Solubilization and Localization of Cholesterol Olate in Egg Phosphatidylcholine Vesicles

A CARBON 13 NMR STUDY*

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Co-sonicated mixtures of egg phosphatidylcholine and small amounts (≤4%, w/w) of [carbonyl-13]C cholesterol olate have been studied by 13C NMR spectroscopy at 50.3 MHz. The carbonyl chemical shift from cholesterol olate solubilized in vesicles was 1 ppm downfield from the carbonyl chemical shift of cholesterol oleate present in a separate oil phase. The maximum solubility of the steroid in vesicles determined by chemical analysis of purified vesicles was 1.6 weight % (~2 mol %), in close agreement with the maximum solubility estimated from NMR peak intensity measurements (1.4 weight %). The downfield shift indicates hydrogen bonding of solvent (H2O) molecules with the cholesterol olate carbonyl group, suggesting that vesicle-solubilized cholesterol olate molecules are located in the phospholipid bilayer with the carboxyl group close to the aqueous interface and the sterol ring and fatty acyl chain approximately parallel to the fatty acyl chains of the phospholipid. Such a folded conformation and localization of the carbonyl group at the aqueous interface may facilitate interactions of sterol esters with cholesterol ester transfer proteins and cholesterol esterases.

In plasma, cholesterol is carried in lipoproteins mainly in an esterified form. This cholesterol ester enters cells through the receptor-mediated pathway(s) (1) and then is hydrolyzed in the lysosome to free cholesterol. Cellular free cholesterol regulates the synthesis of cholesterol and certain lipoprotein receptors and is re-esterified into cholesterol olate, which forms intracellular cytoplasmic droplets (1). The physical properties of cholesterol and its long chain acyl esters are remarkably different (2–4). For example, biologically abundant cholesterol esters exhibit phase transitions near physiological temperature in pure lipid systems (3–8), in lipoproteins (9–13), and in some tissue deposits (4, 13–17). In contrast, cholesterol monohydrate is crystalline below 85 °C (18).

The interactions of CE† and cholesterol with polar lipids contrast markedly. Free cholesterol partitions into phospholipid bilayers up to a 1:1 molar ratio (4), but CE, because of their highly nonpolar character, form separate oil or liquid crystalline phases in the presence of phospholipids (4, 6, 8). However, a small percentage of CE partitions into the phospholipid bilayer (4, 6, 8), and an important question from both a metabolic and structural point of view is how the CE molecules are positioned in the bilayer. It was suggested (6, 8) that the CE could either interdigitate parallel to the chains of the phospholipid or lie in the center of the bilayer. X-ray studies could not distinguish between these positions (6, 8) and subsequent attempts using magnetic resonance techniques to determine the localization of CE in multilayers (19–21) and in vesicles (22) have not provided a definitive answer.

This study determines the quantity and localization of the CE in unilamellar phospholipid vesicles. Recently, we have shown that triolein, another relatively apolar molecule, partitions into PC bilayers and that the triolein carbonyl groups interact with water molecules, indicating a surface location of the polar portion of the triglyceride (23). If CE is positioned in bilayers with the carbonyl group at or near the aqueous interface and if the polar group hydrogen bonds with water molecules, a downfield shift of the carbonyl 13C NMR resonance would be expected (23). In the present study, we use 90% isotopically substituted [carbonyl-13]C cholesterol olate co-sonicated with egg PC to detect the 13C carbonyl resonance from small amounts of CE solubilized in vesicles. In contrast to the nitrooxide probe of spin-labeled CE (19), the 13C nucleus is a nonperturbing probe which can be used to study molecular environment as well as molecular motions.

MATERIALS AND METHODS

Egg yolk PC was obtained from Lipid Products, Nutford, England; 90% [1-13]C oleic acid was from Voronics, Inc., Cambridge, MA; and cholesterol olate was from NuChek Prep Inc., Elysian, MN. Purity of the lipids (≥99%) was verified by thin layer chromatography using the solvent system chloroform/methanol/water/acetic acid (95:25:4:1) and Silica Gel G plates and by 13C NMR spectroscopy. 90% [carbonyl-13]C cholesterol olate was synthesized by first converting the 90% [1-13]C oleic acid to its anhydride (24) followed by acylation of cholesterol (25). The cholesterol olate was purified in a silice acid column and the purity was verified by thin layer chromatography (25). The 13C NMR spectrum of the 13C-enriched product was consistent with the expected structure and showed a single carbon resonance at signal-to-noise ratios of >50:1.

Vesicles were prepared by co-sonication of the appropriate proportions of lipids in 1.8 ml of 0.075 M (0.56%, w/v) aqueous KCI for 30–60 min as described for triolein–PC vesicles (29). Unless noted otherwise, the internal sample temperature was regulated at ~35 °C. The compositions are given as percentage of CE by weight of total lipid. Selected samples were fractionated by ultracentrifugation for 4 h at 140,000 × g and 15 °C. The density (ρ = 1.004) of the 0.56% KCI solution allowed clear separation of the oil phase cholesterol olate.

Samples were analyzed for purity and composition following NMR studies. No (<1%) unesterified fatty acid or lyssolecithin was detected by thin layer chromatography. The CE concentration was determined by the method of Rudel and Morris (26) and the PC concentration by a modified Bartlett method (27).

Fourier transform NMR spectra were obtained at 50.3 MHz with a Bruker WP 290 spectrometer system using a 10-mm 13C probe as detailed previously (23). Chemical shifts (δ), line widths (δv), and peak areas were measured as before (23). Spin lattice relaxation times (T1) were measured by a fast inversion recovery method (28) and calculated using a three-parameter fitting routine (29). NOE was measured as the ratio of integrated intensities with broad band decoupling and with inverse gated decoupling (maximum NOE = 3.0) by the technique described by Opella et al. (30).

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† The abbreviations used are: CE, cholesterol ester; PC, phosphatidylcholine; T1, spin lattice relaxation time; NOE, nuclear Overhauser enhancement.
RESULTS

Solvation effects on the $^{13}$C chemical shift of the carbonyl resonance were investigated in organic solvents of differing polarity. Neat cholesteryl oleate exhibits a carbonyl resonance at 171.15 ppm; 8 values increase with increasing solvent polarity (171.29 ppm in heptane, 172.95 in CD$_3$OD, 173.32 ppm in CDCl$_3$, and 174.56 ppm in 1:1 CD$_3$OD/CDCl$_3$). CE and water are mutually immiscible, and the 8 of the $^{13}$C carbonyl resonance of cholesteryl oleate in the presence of H$_2$O at 82 °C is identical with that for neat cholesteryl oleate.

The spectrum of a sonicated 1.0% [carboxyl-$^{13}$C]cholesteryl oleate, 99.0% egg PC mixture is shown in Fig. 1. This spectrum is identical (δ and ν$_{1/2}$ values, relative intensities) to sonicated PC with no added CE, except for the peak at 172.06 ppm in the carbonyl region, which is attributable to the CE carbonyl carbon. The peaks at 173.90 and 173.61 ppm are from PC carbonyl groups on the outside (designated P$_{o}$) and inside (designated P$_{i}$) of the bilayer, respectively (31). The 8 of the CE carbonyl is ~1 ppm downfield from the value for unhydrated CE carbonyl carbons (neat CE) and reflects carbonyl groups which are hydrogen-bonded with water molecules at the aqueous interface of the bilayer surface (see “Discussion”).

T$_1$ and NOE values for carbonyl resonances were measured in spectra of sonicated 1% CE, 99% PC and 2% CE, 98% PC. The T$_1$ values for the P$_{o}$ and P$_{i}$ resonances were 2.2 ± 0.1 and 2.1 ± 0.1 s, respectively. The NOE was 1.8 ± 0.1 for the P$_{o}$ resonance; that for the P$_{i}$ resonance was similar but could not be measured accurately. The T$_1$ of the CE carbonyl resonance was 1.3 ± 0.1 and the NOE was 1.6 ± 0.2.

Using the NOE results, a theoretical carbonyl peak area ratio of CE/PC can be calculated (23). Based on the T$_1$ data, the pulse interval employed in these studies (8.0 s) gave equilibrium intensities for all carbonyl peaks. The peak area ratio from the spectrum in Fig. 1 (0.47) is in good agreement with the theoretical value (0.45). This formulation can also be used to calculate the CE/PC ratio in the vesicle from the peak area measurement (see below).

Samples were prepared with different initial amounts of cholesteryl oleate (0.50, 1.0, 2.0, 3.0, and 4.0%) under otherwise identical conditions to determine the concentration dependence of the CE carbonyl peak intensity, δ, and ν$_{1/2}$. Samples containing >1% CE showed progressively larger amounts of uniform broadening after sonication. After the low speed centrifugation step to remove titanium fragments, these samples had a small amount of turbid floating material which was resuspended prior to NMR analysis. Fig. 2 shows the carbonyl region of the $^{13}$C NMR spectra for four samples with different initial concentrations of CE. All spectra (obtained under identical conditions) were identical except for the region shown. The peak area of the CE carbonyl resonance relative to the area of the PC carbonyl resonances increased with increasing percentage of CE in the starting mixture up to a maximum value in the 2% spectrum and did not increase with higher initial amounts of CE. The carbonyl δ for these samples and for other preparations was the same (± 0.1 ppm) at different concentrations; the ν$_{1/2}$ of the resonance was 10 ± 3 Hz (median ± range).

Following initial NMR analysis, samples containing >1%

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\begin{align*}
\text{1.0 mg CE} & \times \frac{1 \text{ mmol}}{600 \text{ mg}} \\
\text{99 mg PC} & \times \frac{1 \text{ mmol}}{807 \text{ mg}} \\
\end{align*}
\]

\[
= \frac{\text{90} \times 1 \text{ carbonyl/mmol}}{90} \times 1.1 \times 2 \text{ carbonyl/mmol} \times 1.8
\]

where 90 and 1.1 are the percentage of abundance of the $^{13}$C carbonyl nucleus of CE and PC, respectively, and 1.6 and 1.8 are the NOE values of the CE and PC carbonyl resonances, respectively.

Fig. 1. Proton-decoupled $^{13}$C Fourier transform NMR spectrum at 38 °C of sonicated 1.0% [carboxyl-$^{13}$C]cholesteryl oleate, 99.0% PC vesicles at ~75 mg of PC/ml. The spectrum was recorded after 6,900 accumulations using a recycle time of 8.0 s, a 200 ppm spectral width, 32,768 time domain points, and a line broadening of 1.0 Hz. The carbonyl region is shown in the inset; P$_{o}$ and P$_{i}$ designate peaks from phospholipid carbonyl groups on the outside and inside of the bilayer, respectively, and the cholesteryl oleate carbonyl group gives a peak at 172.06 ppm. Other major peaks in the PC spectrum are as indicated; tCH$_3$ represents the fatty acyl terminal methyl groups (see also Ref. 23).

Fig. 2. Carboxyl region of the $^{13}$C NMR spectrum at 38 °C of sonicated [carboxyl-$^{13}$C]cholesteryl oleate-PC at different starting compositions, as indicated by percentage of CE, and at ~80 mg of PC/ml. Except for the number of scans (1800/spectrum) and applied line broadening (1.5 Hz), conditions are the same as in Fig. 1. Accumulation, processing, and plotting conditions are the same for all spectra. The inset shows a schematic diagram of the proposed location of the cholesteryl oleate molecule in the phospholipid monolayer. 1 and 2 signify sn-1 and sn-2 carbonyl oxygens, P signifies the phosphate group, and N is the choline methyl group.

