric (23, 29), x-ray scattering (30), and other physical-chemical studies the structure of the particle appears to be a bimolecular disk of phospholipid encircled on its perimeter by the apoprotein. The most reasonable structure would have the hydrophobic parts of the amphiphilic alpha helices of the A-I and other apo peptides in contact with the hydrophobic chains of the lecithin. The hydrophilic half of the alpha helix would contact the aqueous system. The analogy of these disks and the disk-like bile salt lecithin micelles formed at high lecithin

![Graph](image)

Fig. 8 (Adapted from Tall et al 27). Diameter of apo-HDL/DML recombinants as a function of DML/apoHDL molar ratio. The individual values shown are mean ± S.D. for 200 to 300 particles measured from six to eight micrographs from three to four grids for each sample. Dimensions were obtained from disks stacked on edge in rouleux. The line shown is the least square regression line, y = 1.37x + 56. The correlation coefficient was 0.94 and the standard error of the estimate of y on x was 8.05.
concentrations is shown in Figure 9. From the physical interactions of a specific lecithin, for instance dimyristoyl lecithin (DML) and apo A-1 (29), one can construct a rough binary phase diagram of the interaction of these two lipids in excess water as a function of temperature. Such a diagram is shown in Figure 10.

Such disk-like apoprotein - phospholipid micelles or aggregates can, like bile salt - lecithin micelles, solubilize a certain amount of cholesterol. The maximum amount of cholesterol solubilized by disks of cholesterol and dimyristoyl and other lecithins appears to be about 1 cholesterol to 2 lecithins (31). Tall and Lange concluded from calorimetric studies that the cholesterol interacted only with the lecithin and was excluded from the protein interface (31). Such lecithin-cholesterol-apoprotein disks can be formed using a number of apoproteins as well as a variety of different phospholipids. What do these disk-like micellar apoprotein-cholesterol apoprotein-lipid aggregates have to do with high density lipoprotein?

**Fig. 9** (Adapted from Small D.M.11). The analogy between the bile salt-lecithin mixed micelle and the A-1 lecithin disk.
Fig. 10 DML - apo A-1 binary "phase diagram". Mixtures were formed in an excess of water. This diagram represents a cut of the ternary system, water - apo A-1 - DML, as a function of temperature. On the left, pure DML undergoes a chain melting phase transition at 23°C. When large amounts of DML are present, the A-1 and DML appear to separate into two phases, one a phase made up of disks saturated with DML, the second the excess DML phase. At about 80% DML/20% A-1, a single phase zone begins made up of disks of DML with A-1; these disks are large on the order of 160 Å diameter. Furthermore, the large disks tend to be thermodynamically the most stable, in terms of the A-1 protein denaturation (29). As the ratio of protein to DML is increased the disks become smaller and at a certain ratio of DML to A-1, the excess DML became present. The upper line represents the denaturation of A-1 in the complex. The temperature of denaturation is much higher when A-1 is present on the disk compared to pure A-1 in solution. The lower line represents the chain melting transition of DML. It is increased slightly (23 to 25°C) in the disk compared to the myelin figures.
The Role of LCAT

In 1935, Sperry (32) described a reaction in plasma which increased the amount of esterified cholesterol as plasma sat at 37°. Glomset rediscovered this reaction and believed that it was important in the formation of esterified cholesterol in plasma (33). He termed this enzyme lecithin-cholesterol acyltransferase (LCAT) and more clearly defined its characteristics. Shortly after, Norum and others (34-38) described a patient with lecithin cholesterol acyltransferase deficiency. These patients had lipoproteins in their serum which had not been altered by the LCAT enzyme. Interestingly, the majority of their HDL were not spherical but disk-like particles. They contained appropriate apoproteins, lecithin and free cholesterol, but no cholesterol ester. It was suggested that these were the newly formed HDL which were secreted into plasma by the action of LCAT. Indeed, the incubation of patients' discoidal particles with LCAT converted them to spherical particles similar to those of normal plasma high density lipoproteins. Interestingly, apo A-I was found to be a co-factor for the LCAT reaction. A schematic representation of this reaction is shown in Figure 11.