CE were fractionated by ultracentrifugation to obtain homogeneously sized unilamellar vesicles (32). Each sample yielded a small pellet, a thin band of floating turbid material (which was removed by pipetting), and a large clear zone containing vesicles. $^{13}$C NMR spectra obtained for the clear zones were identical (see below) with corresponding spectra of the unfraccionated samples except for a small uniform reduction of all peak intensities. Thus, the CE/PC carbonyl peak area ratio did not change following centrifugation.

Chemical analysis of the fractionated NMR samples showed a maximum incorporation of 1.6 ± 0.2% CE into the vesicle fraction, based on data for the 2, 3, and 4% samples. The CE/PC carbonyl peak area ratio reached an average maximum of 0.61 ± 0.07, based on the NMR spectra of unfraccionated samples containing saturating amounts of CE (2±4%). Using this average CE/PC carbonyl peak area ratio, calculation of the composition of the vesicles gave 1.4% CE, 98.6% PC. A second 4.0% CE sample was prepared by sonicating the lipids above the crystal → liquid transition of cholesteryl
Fig. 3. Carbonyl region of the $^{13}$C NMR spectrum at 52 °C of sonicated 4.0% [carbonyl-$^{13}$C]cholesterol olate, 96% egg PC before (A) and after (B) fractionation by ultracentrifugation. Peaks are designated as in Fig. 1; peak at 171.9 ppm is from surface CE and at 171.35 ppm from oil CE.

Oleate (3) at an internal sample temperature of 52 °C. After low speed centrifugation at 52 °C, the sample was transported (without lowering the temperature) to the NMR probe equilibrated at 52 °C. The carbonyl region of the $^{13}$C NMR spectrum of this sample, with the turbid phase resuspended, is shown in Fig. 3A. The spectrum differs from the 4% spectrum in Fig. 2 in that it contains an additional narrow peak (c$_{1/2}$ = 3 Hz) at 171.35 ppm, close to the 6 of neat CE (171.15 ppm). This peak will be designated as “oil” CE. The hydrated (“surface”) CE/PC peak area ratio was slightly higher than that for the sample prepared at 35 °C; the incorporation measured chemically was also ~30% larger. After cooling to 38 °C (9 °C below the liquid → liquid crystalline transition of cholesterol olate; Ref. 3) the peak was no longer detectable and the remaining spectrum was unchanged (spectra not shown). The sample was then fractionated at 15 °C by ultracentrifugation to float up the excess CE, and the resulting clear zone containing vesicles was re-examined by NMR at 52 °C (Fig. 3B). The oil CE peak was not present in this spectrum and there was a small decrease in signal-to-noise ratios of all resonances. Otherwise, the spectrum was very similar to that for the starting material; in particular, the CE/PC carbonyl peak area ratio was not significantly (<10%) different in the two spectra.

Finally, a 4.0% CE sample was prepared below the transition temperature of pure cholesterol olate. The carbonyl region of the $^{13}$C NMR spectrum showed a single peak at 172.0 ppm for CE as in Fig. 2. When the sample was heated to 54 °C (after dispersing the cloudy material by agitation) and examined by NMR, an additional peak appeared at 171.30 ppm (as in Fig. 3A) from the melted oil phase CE. After cooling to 38 °C, the oil CE peak disappeared, as above.

To determine whether CE in vesicles could crystallize, a 0.5% CE sample was stored for 24 h at 4 °C after initial NMR analysis. A spectrum obtained at 38 °C (12 °C below the melting point of crystalline cholesterol olate) was identical with the original, indicating that the CE in the vesicles was still liquid.

**DISCUSSION**

The chemical shift of the cholesterol olate carbonyl carbon has a strong solvent dependence, showing a downfield shift with increasing hydrogen-bonding capacity of the solvent molecule(s). This solvent-induced deshielding effect has been documented for the $^{13}$C carbonyl resonance of organic molecules (33), phospholipids (31, 32), and triglycerides (23). The $\delta$ values of the CE carbonyl in neat form and in CDCl$_3$ solution are the same as the values reported by Sears et al. (35), who suggested that the observed $\delta$ difference was a solvent-dependent effect.

$^{13}$C NMR spectra of sonicated CE/egg PC mixtures exhibit, in addition to the PC carbonyl resonances, a carbonyl resonance at 172.0 ± 0.1 ppm, almost 1.0 ppm downfield from the carbonyl resonance of neat cholesterol olate, indicating an interaction between solvent (water) molecules and CE carbonyl groups. The CE molecules are therefore located in the vesicle with the carbonyl group in close proximity to the aqueous surface of the bilayer. The $\delta$ difference between unhydrated (neat) CE and hydrated CE in the vesicle is very similar to that found for the $\beta$ carbonyl group of triolein (23). Since the chemical shift difference between neat CE and CE in CDCl$_3$ (2.2 ppm) was larger than the corresponding difference for triolein (1.3 ppm), the fractional hydration of CE carbonyls may be somewhat smaller than that of $\beta$ carbonyls of triolein (fractional hydration ~ 0.5; Ref. 23).

Cholesterol olate was chosen for this study not only because of its major biological importance but also because its stable state is crystalline at 38 °C. Neat cholesterol olate melts from the crystalline phase at 50.5 °C; on cooling, it forms two metastable liquid crystalline phases, the cholesteric phase at 47 °C and the smectic phase at 42 °C, from which crystallization occurs (3). Natural abundance $^{13}$C NMR studies of neat cholesterol olate have demonstrated that resonances from steroid ring carbons and the carbonyl carbon are too broad to detect in the liquid crystalline and crystalline states (7). Therefore, only cholesterol olate which is solubilized in vesicles would be expected to produce an observable carbonyl peak at 38 °C and heating to 47 or 51 °C would be necessary to observe CE in an oil phase. Our experimental results are in accord with these predictions. At 38 °C, only one CE carbonyl peak was observed (downfield from neat CE). The intensity (and $c_{1/2}$) of this peak was not affected by increasing the temperature to 54 °C or by prolonged incubation at 4 °C. The maximum solubility of cholesterol olate in vesicles estimated from NMR peak intensity ratios (1.4%) was in good agreement with the maximum solubility determined chemically (1.6%). Thus, all the CE present in the vesicle is surface-oriented CE, and this CE does not exhibit phase transitions characteristic of cholesterol olate, in accord with expectations based on previous studies (8). When ≥2% CE was present in the starting composition, excess CE partitioned into a turbid phase which could be separated by centrifugation. The $^{13}$C NMR spectrum of an unfraccionated 4% CE sample showed only one CE peak at 38 °C; an additional peak at the $\delta$ characteristic of the carbonyl resonance of neat CE was observed at 52 °C; this peak broadened beyond detection when the temperature was decreased to 38 °C. Thus, the phase behavior of the oil phase CE is similar to that for neat cholesterol olate.

Cholesterol olate solubilized in the vesicle must be present in the PC monolayer with the fatty acyl chain folded over the steroid ring, since the carbonyl group is proximal to the aqueous interface and since the ring and fatty acyl chain are too apolar to be extended into the aqueous medium. The folded conformation of CE (illustrated schematically in the Fig. 2 inset) has been previously suggested from x-ray diffraction (6), electron spin resonance (19), and $^2$H magnetic

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3 The exact localization of the cholesterol olate carbonyl relative to the phospholipid carbonyl groups cannot be determined from our results, and the schematic drawing is intended to indicate the proposed conformation and to suggest only an approximate location.

4 Introduction of a nitroxide group close to the CE carbonyl for spin labeling could increase the probability of the folded conformation.
resonance (20, 21) studies; however, none of these studies provided evidence for the localization of the CE carbonyl group at the aqueous interface.

The $v_1/2$, NOE, and $T_1$ results, which can be related to molecular motions, provide indirect evidence supporting the conformation proposed above. The motions of the CE carbonyl may be different from the PC carbonyls or surface-located triglyceride carbonyls (23) because of the steric hindrance of the steroid ring. The motions of oil phase and surface-located CE carbonyl groups will also differ. The average conformation of a CE molecule in the isotropic liquid is probably an extended one, with the chain projecting away from the sterol ring (36); molecular motions will occur in all spatial directions, though at different rates (7). Motions of surface CE will be highly anisotropic, with preferred motion along the long molecular axis and a much slower isotropic component from vesicle tumbling. The $v_1/2$ is larger, and the $T_1$ and NOE values are smaller, for the surface CE carbonyl resonance than for PC and surface triglyceride carbonyl resonances (23), consistent with a slower and/or more anisotropic reorientation of the CE carbonyl. The $v_1/2$ and NOE values for the surface CE carbonyl resonance are similar to the values for neat CE near the liquid → liquid-crystalline transition temperature, where motions are anisotropic (7); however, the $T_1$ is shorter for the surface CE resonance, possibly indicating that the long rotational axis is different in the two cases and that motion about the long axis is more restricted in the case of surface-located CE.

Our finding that CE is present in a PC bilayer with the CE carbonyl group at the aqueous interface has important implications for metabolism of the sterol esters. The CE molecule will be available for hydrolysis, with the second substrate, H$_2$O, present at the hydrolytic site. Thus, CE hydrolases can act at the aqueous interface, and penetration of the enzyme deep into the hydrocarbon interior should not be necessary. Rapid hydrolysis of cholesteryl oleate in egg PC vesicles containing 1.5 weight % CE has been demonstrated using rat liver (37) and rabbit aortic (38) cholesterol esterases. In addition, CE molecules will be available for transfer to other particles, which occurs via carrier proteins because of the extremely low water solubility of CE (39–41).

The maximum incorporation of cholesteryl oleate into egg PC vesicles is −2 mol % (−1.5 weight %) as measured by both chemical and NMR means. A range of solubilities (0.2 to 5 mol %) has been reported previously for various CE in phospholipid vesicles and liposomes (4, 6, 8, 19–21). The maximum CE solubility may depend on the particular CE, the type of phospholipid dispersion, and the temperature at which samples are prepared (6, 8). Our present methodology allows the quantitative determination of surface-located CE in a phospholipid bilayer and a clear distinction between such CE and CE present in an oil phase.

The maximum amount of CE as a surface component is slightly lower than that of trilinolein in egg PC vesicles (2.5 mol %; Ref. 23). Such surface-oriented nonpolar lipid molecules may be present in plasma lipoproteins and certain cell membranes and may be a substrate for many biochemical reactions.

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Microemulsions of Phospholipids and Cholesterol Esters

PROTEIN-FREE MODELS OF LOW DENSITY LIPOPROTEIN*

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Low density lipoproteins (LDL) are ~200 Å diameter microemulsions consisting of an apolar core of cholesterol esters surface stabilized by phospholipid and protein. As models for the lipid organization of LDL, protein-free homogeneous microemulsions have been prepared from specific phospholipids and cholesterol esters. Aqueous dispersions of cholesteryl oleate (CO) or cholesteryl nervonate (CN) with egg yolk (EYPC), dimyristoyl (DMPC), or dipalmitoyl (DPPC) phosphatidylcholine were sonicated for 300 min above the order-disorder transition of both lipid components. Fractionation by ultracentrifugation and agarose gel column chromatography at 25 °C yielded stable homogeneous particles (molar ratio of cholesterol ester to phospholipid = 0.9 ± 0.1) with a Stokes radius of ~100 Å. Electron microscopy showed the particles to be circular (spherical) with a diameter of ~200 Å consistent with the surface/volume ratio of a microemulsion with a cholesterol ester core surface stabilized by a phospholipid monolayer.

Differential scanning calorimetry, x-ray scattering/diffraction, and 1H nuclear magnetic resonance spectroscopy studies showed that the lipids in the microemulsions could undergo at least two specific thermal transitions depending on composition, one arising from core-located cholesterol esters (ΔH = 0.7 cal/g of cholesterol ester), similar to LDL, and the other from the phospholipid forming the surface monolayer (ΔH = 5 cal/g of phospholipid).

EYPC/CO and EYPC/CN microemulsions exhibit an order-disorder transition of the core-cholesterol esters (Tm = 42 °C and 51 °C, respectively) with no transition associated with the phospholipid.

DMPC/CO and DMPC/CN microemulsions show a chain-melting transition of the surface-located phospholipid (Tm = 25 °C for both systems) at a temperature 2 °C higher than pure DMPC multilamellar liposomes in addition to the thermal transition of the cholesterol esters. Elevated transition temperatures for the cholesterol esters (DMPC/CO = 46 °C, DMPC/CN = 54 °C) in the particle core compared to the temperatures for the analogous transition in the neat cholesterol esters (CO = 42 °C, CN = 52 °C) suggest that the core cholesterol esters are stabilized with respect to temperature by the surface phospholipid monolayer.

Microemulsions formed with DPPC exhibit concomitant melting of surface phospholipids and core cholesterol esters (Tm = 41 °C for both DPPC/CO and DPPC/CN) indicative of coupling between the core and the surface.

The results show that stable microemulsions with the size and general organization of LDL can be made from phospholipids and cholesterol esters without protein. These results may be extended to native lipoproteins and suggest that interactions between core and surface phases take place dependent on their lipid composition.

Numerous physical studies of the equilibrium phase behavior of the constituent lipids of the plasma lipoproteins in neat, single component, binary, and ternary lipid systems in both the hydrated and anhydrous states have provided a firm background for structural models of lipoprotein particles based on their composition (1-10). The lipid composition of all of the plasma lipoproteins falls in the two-phase region of the phase diagram of their major lipid components (11, 12). The polar constituents (phospholipids and apoproteins) provide surface stability to a nonpolar neutral lipid phase composed predominantly of cholesterol esters and triglycerides. Thus, phospholipids and apoproteins emulsify cholesterol esters and triglycerides and lipoproteins therefore are stable biological emulsions and microemulsions (13).

Low density lipoprotein is a biological microemulsion of particular interest because of its important role in cholesterol transport and metabolism. LDL1 are quasishperical particles approximately 220 Å in diameter whose predominant lipids, phospholipids, and cholesterol esters, constitute ~70% by weight of the total LDL particle and ~90% of the LDL lipids (14). It has been clearly established in tissue culture that receptor-mediated endocytosis of LDL regulates intracellular synthesis of cholesterol through inhibition of hydroxymethylglutaryl-CoA reductase, in addition to regulating its own uptake and intracellular cholesterol ester synthesis (15).

Chemical modification of LDL has been shown to alter the interactions of LDL with its receptor (16). However, differences in the interaction of LDL with its receptor or in the intracellular catabolism of LDL due to alteration of its lipid composition and physical properties are more difficult to assess. Early studies on dietary fat intake showed that a saturated fat diet can decrease the microscopic fluidity of LDL as measured by TEMPO paramagnetic resonance (17) and that an increase in the saturated fatty acid content in LDL.

The abbreviations used are: LDL, low density lipoprotein; Tm, temperature of the midpoint of calorimetric transition; Ts, temperature of onset of calorimetric transition; Tc, endpoint temperature of calorimetric transition; EM, electron microscopy; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; EYPC, egg yolk phosphatidylycholine; DMPC, dimyristoylphosphatidylycholine; DPPC, dipalmitoylphosphatidylycholine; CO, cholesteryl oleate; CN, cholesteryl nervonate; TLC, thin layer chromatography.

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Protein-free Models of Low Density Lipoprotein

LDL can decrease the fractional catabolic rate of apoprotein B (18). More recently however, Kreiger et al. (19) have developed a method for the replacement of the endogenous core lipids of LDL with either exogenous cholesterol esters or other lipid classes. These reconstituted LDL particles have been shown to have similar biological activity to native LDL in terms of uptake and catabolism by fibroblasts in culture. This reconstitution methodology has also been used to investigate the physical properties of the core lipids (20-23).

In normal human LDL, the core-located cholesterol esters undergo a transition from a "smectic" to disordered state at 33 °C, or slightly below body temperature (12, 24). Normal Macaca fascicularis monkeys also have an LDL transition slightly below their body temperature (25). However, feeding M. fascicularis increased saturated fat and cholesterol has been shown to cause severe atherosclerosis in this species, and to increase the size, molecular weight, and the transition temperature of the cholesterol ester core. The increase in the core transition temperature in the cholesterol-fed monkeys was correlated with the degree of saturation of the cholesterol ester fatty acids in the particle. However, in normal humans and in the monkey control group, the transition temperature (Tm) of the LDL core is correlated not only with the degree of saturation of the cholesterol esters but also with cholesterol ester/triglyceride ratio in the particle (12, 25).

The precise lipid-lipid interactions or lipid-protein interactions which mediate these transitions, however, are not known. A major obstacle to understanding precisely what controls these changes in physical properties is the lack of a good lipid model system for LDL which can be systematically and selectively modified and whose physical properties and biological activity can be studied in a controlled manner.

The present work concerns the development an LDL-like system to serve as a model in which to study lipid-lipid interactions. In this system, the chain length and the degree of saturation of each component can be systematically altered and the lipid-lipid interactions studied. However, domain size and radius of curvature are known to have significant effects on the phase behavior and thermodynamics of both phospholipids (26-28) and cholesterol ester systems (29). Therefore, we emphasize that a homogeneous population of particles the size of LDL will serve as an accurate model for this lipoprotein.

Homogenous phospholipid-cholesterol ester microemulsion systems have been prepared and separated by ultracentrifugation. The size and composition have been studied by column chromatography and electron microscopy and the structural properties by differential scanning calorimetry, x-ray diffraction/scattering, and 1H nuclear magnetic resonance spectroscopy. Each system contained a single phospholipid (egg yolk phosphatidylcholine, dimyristoyl phosphatidylcholine, or dipalmitoyl phosphatidylcholine) and a single cholesterol ester (cholesterol oleate or cholesteryl nervousate). The results show that a variety of lipid-lipid interactions may take place at the core-surface interface dependent on the core and surface compositions of the microemulsion. These results may be extended to native lipoproteins.

MATERIALS AND METHODS

Lipids—EYPC (Lipid Products, Surrey, England) and DMPC (Sigma) were judged >99% pure by thin layer chromatography in chloroform/methanol/water/acetic acid (65:25:4:1) and used without further purification. DPPC (Calbiochem-Behring) was purified by silicic acid column chromatography to remove fatty acid and lysophosphatidylcholine. Purified DPPC was judged 99% pure by TLC in petroleum ether/diethyl ether (50:30) and by analytical 13C NMR spectroscopy. Tritiated cholesterol esters were synthesized by the method of Lenz et al. (30) from [7-14C]cholesterol (10.9 Ci/mol) (New England Nuclear) and the appropriate diacyl anhydride (NuChek Prep). The final reaction mixture was purified by silicic acid column chromatography using heptane (6 column volumes) and 1% benzene in heptane (2 column volumes) as eluants. Radiopurity of the desired [14C]cholesterol ester was assessed by TLC to be >98% in the synthesized [14C]cholesterol ester and in solutions in CHCl3/MEOH of the same unlabeled cholesterol ester. 14C-labeled phospholipid solutions in CHCl3/MEOH were made by adding [1-14C]DPPC (100.0 mCi/mmol) to unlabeled phosphatidylcholines in quantities which totaled less than 0.01 mol% of the total lipid mixture. Specific activity in disintegrations per min per mg were determined for each solution by dividing the disintegrations per min per unit volume by the dry weight (milligrams) per unit volume. Specific activities of all solutions were adjusted to approximately 100,000 dpm/mg to give a counting accuracy of 0.5% in aliquots from column chromatography assayed by liquid scintillation counting (see below). All lipids were stored in chloroform/methanol (2:1) solutions at -20 °C sealed under N2.

Microemulsion Preparation—Aliquots of [14C]cholesterol ester and 14C-phospholipid in amounts appropriate to give the desired molar ratio of the two components were taken from stock solutions and the initial total amount and starting molar ratio of the two lipids was verified by liquid scintillation counting of an aliquot from the mixture. The solvent was removed by evaporation followed by vacuum decantation at 4 °C for 4 to 6 h. The dried lipids were resuspended in 10 ml of 0.1 M KCl, 0.01 M Tris-HCl, 0.025% NaCl at pH 8.0. Total lipid concentrations were approximately 1% w/v in all experiments. The cloudy suspension was sonicated for a period of time (see below) under an N2 atmosphere at a temperature at which both components were in a liquid or liquid crystalline state. The temperature was maintained above 53 °C for CO systems and above 53 °C for CN preparations. Temperature was monitored by a thermocouple inserted directly into the sonication vessel. Sonication was performed using a Heat Systems Sonifier (W-530) equipped with a standard 0.5-inch horn at a power setting of 4 (125 watts output) in the "continuous" operating mode. After sonication, the solution was centrifuged at 195,000 x g for 20 min in a Beckman SW 41 rotor at 4 °C. All centrifugation was done without braking so as to prevent intermixing of fractions. The upper 10% of the solution, containing particles which float at a background density of 1.006 g/ml (designated S1) was removed. The remaining infranatant was adjusted to a background density of 1.22 g/ml with KBr and recentrifuged at 195,000 x g for 2 h at 4 °C. The top 20% of the tube volume was removed including the gelatinous layer of lipid which formed in the top 1 to 2 mm at 25 °C. The lipid was readily resuspended to yield fraction S2 and physical studies were conducted on this fraction. Purity of the lipids following sonication was checked by TLC. Lipids were extracted from aqueous solutions by the method of Folch et al. (61). 50 to 100 µg of lipid was applied to TLC plates and solvent systems of chloroform/methanol/water/acidic acid (65:25:4:1) and petroleum ether/diethyl ether (50:30) were used to assess polar and nonpolar lipids, respectively.

Sonicated Phospholipid Vesicles—Phospholipid vesicles were prepared by the method of Barenholz et al. (32). Sonication was performed so as to maintain the temperature of the solution at 5 °C above the gel to liquid crystal transition (Tm) of the phospholipid. Following sonication, the vesicle dispersion was centrifuged for 30 min at 100,000 x g in a Beckman 40.3 rotor previously equilibrated and kept at Tm + 5 °C to remove titanium fragments as well as large multilamellar liposomes.

Gel FIltration Chromatography—Elution column chromatography was performed on Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) with columns (2.6 x 100 cm) at 25 °C and a flow rate of 20 ml/h. Columns were pre-equilibrated and purged with 0.1 M KCl, 0.01 M Tris-HCl, 0.025% NaN3 at pH 8.0 prior to sample application. All samples were applied in volumes of ~2 ml fractions of ~2 ml were collected. Gel columns were calibrated for Stokes radius and molecular weight with a calibration kit (Pharmacia Fine Chemicals) consisting of Dextran 2000 (V0), thyroglobulin, ferritin, catalase, aldolase, and tryptophan (V1). Keq was determined from the equation Keq = (V1 - V0)/(V1 - V0), where V1 is the elution volume and was used to compare elution profiles from one column to another.

Lipid content of eluted fractions was determined by liquid scintillation counting using narrow windows for both 8H and 14C. Efficiencies and 14C cross-over in the 8H channel were determined using [8H] and [14C]toluene standards in Aquasol (New England Nuclear). Scintillation mixture and the eluant buffer was used as a quencher for quench curve generation. Using the external standard ratio and the specific
activity of the radio-labeled lipids, the data are expressed in nanomoles of lipid per ml of effluent. Fractions which were desired for further analyses were pooled and concentrated by adjusting the background solution density to 1.22 g/ml with KBr and resuspended under conditions identical with those under which S₁₀₀₄ was isolated (210 rotor, 195,000 x g at 4 °C for 2 hr). The upper 1.5 ml of each tube was removed, combined into one tube, and if desired, resuspended under the same conditions to yield a highly concentrated solution in 1.0 to 1.5 ml.