If the newly secreted form of HDL is in fact a disk, where do these disks come from? First, some quantities of discoidal HDL aggregates appear to be secreted. The rat liver (39) secretes discoidal HDL with a high concentration of E apoprotein, an apoprotein with similar physical properties to A-I. The intestine also secretes disks (40). The disk-like particles secreted by the intestine are high in apo A-I. A second source of HDL may occur from a process different from secretion. The process involves a second enzyme, lipoprotein lipase, and its interaction with chylomicrons from the intestine and perhaps very low density lipoprotein (VLDL) from the liver. These lipoproteins (chylomicrons and VLDL) are large triglyceride containing lipoproteins which are secreted from the intestine or from the liver, pass to the peripheral tissues where they are bound to the endothelium and where lipoprotein lipase hydrolyses the triglyceride. Chylomicrons, appear to be secreted with large quantities of the A peptides. In fact the surface is largely covered with A peptides, B peptides (the major apoprotein of LDL) and phospholipids. Once in the plasma they absorb C peptides and some free cholesterol. After the triglyceride has been partly removed by the lipoprotein lipase, the remnant chylomicron seems to have lost most of the C and A peptides while retaining the B peptides. Redgrave has shown that the chylomicron remnant particle is cholesterol-rich and rapidly taken up by the liver (41). What happens to the surface of the chylomicron during the lipoprotein lipase reaction? Redgrave and Small were able to show that about 80% of the phospholipid on the surface of the chylomicron as well as newly synthesized soluble peptides (presumably the A peptides) were transferred en masse into the HDL.
fraction 30 minutes after the chylomicron injection into the hepatectomized rat (40). Furthermore, we were able to show that one hour's chylomicron production produced a quantity of new HDL equivalent to about half to a third of the total high density lipoprotein phospholipid pool. Chemical analysis of the surface of the chylomicron indicated that the newly formed high density lipoproteins are very rich in soluble A peptides, phospholipids, but rather low in free cholesterol and therefore would be excellent acceptors for cholesterol from cell membranes. A similar mechanism appears to involve VLDL metabolism, although in that case the major peptides released would be the C and E peptides (43,44). Thus, we believe that a major quantity of new HDL is derived from the surface components of chylomicrons and VLDL.
These concepts bring us full circle. We know that cholesterol is excreted into bile chemically as bile acids and free cholesterol. The exact mechanism for the secretion of lecithin and cholesterol is not clearly known (45) but presumably it involves bilayered leaflets of lecithin and cholesterol interacting with bile salts in such a way that by the time they reach the gallbladder they have formed mixed bile salt-lecithin-cholesterol micelles. These micelles are secreted into the intestine, where they aid in absorption of fat and cholesterol. Absorbed dietary cholesterol is esterified and secreted in chylomicrons. Thus, esterified cholesterol stays with the chylomicron remnant and is rapidly taken up by the liver. Part of the surface of the chylomicron comes off during lipolysis of the triglyceride and enters the HDL system. This system includes: bilayered disks of phospholipid and apoproteins, spherical high density lipoproteins and any intermediates which may occur. This system is able to scavenge cholesterol from tissue, interact with LCAT to form cholesterol esters and return cholesterol to the remnant or other lipoproteins which transport it back to the liver for the excretion into bile.

The gut and the chylomicron coming from the gut appear to be important sources of not only A peptides (46) but also of newly formed HDL. Thus, is it possible that the enterohepatic circulation and secretion of cholesterol and lecithin into the gut can influence the production of chylomicrons in such a way that it can increase or decrease the production of new HDL. These new findings suggest that the enterohepatic circulation of bile acids as well as the absorption of the lipids may play an important role in governing the formation of new HDL and thus tissue removal and return of cholesterol back to the liver. Clearly, this field is worthy of more detailed research.