Differential Scanning Calorimetry—Calorimetry experiments were performed on a DSC-2 (Perkin-Elmer) at full scale sensitivity of 0.1 to 0.2 mcal/s. Heating and cooling rates from 1.25–10 °C/min were used and all transition temperatures were corrected to a rate of 0 °C/min. Seventy-five-pl samples (2 to 4 mg of lipid) were hermetically sealed in stainless steel sample pans (Perkin-Elmer) and 76 pl of 0.1 M Tris-HCl, pH 8.0, was used as a reference. Temperature calibration and enthalpy calculations were performed as described previously (12).

X-Ray Scattering/Diffraction—X-ray scattering or diffraction patterns of microemulsion systems were obtained using a Jarrell-Ash microfocus x-ray generator and a slit collimated Laue-β-BaSO₄ x-ray camera modified to include a single mirror focusing system. The diffraction patterns were recorded using a linear position-sensitive counter (P.S.D. 1100, Tennelec, TN) coupled to a computer-based analysis system (TN 1710, Tracor Northern, WI). Samples were sealed in 1-mm Lindemann glass tubes (Lindemann Glass Corp., Indianapolis, IN) and exposed to x-rays in vacuo.

Proton Nuclear Magnetic Resonance Spectroscopy—Fourier transform 'H NMR spectra were obtained at 47 K (200.13 MHz for 'H) with a Bruker WP-200 spectrometer (Bruker Instruments, Billerica, MA) equipped with an Aspect 2000 data system. All samples were placed in 5-mm tubes with 0.1 M KCl in D₂O as the solvent and lock. Temperature was regulated using a Bruker BVT-1000 variable temperature unit equipped with a liquid N₂ adapter for low temperature studies. Samples were allowed to equilibrate 20 min at each temperature prior to data acquisition. Fifty scans were accumulated at each temperature with 1.85-s acquisition time and a 2.5-s delay between acquisitions (recycle time (4.35 s) = 1.85 s + 2.50 s).

Electron Microscopy—Microemulsions were negatively stained with 2% sodium phosphotungstate, pH 7.4, on Formvar-coated copper grids. Electron micrographs were obtained with a Hitachi HU-11C electron microscope, calibrated with a grating replica (Pelco, Tustin, CA) at a magnification of approximately ×100,000.

RESULTS AND INTERPRETATION

Characterization of Microemulsions—Initial studies of the effect of sonication time and sonifier power output on the turbidity (A₄₅₀) of aqueous dispersions of cholesterol ester and phospholipid at an initial molar ratio of 2 to 1 showed that turbidity fell for 20 min and then remained constant during sonication at 130 watts output. Although turbidity appeared to have attained a constant value after 20 min of sonication, the dispersions were not clear. Fig. 1, A to D, shows the gel chromatography elution profiles of fractions S₁₀₀₄ (see "Materials and Methods") isolated from EYPC/CO dispersions following different periods of sonication. These dispersions had identical starting molar ratios and concentrations. The elution profile of Fig. 1A resulted from the conditions determined from the turbidity studies to represent the condition of sonication required to reach constant low turbidity (20 min of irradiation, 130 watts power output).

EYPC (expressed as nanomoles per ml effluent; see "Materials and Methods") elutes as a fairly symmetrical peak well included in the column (Vₚ = 240 g) at approximately the same elution volume as that of LDL and EYPC unlamellar vesicles formed by sonication (Fig. 1G). The peak of cholesterol ester (expressed as nanomoles per ml of effluent) is asymmetric and broad with the majority of cholesterol ester mass appearing close to or at the void volume (V₀). The molar ratio of cholesterol ester to phospholipid is low (0.2) over the descending portion of the phospholipid peak with much higher ratios toward Vₚ. The effect of increasing the sonication time from 20 min (Fig. 1A), to 40, 60, and 150 min, on the column elution profile of fraction S₁₀₀₄ is demonstrated in Fig. 1B to D. The phospholipid elution profile remains symmetrical and the Vₚ is independent of the period of sonication although, at 150 min, a slight shift to larger Vₚ is apparent. In contrast, the cholesterol ester peak becomes more symmetrical as the sonication time is increased. The cholesterol ester mass gradually

![Fig. 1. Column chromatography of ultracentrifugation fraction S₁₀₀₄ (1.006 g/ml < ρ < 1.220 g/ml) as a function of sonication time. A to D, elution profile of S₁₀₀₄ of EYPC/CO (initial molar ratio = 2:1) sonicated for 20 (A), 40 (B), 80 (C), and 150 (D) min. Fraction S₁₀₀₄ (ρ < 1.006) was isolated and removed and fraction S₁₀₀₄ (ρ > 1.220) was applied to a Sepharose 4B column (2.6 x 100 cm) at room temperature. E, elution profile of ultracentrifugation fraction S₁₀₀₄ resulting from the sonication of CO/DMPC (initial molar ratio = 1:1) sonicated for 300 min. F, elution profile of concentrated fractions comprising the effluent between 220 and 300 g from E (1:1 DMPC/ CO, 300 min sonication). Fractions were pooled, concentrated by ultracentrifugation, and applied to the same column. G, elution profile of EYPC small unlamellar vesicles prepared by sonication (S₂₀). V₀ indicates the void volume, Vₚ represents cholesterol ester (CE) in nanomoles per ml of effluent, Pₚ phospholipid (PL) in nanomoles per ml of effluent, and CE/PL is the molar cholesterol ester/phospholipid ratio indicated on the descending portion of the elution curves as A.](image-url)
becomes more included in the column so as to co-elute with the phospholipid. Consequently, the molar ratio of cholesterol ester to phospholipid increases to approximately 0.8. Accompanying these changes in the elution profiles as a function of sonication time, a decrease was observed in the weight per cent of lipid isolated in S1, based on the initial total lipid while a complementary increase was observed in the weight per cent of lipid isolated in S2 (data not shown).

Increasing the sonication time beyond 150 min at high cholesterol ester/phospholipid ratios (>1:5:1) did not significantly alter the elution profile, the amounts of material distributed between S1 and S2, or the cholesterol ester/phospholipid molar ratio in fractions of constant molar ratio on the descending portion of the elution curves. Co-sonication of the cholesterol esters CO or CN with the phospholipids EYPC, DMPC, or DPPC (the lipids used in this study) gave similar results under the same conditions. These data suggest that particles with a cholesterol ester/phospholipid molar ratio of approximately 0.8 are preferentially formed with prolonged periods of sonication (150 min).

In confirmation of this suggestion, that a particle with a fixed stoichiometry is formed, Fig. 1E shows the elution profile of fraction S2 isolated from a 1:1 molar ratio of CO/DMPC sonicated for 300 min. Greater than 95% of the lipid was isolated in S2, while the remaining 10% was distributed in S1 and the infranatant of S2. A homogenous population of particles (cholesterol ester:phospholipid = 0.9) is clearly demonstrated by the elution profile. Fractions constituting greater than 90% of the peak were pooled, reisolated by ultracentrifugation (see “Materials and Methods”), and replotted to the column to give the elution profile shown in Fig. 1F. Cholesterol ester and phospholipid can be seen to co-elute at the same Vc with a constant molar ratio (0.9) throughout the majority of the peak.

No degradation of lipid occurred under these conditions of sonication as assessed by TLC (see “Materials and Methods”). Particles formed with EYPC showed no degradation of the phospholipid even with 120 min of sonication. However, following 300 min of sonication, lysophosphatidyldcholine and fatty acids were detectable in small (<5%) amounts. Therefore, for particles formed with EYPC, we have used sonication periods of no more than 120 min and isolated constant molar ratio cholesterol ester/phospholipid fractions. Phosphatidyldcholine with saturated fatty acid chains showed no degradation even at 300 min of sonication. Therefore, a prolonged period of sonication was used with these phospholipids.

Table I summarizes data on the size and composition of the particles isolated from column fractions having constant cholesterol ester/phospholipid ratios. Surface area/volume ratios were determined assuming the particle to be a microemulsion with a cholesterol ester core, surface-stabilized by a phospholipid monolayer. Additionally, it was assumed that the microemulsion stoichiometry was fixed by the conditions of sonication, i.e. above the Tm of both components. In these calculations, therefore, it was assumed that any changes in the physical state of the components could be compensated for by structural changes which maintain the particle size and do not alter the composition (see “Discussion”). A surface area of 65 Å2 was used for the area occupied by a phospholipid head group (33) and a negligibly small contribution of cholesterol ester to the surface area was additionally assumed. Volume was calculated using the partial specific volumes given by Rand and Luzzatti (34). EYPC fatty acid content was determined by gas-liquid chromatography to be 39% 16:0, 2% 16:1, 12% 18:0, 2% 18:1, and 18% 18:2. Estimated particle radii were then interpolated from a surface area/volume (3r/r) versus radius (r) plot. Stokes radius was calculated from K, (see

**Table I**

<table>
<thead>
<tr>
<th>System</th>
<th>Cholesterol ester to phospholipid molar ratio</th>
<th>Surface area/volume ratio</th>
<th>Estimated particle radius (Å)</th>
<th>Stokes radius (Å)</th>
<th>A ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYPC/CO</td>
<td>0.77 ± 0.02</td>
<td>0.031</td>
<td>96</td>
<td>108 ± 2*</td>
<td>225 ± 21</td>
</tr>
<tr>
<td>EYPC/CN</td>
<td>0.82 ± 0.01</td>
<td>0.029</td>
<td>102</td>
<td>122 ± 2*</td>
<td>221 ± 34</td>
</tr>
<tr>
<td>DMPC/CO</td>
<td>0.89 ± 0.01</td>
<td>0.031</td>
<td>96</td>
<td>94 ± 2*</td>
<td>198 ± 19</td>
</tr>
<tr>
<td>DMPC/CN</td>
<td>0.93 ± 0.01</td>
<td>0.029</td>
<td>102</td>
<td>108 ± 2*</td>
<td>218 ± 32</td>
</tr>
<tr>
<td>DPPC/CO</td>
<td>1.04 ± 0.01</td>
<td>0.028</td>
<td>107</td>
<td>100 ± 2*</td>
<td>208 ± 23</td>
</tr>
<tr>
<td>DPPC/CN</td>
<td>1.00 ± 0.01</td>
<td>0.026</td>
<td>114</td>
<td>114 ± 2*</td>
<td>231 ± 39</td>
</tr>
</tbody>
</table>

* Mean molar ratio across descending portion of peaks in the elution profile.
* Surface area/volume for a sphere = 4πr³/(3r) = 4/3r. Estimate from 3r/r versus r plot.
* Determined from K, (see “Materials and Methods”).
* Significant differences in size using Student’s t-test: p < 0.001, 1v versus 2v; 3v versus 4v; 5v versus 6v.

**Materials and Methods** of the phospholipid elution peaks. Negatively stained preparations of phospholipid/cholesterol ester dispersions examined by EM showed these particles to be homogenous with a quasi-circular morphology presumed to be representative of quasi-spherical particles with the dimensions shown in Table I. The mean particle diameters ± 1 S.D. were independent of composition; however, the diameters measured were in close agreement with particle size determined from column chromatography and that estimated from the surface area/volume ratio. These data clearly point to the structure of the cholesterol ester/phospholipid particles, formed by this methodology, as being that of a microemulsion.

Proton nuclear magnetic resonance was used to confirm that the cholesterol ester/phospholipid dispersions prepared by these procedures represented a population of microemulsion particles and to exclude specifically the possibility that a population of vesicular particles was present in the dispersions. Representative data for the DMPC/CO system is presented here.

Similar to observations on sonicated distearoyl phosphatidylcholine (35) 1H NMR experiments at 200 MHz on sonicated DMPC vesicles at 30 °C in 0.1 M KCl in D2O shows the existence of two choline methyl proton resonances (corresponding to the external and internal leaflets of the bilayer) separated by approximately 0.05 ppm. At 30°C, DMPC/CO particles showed a single choline resonance at approximately the same chemical shift as the resonance from choline on the external surface of the bilayer in a vesicle. Addition of Yb³⁺, a shift reagent, in 10:1 molar ratio to phospholipid in the vesicle system resulted in a broadening and upfield shift of the resonance from external choline groups while the resonance from the internal choline groups remained unchanged. The same amount of shift reagent added to the cholesterol ester/phospholipid particles broadened and shifted the single choline resonance leaving no resonance at the chemical shift of either the external or the internal choline methyl protons as seen in vesicles. Thus, the choline groups of the phospholipid are present in a single environment consistent with a monolayer at the surface of a microemulsion. Additionally, these data clearly demonstrate the absence of a population of a single bilayered vesicles co-existing with the microemulsions.

Physical Studies—Microemulsions (S0) were isolated from column fractions and concentrated by ultracentrifugation and vacuum dialysis to a concentration of 25 to 50 mg/ml (ap-
proximately the same concentration at which the lipids were applied to the agarose column). Vacuum dialysis was carried out against 0.1 M KCl, 0.01 M Tris-HCl, pH = 8.0 at 4 °C, for EYPC microemulsion systems, at 4 °C or 25 °C for DMPC systems, and at 25 °C for DPPC systems. Samples concentrated in this manner gave an elution profile identical with that in Fig. 1P when subjected to column chromatography. Seventy-five microliters of the concentrate (1 to 2 mg of phospholipid and 1 to 2 mg of cholesterol ester) were added to the DSC pans.

**Microemulsions of Egg Yolk Phosphatidylcholine and Cholesterol Esters**—The DSC heating and cooling scans of neat CO and CN are given in Fig. 2 for reference. Neat CO (Fig. 2A) exhibits a smectic → cholesteric transition at 42 °C (ΔH = 0.56 cal/g) followed by a cholesteric → isotropic liquid phase transition at 47 °C (ΔH = 0.25 cal/g). Neat CN (Fig. 2B) undergoes only a smectic → isotropic liquid transition at 52.5 °C (ΔH = 1.70 cal/g).

EYPC/CO microemulsions (Fig. 3A) demonstrate a single endothermic transition at 48 °C (ΔH = 0.58 cal/g of cholesterol ester) when heated from −10 to 50 °C. The transition peak (Tm) occurs at 42 °C (Tm = 32 °C, Tc = 50 °C) and is fully reversible with slight undercooling. Microemulsions formed with EYPC/CN (Fig. 3B) exhibit a similar endothermic transition. However, for particles formed with this ester (CN) the peak of the transition occurs at 51 °C (Tm = 44 °C, Tc = 56 °C, ΔH = 0.89 cal/g) when heated from −10 to 60 °C. Small angle x-ray scattering profiles for EYPC/CO microemulsions below the transition temperature (T = 10 °C) exhibit a clearly defined scattering maximum centered at 1/36 Å⁻¹ (Table II) which decreases in intensity on heating and virtually absent at 50 °C (above the transition). This scattering maximum reappears when the sample is cooled below the transition (10 °C). Similarly, x-ray scattering profiles for EYPC/CN microemulsions also show pronounced scattering with a maximum at small angles (Table II). However, in this instance, the intensity maximum is centered at 1/43 Å⁻¹ with no fringe at 1/36 Å⁻¹ as was observed with EYPC/CO microemulsions. On heating through the transition the intensity maximum at 1/43 Å⁻¹ decreases to its minimum at 57 °C (above Tm).

The x-ray diffraction pattern of neat CO in the smectic phase is characterized by a relatively sharp diffraction maximum of 1/36 Å⁻¹ attributed to the layered organization of the smectic state. At higher temperatures where the more disordered cholesteric or isotropic phases are formed this diffraction of 1/36 Å⁻¹ is absent and the diffraction pattern shows only broad diffuse low intensity scattering in the region of 1/33 Å⁻¹. Similarly, neat CN in the smectic phase exhibits a sharp diffraction maxima at 1/43 Å⁻¹ which is not seen at higher temperatures.

Wide angle diffraction patterns of the EYPC/cholesterol ester microemulsion systems below the transitions (10 °C) showed a broad scattering maximum at 1/4.9 Å⁻¹ and 1/4.8 Å⁻¹ for CO and CN, respectively. These diffuse maxima in the wide angle diffraction pattern are typical of cholesterol esters.

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**Fig. 2.** Differential scanning calorimetry curves for neat cholesterol esters. A, cholesteryl oleate. Cooling then heating through the liquid crystalline transitions at 5 °C/min after the initial crystal melt (51 °C, not shown). On cooling from the isotropic liquid, the isotropic liquid ↔ cholesteric phase transition occurs at 47.5 °C and the cholesteric phase ↔ smectic phase transition at 420 °C. B, cholesteryl nervonate. After the initial crystal melt (37 °C, not shown) heating and cooling at 5 °C/min through the smectic ↔ isotropic liquid phase transition at 52.5 °C. Transition enthalpies are given in Table III.

**Fig. 3.** Differential scanning calorimetry curves of EYPC/cholesterol ester microemulsion systems. A, EYPC/CO. Heating and cooling scans at 5 °C/min from −5 to 60 °C. Vertical dashed line denotes the smectic ↔ cholesteric phase transition temperature of neat CO (see Fig. 2A) for reference. B, EYPC/CN. Heating and cooling scans at 5 °C/min from −5 to 60 °C. Vertical dashed line denotes the smectic ↔ isotropic liquid phase transition temperature of neat CN (see Fig. 2B) for reference. Enthalpies of the transition are given in Table III.
in the smectic phase and are attributed to relatively disordered inplanar intermolecular packing distances. Above the transition, these broad maxima are less well defined in both the microemulsion system and the neat esters. Thus, the single endothermic transition observed in these microemulsion systems (EYPC/CO and EYPC/CN) is attributed to the cholesterol ester in the core of the particle undergoing a phase change from an ordered smectic-like packing to a more disordered structure.

**Microemulsions of Dimeristoyl Phosphatidylcholine and Cholesterol Esters**—Calorimetry experiments on microemulsions of DMPC and CO or DMPC and CN (Fig. 4, A and B) showed transitions similar to those observed with EYPC/cholesterol ester microemulsions which are attributed to structural changes of the core-located cholesterol ester. However, the transition temperatures (T<sub>e</sub>) were slightly higher in these systems; 46 °C for DMPC/CO (T<sub>e</sub> = 42 °C, T<sub>μ</sub> = 51 °C) and 54 °C for DMPC/CN (T<sub>e</sub> = 50 °C, T<sub>μ</sub> = 58 °C). Enthalpies of the transition were similar to EYPC/cholesterol ester microemulsions, 0.71 cal/g and 0.70 cal/g for DMPC/CO and DMPC/CN, respectively. An additional small high temperature endotherm was observed at 63 °C in calorimetry experiments on DMPC/CN microemulsions. The low enthalpy (0.14 cal/g of CN) of this transition is suggestive of a second liquid crystalline transition of the core-located cholesterol esters, perhaps a nematic ↔ isotropic liquid phase transition. Both transitions are reversible with 10 °C of undercooling and with the higher temperature exotherm appearing as a shoulder (51 °C) on the lower temperature transition (45 °C). The total enthalpy on cooling (0.65 cal/g CN) is equal to the sum of the two transition enthalpies on heating, but as in EYPC microemulsions, it is less than the enthalpy of neat CN undergoing a smectic ↔ isotropic liquid phase transition.

In contrast to microemulsions formed with EYPC, a large broad endotherm with a peak temperature at 25 °C (T<sub>e</sub> = 10 °C, T<sub>μ</sub> = 36 °C) was also observed in calorimetry experiments on DMPC/cholesterol ester systems. This transition was not seen with EYPC/cholesterol ester systems. The large enthalpy (4 to 5 cal/g of cholesterol ester or DMPC) of this transition is suggestive of the melting of a partially crystalline component of the microemulsion. However, the T<sub>e</sub> is significantly different from the crystal melting temperature of the isolated neat esters (CO, T<sub>e</sub> = 51 °C; CN, T<sub>e</sub> = 37 °C), and the transition occurs at the same T<sub>e</sub> (25 °C) independent of the ester component forming the particle core. Additionally, wide angle x-ray diffraction patterns recorded below this transition (4 °C) showed no diffraction maxima indicative of crystalline cholesterol esters (see Table II). The transition enthalpy (4.89 cal/g of DMPC, DMPC/CO system; 4.52 cal/g of DMPC, DMPC/CN system) is, however, similar to that for DMPC single bilayer vesicles (4.74 cal/g of DMPC) undergoing a gel ↔ liquid crystalline transition. The transition temperature (25 °C) is slightly elevated compared to the transition observed in DMPC vesicles (T<sub>e</sub> = 18 °C) (Fig. 5A), and to aqueous multilamellar dispersions of DMPC (T<sub>e</sub> = 23 °C).

**TABLE II**

<table>
<thead>
<tr>
<th>Microemulsion system</th>
<th>Temperature °C</th>
<th>Small angle spacing* Å</th>
<th>Wide angle spacing* Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYPC/CO</td>
<td>4</td>
<td>36</td>
<td>4.9 (broad)</td>
</tr>
<tr>
<td>EYPC/CN</td>
<td>4</td>
<td>43</td>
<td>4.8 (broad)</td>
</tr>
<tr>
<td>DMPC/CO</td>
<td>4</td>
<td>36</td>
<td>5.1 (broad), 4.2 (broad)</td>
</tr>
<tr>
<td>DMPC/CN</td>
<td>4</td>
<td>43</td>
<td>4.9 (broad)</td>
</tr>
<tr>
<td>DMPC vesicles</td>
<td>4</td>
<td>43</td>
<td>4.2 (broad)</td>
</tr>
<tr>
<td>DPPC/CN</td>
<td>50</td>
<td>5.1 (broad), 4.2 (broad)</td>
<td></td>
</tr>
<tr>
<td>Neat CO</td>
<td>50</td>
<td>5.0 (broad)</td>
<td></td>
</tr>
<tr>
<td>Neat CN</td>
<td>45</td>
<td>5.0 (broad)</td>
<td></td>
</tr>
<tr>
<td>DMPC vesicles</td>
<td>37</td>
<td>5.0 (broad)</td>
<td></td>
</tr>
<tr>
<td>DPPC vesicles</td>
<td>25</td>
<td>4.2 (broad)</td>
<td></td>
</tr>
</tbody>
</table>

* Accurate ± 2 Å based on sample-detector distance.
* Broad wide angle spacings were taken as the scattering maximum ±0.1 Å.
* At 55 °C, some residual intensity remained as compared to 63 °C diffraction patterns, however there was no clearly defined maximum.
* Ref. 3.