ACKNOWLEDGEMENTS

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CURRENT CONCEPTS
Plasma High-Density Lipoproteins

Alan R. Tall, M.B., B.S.,
and Donald M. Small, M.D.

Why all the recent excitement about high-density lipoproteins (HDL)? Because of the strong inverse relation between plasma levels of HDL and mortality from cardiovascular disease.1,2 Increased serum levels of HDL protect against atherosclerosis, and decreased levels predispose to it.3 In this review, we discuss the measurement and normal levels of HDL, their chemical composition, the properties of the molecules making up HDL and our view of the structure of different HDL particles. The present evidence suggests that HDL or its precursors are produced by both the intestine and the liver. Furthermore, HDL precursors are both directly secreted into plasma and derived from the surface components of chylomicrons and very-low-density lipoproteins (VLDL). We propose a possible molecular mechanism by which HDL precursors can be produced from the surface of chylomicrons or VLDL during their catabolism in peripheral tissue. Finally, we discuss potential clinical implications of enhanced or defective chylomicron and VLDL catabolism.

We measure HDL by centrifuging plasma at a density greater than the lipoproteins but less than the other plasma proteins (1.21 g per milliliter). The buoyant lipoprotein fractions are recovered, and the high-density lipoproteins are separated by further centrifugation or by selective precipitation of the non-HDL lipoproteins by means of heparin and manganese chloride. HDL concentrations may be expressed in terms of the total amounts of lipoprotein in milligrams per deciliter, or by the concentration of cholesterol contained in the HDL fraction. The normal quantities for HDL cholesterol are shown in Table 1.4 Men have fairly constant values whereas women appear to reach peak levels in the fifth decade. Women taking estrogens have about 15 per cent higher levels of HDL cholesterol than those on combined estrogen-progesterone therapy or on no medication.5

Composition and Probable Structure of HDL Particles

What are HDL? HDL are very small aggregates of lipids and proteins that circulate in the lymph and blood plasma. To understand their structure and function, we need to understand the properties of the different lipids and proteins that make up HDL. About 90 per cent by weight of the protein of human HDL are the A apoproteins, A-I and A-II. The A-I accounts for 70 per cent of the total protein, and A-II for about 20 per cent; other peptides, called C and E peptides, make up the remaining few per cent. A-I has a molecular weight of about 28,000 whereas A-II and C peptides are smaller. The major lipids of HDL are cholesterol esters, cholesterol and phospholipids, principally lecithin (Fig. 1). Cholesterol esters are completely insoluble in aqueous systems and separate as an oily phase. Both phospholipid and free cholesterol are insoluble in water, but together they form a lamellar structure composed of bilayers of phospholipid and cholesterol separated from each other by layers of water, as shown in Figure 1. Thus, in a pure aqueous system these lipids would separate as large globs of insoluble lamellar or oily structures. How, then, are they packaged in HDL as particles so tiny — approximately 10 nm (100 Å) — that they pass easily from plasma to extracellular fluid? The secret is in the apoproteins. These remarkable peptides are protein detergent molecules capable of solubilizing phospholipids and cholesterol in large quantities. In fact, 1 g of HDL apoprotein, or specifically A-I, can solubilize up to 2.5 g of phospholipid in a disklike micelle. Some cholesterol can be dissolved in the phospholipid bilayer (Fig. 1). These structures are analogous to the lecithin-cholesterol-bile-salt micelles found in bile. However, in bile the bile salt is the detergent, whereas the apoproteins are the detergent in plasma. Although apoproteins dissolve phospholipids and cholesterol, they do not dissolve pure cholesterol esters. How, therefore, do these insoluble molecules get into HDL?

The important studies of Glomsø7 and Norum8 on patients with lecithin cholesterol acyltransferase (LCAT) deficiency implicate this enzyme in the production of HDL cholesterol esters. LCAT requires A-I protein as cofactor and catalyzes the reaction

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<th>Table 1. Normal HDL Cholesterol Values.*</th>
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* Reproduced in part from Fredrickson et al with the permission of the publisher.

thin + cholesterol → cholesterol ester + lysolecithin. Patients lacking LCAT have little or no cholesterol esters, and their HDL are discoidal. The composition and structure of these disks are quite similar to those of disks formed in vitro by incubation of HDL apoproteins with lecithin and cholesterol (see Fig. 1). Incubation of these discoidal lipoproteins with normal plasma or purified LCAT produces cholesterol esters within the particle and converts the disk to a sphere. The physical properties of the lipids determine this change. Both substrates, lecithin and cholesterol, are present in the bilayer, with their reaction group facing the water (Fig. 1). One of the products, lysolecithin, is removed by binding to plasma albumin. The other product, cholesterol ester, is almost entirely hydrophobic and therefore seeks the only hydrophobic environment available — between the fatty acyl chains of the lecithins. Thus, as cholesterol esters are formed, the lecithin-cholesterol bilayer is split by the intruding esters, and the discoidal particle becomes a sphere. Since the cholesterol leaves the surface as cholesterol ester, more cholesterol can enter the particle and can be converted to cholesterol ester until the available source of HDL lecithin is exhausted. The HDL particles isolated from normal plasma are the spherical cholesterol-ester-containing particles. The preponderance of discoidal HDL in LCAT-deficient patients indicates that spherical cholesterol-ester-containing HDL are not directly secreted but are probably derived from the discoidal apoprotein-lecithin-cholesterol precursors by the action of LCAT.

**Probable Sources of Plasma HDL**

If spherical HDL found in normal plasma are derived from lecithin and cholesterol present in bilayers or discoidal aggregates, where are these bilayered precursors produced? HDL precursors appear to come from two sources: from direct secretion of discoidal HDL from liver and intestine into plasma or lymph; and from the surface components of triglyceride-rich lipoproteins such as chylomicrons. In the rat, discoidal HDL is produced by perfused liver and small intestine. The intestine can synthesize A peptides, and intestinal lymph contains discoidal HDL rich in apo-A peptides and lecithin but poor in cholesterol and its esters. The quantity of discoidal HDL secreted into the lymph appears to depend on what is in the digestive tract; however, specific details of how diet affects apoprotein synthesis and intestinal HDL secretion are needed.

Chylomicrons from the intestine and VLDL from the liver are triglyceride-transporting emulsion particles, stabilized by a monomolecular surface film of phospholipid, apoproteins and a small amount of cholesterol. The core contains triglyceride, cholesterol esters and some of the free cholesterol. Chylomicrons and VLDL are partially degraded in skeletal muscle, adipose tissue and other tissues by lipoprotein lipase, an enzyme on the capillary endothelium. Lipoprotein lipase selectively hydrolyzes the lipoprotein triglyceride, forming monoglyceride and fatty acid that are partly taken up by the tissues and partly removed by albumin. As triglyceride is removed, the chylomicron shrinks and is released back into the circulation as a remnant particle that is rapidly removed by the liver.

During the transformation of chylomicron to remnant not only triglyceride but also soluble apoproteins (A + C) and phospholipids are lost from the chylomicrons and are rapidly transferred into the plasma HDL fraction. Fresh chylomicrons isolated from intestinal lymph contain appreciable amounts of A-apoproteins. Fat feeding causes increased intestinal mucosal synthesis of A peptides, increased formation of chylomicrons and a subsequent rise in plasma apoA-I levels; however, chylomicrons allowed to circulate in the plasma for a short time contained no apoA-I, implying rapid transfer of chylomicron apo-A-I mass into HDL. In man, >90 per cent of labeled A peptides of chylomicrons were found in HDL one hour after intravenous injection, and in the rat a similar transfer of radioactivity of newly synthesized chylomicron-soluble apoproteins (mainly A peptides) to HDL was found in 30 minutes.