Fig. 4. Differential scanning calorimetry curves for DMPC/cholesterol ester microemulsion systems. A, DMPC/CO. Heating and cooling scans at 5 °C/min from -10 to 70 °C. Thick vertical dashed line indicates the temperature of the gel ↔ liquid crystal transition temperature of the DMPC vesicles (Fig. 5) for reference. Thin vertical dashed line denotes the smectic ↔ cholesterol transition temperature for neat CO (Fig. 2A) for reference. B, DMPC/CN. Heating and cooling scans at 5 °C/min from -10 to 70 °C. Thick vertical dashed line indicates the T<sub>e</sub> of DMPC vesicles as above for reference. Thin vertical dashed line denotes the smectic ↔ isotropic liquid phase transition temperature of neat CN (Fig. 2B) for reference. Enthalpies of the transition are given in Table III.
observed. However, scattering due to particle structure (see “Discussion”) is still present in the small angle region. Similar behavior is observed for both DMPC/CO and DMPC/CN with the exception of the position of the low angle-scattering maximum (1/36 Å⁻¹ for CO, 1/43 Å⁻¹ for CN).

The results of ¹H NMR temperature studies on DMPC/CO are shown in Fig. 6. The total peak area of the aliphatic region from 0 to 2.5 ppm from tetratmethylsilane was measured as a function of temperature. The resonances in this region of the spectrum result from all fatty acyl protons from the two acyl chains of the phospholipid (DMPC = 54) and all the protons from the cholesterol ester ring and acyl chain less its unsaturated protons and that on cholesterol ring carbon C-3 (CO = 76). The results on heating show that the integrated peak area reaches a maximum at 60 °C in two distinct steps; between 5 °C and 30 °C ~48% of the maximum is attained and the second 51% is accounted for between 35 °C and 60 °C. Peak area or intensity is related to the molecular dynamics of the system in the sense that attenuated molecular motions are associated with loss of signal. Thus the changes in area shown in Fig. 6 represent two distinct events (corresponding to those observed calorimetrically) in which molecular motions increase, i.e., the melting of one phase to another. Since CO accounts for 55% (DMPC = 45%) of the total number of protons in this region of the spectrum when corrected for the molar ratio of the two components of the microemulsion, these data support the hypothesis of the sequential melting of DMPC at 25 °C and CO at 46 °C observed calorimetrically and in the x-ray diffraction experiments.

On cooling, the transitions at Tm = 46 °C, CO and Tm = 54 °C, CN associated with the cholesterol esters were fully reversible with some undercooling. However, two low temperature exotherms were present. The total enthalpy of these transitions was however equal to that of the endotherm at 25 °C observed on heating. In DMPC/CO microemulsions, exotherms occurred at 18 °C and 5 °C and in DMPC/CN microemulsions, these exotherms occurred at 17 °C and 7 °C.

The peak area ratios of the two exotherms were variable from scan to scan, with the higher temperature transition increasing in area at the expense of the lower temperature transition. However, independent of the variability of the exotherms observed on cooling, the full enthalpy of the endotherm at 25 °C was present on subsequent reheating, suggesting a kinetic phenomenon.
Microemulsions of Dipalmitylophosphatidylcholine and Cholesterol Esters—Representative calorimetry experiments on DPPC/CO and DPPC/CN microemulsions are illustrated in Fig. 7. A and B, respectively, and exhibit different behavior from both the EYPC and the DMPC systems. A single large endotherm, broad reversible transition was observed in scans from 10-60 °C in both the DPPC/CO and the DPPC/CN microemulsion systems. On heating, the endotherm begins at 28 °C, peaks at 41 °C (Tm) and returns to base-line at 50 °C for both systems. On cooling, the Tm of the exotherm is undercooled 6 °C, to 35 °C. The enthalpy of this transition was ΔH = 3.05 cal/g of total lipid for DPPC/CO and ΔH = 3.75 cal/g of total lipid for DPPC/CN. The large enthalpy of this transition suggests the melting of a crystalline component of the microemulsion.

However, the Tm of 41 °C is inconsistent with the Tm observed for the crystal melting transition of the cholesterol ester component (CO, 51 °C; CN, 37 °C). The corresponding gel → liquid crystal transition of DPPC multilamellar liposomes or vesicles (Fig. 5B) occurs at 42 °C and 59 °C, respectively, thus, suggesting that the enthalpy of the endothermic transition is primarily associated with a transition of the surface-located phospholipid component.

In the case of microemulsions formed with DPPC and CO, the endothermic transition observed experimentally also encompasses the temperature range of the liquid crystalline transition temperatures of the neat cholesterol esters (Fig. 2A) and of the cholesterol esters in microemulsions formed with EYPC (Fig. 3A) and DMPC (Fig. 4A), suggesting that the observed enthalpy may also contain a contribution from the liquid crystal transition of the cholesterol ester. For CN, the liquid crystalline transition in either the neat state (Fig. 2B) or in the microemulsions (Figs. 3B and 4B) occurs at ~52 °C. However, the width of the observed transition in the DPPC/CN microemulsions (Tm - Tw = 20 °C) is large and the end point (Tm = 49 °C) is close to 52 °C, the expected Tm of the liquid crystalline transition for the ester component. Thus, the endothermic transition for the phospholipid and cholesterol ester components might be expected to partially overlap. However, in the DPPC/CN system, slow heating rates (0.5 °C/min) showed no indication of a shoulder on the high temperature side of the endotherm nor was an additional transition at ~52 °C observed.

At 25 °C, below the transition, small angle x-ray scattering demonstrates an intense fringe (CO, 1/36 Å⁻¹; CN, 1/43 Å⁻¹) characteristic of cholesterol esters in a smectic array and similar to the scattering from cholesterol esters in DMPC and EYPC microemulsion systems (see Table II). At 50 °C, above the transition, this fringe is not present in the small angle scattering pattern. As was observed in the DMPC system, a wide angle diffraction maximum was observed in both DPPC/ cholesterol ester systems below the transition (25 °C) at 1/4.2 Å⁻¹, indicative of the crystallization of the phospholipid fatty acyl chains. Above the transition at 50 °C, this diffraction maximum was not observed. Thus, these x-ray data clearly demonstrate that changes in physical state of both the cholesterol ester and the phospholipid component of the microemulsion system are associated with the endothermic transition at Tm = 41 °C. Small angle scattering at 46 °C on DPPC/CN microemulsion (midway between Tm and the end of the transition) failed to produce any intensity at s = 1/43 Å⁻¹ demonstrating that the core cholesterol esters were fully melted even before the phospholipid had completed the transition.

Thus, the total enthalpy of the observed transition reflects contributions from both the surface phospholipid and core cholesterol esters as a single endothermic event. Enthalpy calculations were therefore made assuming that the enthalpy for the cholesterol ester transition was the same for the DPPC microemulsion system as in the EYPC and DMPC systems. Thus, the contribution to the peak area of the endotherm from the cholesterol ester and phospholipid transitions could be determined and an enthalpy for the phospholipid transition was calculated. For DPPC/CO, the enthalpy was ~5.2 cal/g of DPPC, for DPPC/CN, the enthalpy was ~6.9 cal/g of DPPC.

DISCUSSION

Extensive sonication (300 min) of aqueous dispersions of equimolar amounts of cholesterol esters and phospholipid above the Tm (order-disorder transition temperature) of both lipids yields microemulsion particles. The microemulsion particles prepared under these conditions have a cholesterol ester/phospholipid molar ratio of 0.9. At equilibrium, a ternary system with this composition must exist as three separate phases (9): 1) phospholipid saturated with a small amount of cholesterol ester (2 to 3%, w/w); 2) pure cholesterol ester; and 3) water. Surface/volume calculations assuming that the parti-
Protein-free Models of Low Density Lipoprotein

ticle organization is that of an emulsion and based on the particle composition and stoichiometry yield a size of approximately 200 Å in diameter which is consistent with the particle size independently determined by column chromatography and electron microscopy (Table 1). The morphology observed in negative staining electron micrographs is also consistent with this model. Thus, the overall particle organization reflects the equilibrium phase behavior of its components in bulk systems and the microemulsion particles consist of cholesterol ester core surface stabilized by phospholipid monolayer presumably saturated with cholesterol ester.

Although turbidity (Δε) reaches a constant low value after 20 min of sonication at an initial cholesterol ester/phospholipid molar ratio of 2:1, an optically clear solution is not obtained, contrary to an earlier report which suggested that stable microemulsions of CO and EYPC could be formed under these sonication conditions (38). The agarose gel column chromatography elution profile of ultracentrifugation fraction S1 isolated from this experiment (Fig. 1A) is indicative of a heterogeneous system of particles. The symmetrical phospholipid peak well included in the column with a similar Vc to sonicated EYPC vesicles (Fig. 1F) suggests a homogeneous population of phospholipid vesicles. A heterogeneous population of large emulsions is reflected in the high (>2:1) cholesterol ester/phospholipid molar ratio close to or at Vc in the elution profile and in fraction S1 isolated in the ultracentrifuge separation. Particles of this composition would be large enough to scatter light accounting for the absence of clearing. Finally, a small population of microemulsion particles is suggested by the 0.2 cholesterol ester/phospholipid molar ratio on the descending portion of the phospholipid elution curve since at this molar ratio cholesterol ester should be present as a separate phase, and at these elution volumes the particle size must be small. The effect of extending the sonication time is to form microemulsion particles at the expense of the large emulsions and small unilamellar vesicles. Fig. 1, A to E demonstrate that the phospholipid elution profile remains virtually unchanged as a function of sonication time perhaps shifting to slightly larger Vc. The cholesterol ester peak becomes gradually symmetrical, included in the column, and elutes at the same Vc as the phospholipid after 300 min of continuous sonication, to yield microemulsions. Under these conditions, 90% of the starting lipids are isolated in this homogeneous fraction.

Table III summarizes the results and interpretation of the DSC and x-ray diffraction/scattering experiments on the isolated microemulsions. All six systems studied exhibit a broad reversible low enthalpy endothermic transition on heating. In microemulsions formed with EYPC, the Tm of this endotherm is dependent on the cholesterol ester and identical with that observed for the smectic ↔ cholesteric and smectic ↔ isotropic liquid phase transitions for CO and CN, respectively, suggesting that this transition is associated with the order-disorder transition of the cholesterol ester in the particle core.

An additional transition is observed in DMPC and DPPC microemulsions associated with chain melting of the phospholipid surface monolayer. X-ray diffraction experiments substantiated these interpretations. The transition enthalpies for both the phospholipids and cholesterol esters in the microemulsion systems are lower than those observed for the neat components of these systems at equilibrium. The lower enthalpy values suggest that the microemulsions (similar to unilamellar phospholipid vesicles) are systems with higher free energies than their isolated neat components. These particles are thus metastable, non-equilibrium systems.