The transfer of soluble peptides from chylomicrons to HDL is paralleled by a similar rapid transfer of phospholipid in the rat. This transfer may explain

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**Figure 1. Lamellar Bilayered Structures, the LCAT Reaction and Spherical Plasma HDL.**

Above, the different molecules in HDL are shown. Left, phospholipids, such as lecithin and free cholesterol, form lamellar bilayers in aqueous systems, which, in the presence of apo A-I or apo A-II, form small disklike complexes. The apoproteins surround the hydrophobic parts of the phospholipids to render the whole complex soluble. These discoidal apoprotein-lecithin-cholesterol complexes are good substrates for the LCAT reaction, in which cholesterol and lecithin are converted to cholesterol ester and lysleicithin. In the plasma, the lysleicithin formed by the reaction is removed from the particle surface by albumin, whereas the cholesterol ester is insoluble in the interface and partitions into the oily parts of the particle. As the reaction proceeds, the discoidal particle becomes a sphere with a cholesterol ester core. Such spherical particles are consistent with the structure of circulating plasma HDL.
previous observations made in man that HDL phospholipid rises after fat ingestion. Although the case for VLDL is not as clear as that for chylomicrons, C peptides are transferred to HDL during VLDL catabolism, and in vitro lipolysis of VLDL leads to transfer of phospholipid into HDL.

A Possible Mechanism for Formation of HDL Precursors from the Surface of Chylomicrons or VLDL

We can suggest a molecular mechanism for the formation of bilayered HDL precursors from the surface of triglyceride-rich particles, such as chylomicrons and VLDL, because of the following facts. As noted above, phospholipids and some soluble apoproteins are transferred en masse from chylomicrons to HDL in vivo. Lipolysis of VLDL in the perfused rat heart leads to formation of a discoidal HDL particle containing the VLDL phospholipid and VLDL C-apoproteins. Selective delipidation of the chylomicron core in vitro causes the surface components to form phospholipid-cholesterol-A-protein lamellar aggregates appearing as bilayered phospholipid vesicles; similar vesicles appear in the HDL fraction after intravenous injection of chylomicrons in the rat. Folds of lipid bilayer have been observed to arise from the surface of chylomicrons during in vitro lipolysis. Finally, lipoprotein lipase leads to selective removal of triglyceride from chylomicrons and VLDL that causes shrinkage of the lipoprotein core. Figure 2 shows the probable result: shrinkage causes redundancy of the polar surface constituents — phospholipids, unesterified cholesterol, and apoproteins. The increase in lateral pressure occurring in the surface of the particle causes the monolayer to fold into bilayers that protrude from the particle surface. Some of the bilayer may be dissolved as discoidal micelles in association with the detergent A or C apoproteins present in chylomicron surface. However, since there are not enough A or C apoproteins to convert all the bilayer to disks, most of the bilayer fragments probably leave the surface as larger sheets that can seal into phospholipid vesicles (hollow spheres). Subsequently, these sheets and vesicles are converted into spherical HDL by the interaction with the circulating pool of HDL and the action of LCAT.

Since apo A-I is loosely integrated into the spherical HDL particle, plasma HDL can act as a source of apo A-I. Intact HDL releases part of its apo A-I when incubated with model phospholipid membranes or vesicles and leads to the formation of soluble discoidal phospholipid-apoprotein complexes. Thus, the incorporation of chylomicron-derived phospholipid into HDL probably depends on the pre-existing pool of apo A-I in the circulating spherical plasma HDL. As a consequence of losing some of its emulsifier, apo A-I, the spherical HDL particle becomes thermodynamically unstable and could fuse with another lipoprotein.

Since chylomicron remnants are avidly taken up by the liver their fusion with unstable HDL could provide a possible route for transfer of HDL cholesterol ester to the liver.