Both DMPC/CO and DMPC/CN microemulsion systems show elevated transition temperatures, associated with the core cholesterol ester, compared to the analogous transition of the isolated neat cholesterol esters. Thus, in these particles, the ordered smectic liquid crystalline phase exists at temperatures where the corresponding phase in the neat ester would be either a cholesteric or isotropic liquid phase. Thus, with respect to temperature, the smectic phase appears to be stabilized in these microemulsions. The existence of a second,

<table>
<thead>
<tr>
<th>System</th>
<th>Tm (°C)</th>
<th>ΔH (cal/g)</th>
<th>Structural interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYPC/CO</td>
<td>42</td>
<td>0.60 ± 0.01°</td>
<td>Radial smectic ↔ disordered</td>
</tr>
<tr>
<td>EYPC/CN</td>
<td>51</td>
<td>0.89 ± 0.01°</td>
<td>Radial smectic ↔ disordered</td>
</tr>
<tr>
<td>DMPC/CO (1)</td>
<td>25</td>
<td>4.89 ± 0.05</td>
<td>Gel → liquid crystal (monolayer)</td>
</tr>
<tr>
<td>DMPC/CO (2)</td>
<td>46</td>
<td>0.71 ± 0.01</td>
<td>Radial smectic ↔ disordered</td>
</tr>
<tr>
<td>DMPC/CN (1)</td>
<td>25</td>
<td>4.52 ± 0.02</td>
<td>Gel → liquid crystal (monolayer)</td>
</tr>
<tr>
<td>DMPC/CN (2)</td>
<td>54</td>
<td>0.70 ± 0.04</td>
<td>Radial smectic ↔ ordered fluid (nematic)</td>
</tr>
<tr>
<td>DMPC/CN (3)</td>
<td>63</td>
<td>0.14 ± 0.03</td>
<td>Ordered fluid (nematic) ↔ disordered</td>
</tr>
<tr>
<td>DPPC/CO</td>
<td>41</td>
<td>~5.2 ± 0.1</td>
<td>DPPC: gel ↔ liquid crystal (monolayer)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.6 ± 0.1)</td>
<td>CO: radial smectic ↔ disordered</td>
</tr>
<tr>
<td>DPPC/CN</td>
<td>41</td>
<td>~6.9 ± 0.1</td>
<td>DPPC: gel ↔ liquid crystal (monolayer)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.9 ± 0.1)</td>
<td>CN: radial smectic ↔ disordered</td>
</tr>
<tr>
<td>Neat CO (1)</td>
<td>42</td>
<td>0.56 ± 0.01°</td>
<td>Smectic ↔ cholesteric</td>
</tr>
<tr>
<td>Neat CO (2)</td>
<td>47.5</td>
<td>0.25 ± 0.00°</td>
<td>Cholesteric ↔ isotropic liquid</td>
</tr>
<tr>
<td>Neat CN</td>
<td>52.5</td>
<td>1.70 ± 0.00°</td>
<td>Smectic ↔ isotropic liquid</td>
</tr>
<tr>
<td>EYPC vesicles</td>
<td>&lt;10</td>
<td>0.25 ± 0.00°</td>
<td>Gel → liquid crystal</td>
</tr>
<tr>
<td>DMPC multilamellar liposomes</td>
<td>23</td>
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<tr>
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</tr>
<tr>
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<td>Gel → liquid crystal</td>
</tr>
<tr>
<td>DPPC vesicles</td>
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<td>Gel → liquid crystal</td>
</tr>
<tr>
<td>LDL</td>
<td>30</td>
<td>0.69 ± 0.01°</td>
<td>Radial smectic ↔ disordered</td>
</tr>
</tbody>
</table>

*Mean ± S.E. for at least four independent measurements.

* Assumed enthalpies of CO and CN based on EYPC/cholesterol ester system. See text for explanation.

* Enthalpy values from Ref. 44.

* Enthalpy values from Ref. 45.

* CE = total LDL cholesterol esters.
ordered cholesterol ester phase in DMPC/CN microemulsions with a transition at 63 °C (well above the transition temperature to the isotropic liquid in the neat ester (52.5 °C)) suggests that intermolecular interactions between surface and core stabilize the microemulsion core.

In DPPC microemulsion systems, no information is available by DSC on the \( T_m \) or enthalpy of the cholesterol ester transition since a broad high enthalpy transition associated with the surface phospholipid spans the temperature range of the expected ester transition. However, x-ray-scattering studies have clearly shown that a cholesterol ester transition does occur. Interestingly, DPPC/CN microemulsions exhibit no transition at or above the \( T_m \) of the smectic ↔ isotropic liquid phase transition of neat CN (52.5 °C). X-ray-scattering experiments further indicate that the CN transition in the microemulsion is completed at 46 °C indicating that the \( T_m \) in this system is depressed by as much as 10 °C.

Thus, three distinct lipid-lipid interactions are evident dependent upon the phospholipid component of the system (the results are schematized in Fig. 8, and the transition temperatures and enthalpies are in Table III). In EYPC microemulsions the core esters behave as a phase independent of the phospholipid monolayer which surrounds it (\( T_m, \) core cholesterol ester = \( T_m, \) neat cholesterol ester). In contrast, cholesterol

A) EYPC/CO:
EYPC/CN :

B) DMPC/CO :

C) DMPC/CN :

D) DPPC/CO:
DPPC/CN :

INCREASING TEMPERATURE

Fig. 8. Models for the structural transitions of phospholipid/cholesterol ester microemulsions as a function of increasing temperature. Table III is a summary of the temperatures and the enthalpies of the transitions shown here. The model for all systems is that of a core of cholesterol esters surface stabilized by a monolayer of phospholipids. A, EYPC/CO, EYPC/CN. For both systems, below \( T_m \) the phospholipid acyl chains are fully melted and the cholesterol esters are organized in a radial smectic-like array. A small amount of cholesterol esters are shown in the phospholipid monolayer (Refs. 6, 9, and 41). The model for the core below the transition is depicted as a radially oriented bilayer based on preliminary small angle x-ray-scattering studies and column chromatography (see text). Above the transition, the cholesterol esters are disordered. B, DMPC/CO. Below the transition, the surface phospholipid monolayer is in the gel state and the core cholesterol ester is in a radially oriented smectic array. The phospholipids are shown in the model in a tilted orientation to account for changes in the surface area of the core and the surface that must occur when the microemulsion is cooled below its transitions from its temperature of formation (above the \( T_m \) of both components). The melting of the phospholipid acyl chains and of the cholesterol esters occurs as two well separated calorimetric events. C, DMPC/CN. The temperature-dependent structural changes in this system are similar to those for DMPC/CO microemulsions. The surface phospholipid melts at a \( T_m \) which is well separated and distinct from the \( T_m \) of the core cholesterol ester. However, in this system, the smectic-like phase melts to a second ordered, perhaps radial nematic state of the core and finally to an isotropic liquid. D, DPPC/CO, DPPC/CN. The model for these two systems is that of a gel state phospholipid monolayer and a radial smectic phase of cholesterol esters below \( T_m \). In contrast to other systems, however, the core and surface phases melt simultaneously as a single calorimetric event. Precise details of this core-surface coupling phenomenon have been omitted from this representation since several factors may be responsible (see text for details).
esters in DMPC microemulsions, although clearly an independent phase, appear to have a more stable smectic phase (shown by their elevated $T_m$). This result may be attributed to the effects on the cholesterol ester of chain packing of heterogeneous (EYPC) versus homogeneous (DMPC) phospholipid fatty acyl chains at the interfacial region, and the 50 °C difference in $T_m$ between phospholipid and cholesterol ester in the EYPC system versus the 20 °C difference in the DMPC system. Differences in the miscibility of smectic cholesterol ester and liquid crystalline phospholipid at the interfacial region may exist between the two systems.

Distinct from EYPC and DMPC systems, DPPC microemulsions exhibit simultaneous melting of the core cholesterol ester phase and the surface phospholipid. Compared to microemulsions with DMPC, smaller differences in $T_m$ exist between the components of the DPPC microemulsions, which may promote incomplete phase separation at the interfacial region and enhance interactions between the core cholesterol esters and the surface phospholipid monolayer. In binary mixtures of phospholipids, differences in miscibility have been shown to depend on the differences in the transition temperature of the individual components (39). In blayers of sphingomyelin, coupling phenomena have been observed and have been attributed to interpenetration of acyl chains of unequal length from one monolayer of the bilayer to the other (40). Although the precise details of the intermolecular interactions at the interfacial region of the microemulsion are not presently known, interdigitation of the acyl chains of the core located cholesterol ester with those of the phospholipid monolayer may occur.

Regardless of whether the core cholesterol ester is CO or CN, DMPC and DPPC microemulsions exhibit a gel ↔ liquid crystal phase transition of the surface phospholipid monolayer, whereas EYPC microemulsions show no transition of the surface lipids above -10 °C. Interestingly, the phospholipid transition temperature is the same (DMPC, $T_m = 25 °C$; DPPC, $T_m = 41 °C$) independent of the core cholesterol ester. In addition, both phospholipid monolayers have transition temperatures above the analogous transition in unilamellar vesicles (DMPC, $T_m = 18 °C$; DPPC, $T_m = 39 °C$). The elevated $T_m$ may be due to either the small amount of cholesterol ester known to be soluble in phospholipid (9, 41), a direct interaction in which core cholesterol ester stabilize the surface phospholipid, or an inherently more stable phospholipid monolayer at the surface of the microemulsion with respect to a vesicle in which the outer leaflet has approximately the same radius of curvature as the microemulsion, but whose inner leaflet may assume a less stable, smaller radius of curvature.

Low density lipoprotein is essentially a microemulsion of cholesterol esters surface stabilized by phospholipids and protein. The model systems developed here provide information relevant to the lipid-lipid interactions in LDL in the absence of the influence of the protein moiety. DSC on LDL from -10-50 °C exhibits a low enthalpy-reversible endotherm similar to that observed in all systems studied here, associated with the core cholesterol esters undergoing a transition from a smectic-like to a disordered phase (12). Although a full structural study by small angle x-ray scattering is not completed, Fig. 9 shows the small angle x-ray-scattering profile of DMPC/CO microemulsions at 4 °C. The profile closely resembles that of LDL at the same temperature.

Model analysis based on small x-ray-scattering profiles has been used to describe the packing of cholesterol esters in the core of LDL below its calorimetric transition and suggest that a single radially oriented bilayer of cholesterol esters is present in the core of the particle (12, 42). A similar model for the organization of the central core of the microemulsion system seems probable (Fig. 8).

Inspection of the data from Table I shows not only that particle size of the microemulsion increases linearly with the chain length of the phospholipid, but also that a constant size difference of 14 Å ($p < 0.001$) exists between microemulsions with CO and microemulsions with CN (with a given phospholipid). This constant difference in particle radius indicates that not only is the core size determined by the dimension of the cholesterol ester, but also that since 14 Å is twice the difference between the small angle spacing of neat CO (36 Å) and neat CN (43 Å) in the smectic phase, the core of the particle contains 2 repeating units of smectic cholesterol ester molecules. Thus, the size data, for these microemulsions derived from column chromatography and EM support the model for LDL derived from small angle x-ray scattering.

DMPC and DPPC microemulsion systems show a phase transition associated with the surface monolayer and direct interaction between the core-located components and the surface. Although similar phase transitions have not been observed in LDL, these studies have demonstrated that direct interactions between the core and the surface components probably occur in lipoproteins. Recently, using 1H NMR, Kroon (43) has proposed that a fluid phospholipid monolayer in LDL disorders the molecular packing of the core cholesterol ester in the smectic-like phase (below $T_m$) suggesting an interaction between core and surface in native LDL.

The protein at the surface of the native lipoproteins is likely to play a role in the interactions between the core and surface while providing additional surface stability to the lipoprotein microemulsion. Studies on apoprotein-microemulsion complexes are currently being pursued in order to better understand the structural role of proteins in native lipoproteins.

Acknowledgments—We gratefully acknowledge Dr. James Hamilton and Dr. G. Graham Shipley for their valuable discussions. We also wish to thank Cynthia McCormick for her excellent technical assistance in electron microscopy and Anne Gibbons for her expertise in preparing the manuscript.

REFERENCES
Protein-free Models of Low Density Lipoprotein

Our task is to summarize briefly the biochemical constituents of the vessel walls and how these change during the progression from normal intima to an atherosclerotic plaque. The intima is made up of a variety of cellular and noncellular components, including endothelial and occasional smooth muscle cells, glycosaminoglycans, collagen, and elastin ground substances. There is evidence that all of these intimal constituents undergo some changes during the progression from normal intima to atherosclerotic lesion. The major changes in mass and therefore volume during the progression to atherosclerotic lesions in man are in the lipid constituents of the vessel wall. In the normal vessel wall at different ages the total dry weight of the intima contains only a very few percent of its total mass as lipid (1); in the discrete small lesions called fatty streaks the lipid content of the intima in that lesion has increased to approximately 20% of the total mass. However, in carefully dissected, large, raised lesions described as atherosclerotic plaques, the percent dry weight is often greater than 50% (2). Since the density of the lipids is some 30-40% less than the density of the other constituents (proteins, polysaccharides),

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absolute volume occupied would be greater than the mass. Thus in
human atherosclerotic lesions lipids account for a major fraction
of the nonwater volume occupied by the lesion and therefore must
be considered in the pathogenesis. The rest of this discussion
will be directed mainly towards lipids, although other substances
which increase in mass during the development of atherosclerotic
lesions also include some cellular and connective tissue elements.