During the transformation of precursor particles into spherical HDL there is probably a large influx of cholesterol into the HDL fraction. The driving force in this input is the relative deficiency of cholesterol in the chylomicron surface and in the chylomicron-derived precursors of HDL, as compared to other circulating apoproteins and cellular elements, which would result in a chemical gradient for movement of cholesterol into HDL precursors. The LCAT reaction would gradually transform this free cholesterol into esters and thus form spherical HDL (Fig.1). An unsolved problem is the metabolic fate of HDL cholesterol ester. If some is transferred to larger lipoproteins by fusion mechanisms, as suggested above, or by way of a specific cholesterol ester-triglyceride exchange protein, HDL could protect against atherosclerosis by providing a mechanism for transfer of cholesterol from the tissues to the liver. However, HDL may simply transport cholesterol from one tissue to the other. In fact, the exact metabolic link between HDL and atherosclerosis is not yet clearly understood.

HDL FORMATION IN HEALTH AND DISEASE

Since HDL can be formed as a result of lipolysis, HDL formation is probably accelerated when increased flux of triglyceride-carrying lipoproteins is associated with normal or enhanced lipoprotein lipase activity. For example, increased VLDL flux and normal lipase activity occur with alcohol consumption and are associated with increased HDL concentration. Exercise results in increased triglyceride extraction from chylomicrons that is probably associated with increased lipoprotein lipase activity and exercise-increased HDL levels. Since insulin stimulates lipoprotein lipase, diabetic patients receiving adequate insulin therapy should have higher lipoprotein lipase activity and increased levels of HDL as compared to diabetic patients receiving inadequate insulin.

Conversely, impaired peripheral catabolism of triglyceride-rich lipoproteins may cause a partial block in HDL formation. In some hypertriglyceridemic patients (for example, those with congenital deficiency of lipoprotein lipase or uremic patients with deficient hepatic lipase), accumulation of triglyceride-carrying lipoproteins reflects defective lipoprotein catabolism and is associated with low HDL.

In the future, it may be possible to influence HDL levels by altering production of HDL derived by lipolysis. Drugs such as heparin might increase the rate of chylomicron hydrolysis and lead to increases in HDL. In addition, since the small intestine is a source of HDL components, modifications of diet or the enterohepatic circulation may affect HDL production.
Lipoprotein lipase situated in the capillary endothelium hydrolyzes chylomicron triglyceride (lower left). The surface of the chylomicron contains phospholipids, free cholesterol, soluble A and C apoproteins (shown as hatched bodies) and apo B protein (labeled B). In 1, as triglyceride is removed, during lipolysis, the core shrinks, and the redundant surface constituents form lipid bilayer folds projecting from the chylomicron. In 2, a disklike particle could be formed directly from the apoprotein and phospholipids of the surface of the chylomicron. In 3, however, most of the excess material probably comes off as unstable bilayered sheets. In 4, these sheets seal to form vesicles — a more stable form of the bilayer. In 5, the circulating spherical high-density lipoprotein may interact with any of these bilayered lamellar structures (redundant folds, sheets or vesicles), denaturing A-I apoprotein. In 6, any of these bilayered fragments (sheets, vesicles or disks) are good substrates for the LCAT reaction if some A-I apoprotein is present. Thus, the pool of high-density lipoprotein helps to convert the bilayer fragments to new spherical high-density lipoprotein. In 7, however, the apo A-I-depleted spherical HDL has lost a large fraction of its surface and is thus unstable. Such particles could potentially fuse with various other lipoproteins disgorging their cholesterol ester core into such particles. In 8, if fusion occurred with the chylomicron, cholesterol esters, originally present in the circulating HDL, could be transported back to the liver in the remnant.

For instance, if the diet could be modified so that more phospholipids and HDL apoproteins were secreted on chylomicrons, increased levels of chylomicron-derived HDL precursors might be produced, leading to increased levels of circulating HDL. Since interventions that increase HDL levels cannot yet be equated with protection against atherosclerosis, there will be a need for prospective studies evaluating the effect of such interventions on atherosclerotic vascular disease.

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