PHYSICAL CHEMISTRY OF LIPIDS OF Atherosclerotic Lesions

Matter can exist in a number of physical states such as gas, 
liquids, solids, and some intermediate forms between liquids and 
solids called liquid crystals. Thus it is possible that lipids
in the arterial wall during the progression of atherosclerosis
could be present in different states (liquids, liquid crystals,
or solids), and that the reversibility of these lesions might be
related to the state(s) of the deposited lipids. Therefore, many
years ago we undertook to understand the physical interactions
of the major lipids found in atherosclerosis (3).

The major lipids that accumulate in atherosclerosis are
phospholipids, cholesterol, and cholesterol esters. The inter-
actions of equilibrated mixtures of these substances in aqueous
systems can be represented on a triangle, in which there are four
major zones (Fig. 1). Zone I, a lamellar liquid-crystalline
phase, contains bimolecular leaflets of phospholipid into which
free cholesterol and small amounts of cholesterol ester can be
incorporated. The maximum amount of free cholesterol incorporated
is about 35% of the amount of phospholipid by weight, or approxi-
mately 1 mol free cholesterol per mol phospholipid. The maximum
amount of cholesterol ester incorporated is about 2%, or approxi-
mately 1 mol cholesterol ester per 40 mol phospholipid. In this
phase, which is analogous to the lipid regions of many cell
membranes and cell-organelle membranes (4), the lamellar liquid
 crystals may be more or less fluid, depending on the type of
phospholipid, the fatty acyl chains within it, and the other

Fig. 1. Phase diagram of three-component system cholesterol (C),
phospholipid (PL), and cholesterol ester (CE) in excess water at
37°C and 1 atmosphere pressure. Zone I has a single phase of
phospholipid lamellar liquid-crystal with up to 33% cholesterol
and 2% cholesterol ester. Zone II also contains a single phase
of liquid or liquid-crystalline cholesterol ester. Zone III
contains both the cholesterol ester and phospholipid phases. Zone
IV has a third phase, cholesterol monohydrate crystals. Schematic
molecular representation of the phases are shown near each apex
of the triangle. The irregular lines denote phospholipid mole-
cules, the solid symbols cholesterol molecules and the solid,
tailed symbols cholesterol ester.

membrane components present, such as cholesterol, glycosphingo-
 lipids or proteins.

Zone II contains a single phase of cholesterol ester, which
incorporates small amounts of free cholesterol--about 4% at 37°C.
Depending on the fatty acid that is esterified to cholesterol,
this phase may be in one of four states: a liquid state, in which
the molecules are randomly associated; a cholesteric liquid-
 crystal state, in which the molecules are nearly oriented along
their long axes; a smectic liquid-crystalline state, in which the
molecules form a layered structure perpendicular to the long axis
(Fig. 1); or a crystalline solid state. Equivalent amounts of
cholesterol--4% by weight--are incorporated by the liquid,
cholesteric, and smectic phases, but crystalline ester excludes free cholesterol from its lattice.

In Zone III, mixtures of these lipids separate into the cholesterol-ester phase (Zone II), which floats in water, and the lamellar liquid-crystalline phase (Zone I), which sinks. In Zone IV, three phases with fixed composition coexist: the lamellar phospholipid liquid-crystalline phase saturated with cholesterol and cholesterol ester; an oily cholesterol-ester phase saturated with free cholesterol; and a cholesterol-monohydrate-crystal phase (3,5).

Each of these phases has physical characteristics that can be identified with polarizing or electron microscopy, by colorimetry, or by x-ray diffraction. The liquid-crystalline phase in Zone I has a characteristic appearance under polarizing and electron microscopes, and its x-ray diffraction is typical of a one-dimensional lamellar lattice. The first-order lamellar spacing reflects the thickness of the bilayer and its accompanying layer of water. This thickness, determined by the lipids and the amount of water present, may vary from about 6 to 9 nm (60 Å to 90 Å), but it is characteristic for a given lipid at a given temperature.

Zone II, the cholesterol-ester phase, may be liquid, liquid crystalline, or crystalline. If liquid, it is isotropic (non-birefringent between crossed polars) and produces x-ray scattering with very diffuse maxima at approximately 0.5 nm (5 Å) and 3 nm (30 Å). The cholesteric and smectic phases have different birefringent textures under the polarizing microscope (6).

Furthermore, the cholesteric phase scatters x-rays like the liquid, but the smectic phase produces very sharp maxima from which the organization of ester molecules in layers has been deduced (Fig. 1) (7). Each ester has a characteristic transition temperature that can be quantitated by microscopy and colorimetry, and mixtures of esters undergo transitions in a predictable manner—for example, the more double bonds in the acyl chains of the mixture, the lower the transition temperature (6). Thus the transition temperature provides evidence of the chemical composition of the esters in Zone II.

The cholesterol-monohydrate-crystal phase in Zone IV has been characterized extensively in plaques (3,5). The crystals show characteristic x-ray spacing and appearance under the microscope; they melt at 85°C (8); and their crystal structure has been identified (9).

PHYSICAL CHEMICAL PROPERTIES OF THE NORMAL INTIMA

Katz (10) has recently shown that the normal intima of newborn children contains virtually no cholesterol ester as indicated in Fig. 2. As the person passes through the various decades of life the mean intimal lipid composition increases, a separate cholesterol ester phase is formed, which is largely extracellular and progresses down the line toward LDL composition as noted in Fig. 2. In the fifth decade this line changes its direction as the intima becomes enriched in free cholesterol and deviates from the direction of the lipid deposition in the younger intima. Lipid deposition in the intima up to 40 years looks as if lipids from low-density lipoproteins are added to the vessel wall. However, the composition at older ages, richer in cholesterol, is different and thus appears to have undergone biochemical changes. It should be stressed that the intima in all these cases would look grossly normal and be considered pathologically normal.

PHYSICAL PROPERTIES OF ATHEROSCLEROTIC LESIONS

The first grossly recognizable lesions are the fatty streaks, slightly raised yellow lesions from 1 mm up to 1 cm in size, which contain large masses of foam cells and whose composition is plotted on Fig. 3. The origin of the lipid in foam cells has stimulated great interest recently and several reviews concerning the cellular metabolism of cholesterol and lipid deposition have been worth consulting (11,12,13). Most of the lipids in fatty streaks are present in the foam cells and are present as
Fig. 2 Compositions of normal intima lipids of newborns and people of subsequent decades as plotted on the cholesterol, phospholipid, and cholesterol ester phase diagram (see Fig. 1). Plots 1-9 represent intima lipids, whereas point 10 is the composition of human LDL. Intima lipid compositions up to the fourth decade fall on a straight line joining newborn to LDL (From Ref. 10, with permission.)

cholesterol ester phase, readily observable under the polarizing microscope as shown in Fig. 4A. The intermediate lesions appear rather similar although perhaps slightly larger than fatty streaks, but they fall in a region where three phases are predicted to coexist, that is, the cholesterol ester phase, the membrane phase, and cholesterol monohydrate crystals. However, only a few of the lesions in this zone actually contain cholesterol monohydrate crystals and the number of crystals is very small (5), which suggests that these lesions contain cholesterol in a supersaturated system.

The gruel-containing plaques have a composition having much greater free cholesterol. Unlike fatty streaks and intermediate lesions the plaques are necrotic and contain much dead cellular material, and most of the lipid is extracellular (2,5). The predicted lipid physical state should contain large numbers of crystals of cholesterol monohydrate and these are in fact present.

Fig. 3 Mean composition of fatty streaks, intermediate lesions, and gruel-containing plaques plotted on the phase diagram (see Fig. 1). (From Ref. 5, with permission.)

In human lesions, the cholesterol monohydrate crystals may reach the size of 1 mm in length. They are present as large plates as indicated in Fig. 4B.

A simplified thesis of the development of the plaque (13,14) is outlined in Fig. 5. First, during the early period of life the intimal cells and the extracellular intima contain very little cholesterol-ester-rich lipid material. Most of the lipid is present as membranes in cells and has a composition lying near the phospholipid zone. With increasing age the composition of the

Fig. 4 A: Polarized light photomicrographs of a foam cell. B: Cholesterol monohydrate crystals at 22°C (x100, multicrossed polarizers).
foam cells and a group of such cells is called a fatty streak. These lesions are obviously reversible if cholesterol balance can be reversed. In time some of these lesions may develop a lysosomal defect that allows the continued intake of cholesterol ester into lysosomes and the hydrolysis into free cholesterol, but blocks exit out of lysosomes either due to cellular mechanisms being saturated with cholesterol or a relative deficiency in cholesterol carrier protein. This allows free cholesterol concentration to increase within lysosomes to the point where nucleation may occur, crystals form, the lysosomes rupture, cells autolyze, and cell death and necrosis begin. Such a progression would lead to a relative decrease in cholesterol ester and an increase in free cholesterol as is seen in the atherosclerotic plaque (Fig. 3). Furthermore, such necrosis would allow other elements such as thrombosis, fibroblast, collagen deposition, and calcium precipitation to accompany the necrosis and give rise to the other elements found in advanced calcified atherosclerotic plaques.

THE CORRELATION OF THE HISTOLOGICAL APPEARANCE OF LESIONS AND THE POSITION OF THE LIPIDS IN THE LESIONS

In lesion fragments as shown in Fig. 4, it is possible to dissect unfixed lesions and observe foam cells, lipid droplets, and cholesterol monohydrate crystals. Dissection and removal of parts of lesions to microscopic slides were necessary because the usual fixation techniques for vessel walls involve changes in temperature and medium, particularly organic solvents and dyes, which alter the physical state of the lipids. However, the techniques utilized by us to observe the native physical state does not allow fixation of the tissue or preservation of the normal architecture of the vascular wall. Thus we would like to know where the cellular and extracellular lipids are in respect to the vessel wall architecture and whether the physical state varies in different regions of the arterial wall.

In normal histological sections of atherosclerotic lesions the presence of lipid is either inferred from empty spaces or detected...
with dyes such as oil-red-O and Sudan black (17), both of which alter the physical properties of lipid. Polarized light microscopy (PLM) however has the advantages of not only detecting lipid in its natural state, but also by determining some of the physical properties of the lipid (such as birefringence, morphology and melting) one can deduce the biochemical nature of the lipid (5). For example, what is thought to be cholesterol in plaques as inferred from "clefts" can really be confirmed only by PLM. In certain cases the clefts could well be artifacts.

As it has been mentioned, although PLM has been used to detect the presence of lipid in its various phases within lesions, it has been done in squashed tissue, thus destroying any chance of knowing the original site of the lipid. However, we have found that by rapidly freezing tissue in hexane at -70°C excellent sections could be obtained with good definition of the lipid and maintenance of its physical properties. This allows correlations to be made between histology and biochemistry at a level not possible with other techniques.

An example of the technique can be seen in the micrographs of a lesion taken from an atherosclerotic rabbit (Fig. 6). A section stained with hematoxylin & eosin (HE) shows marked intimal thickening with smooth muscle cells and early foam cell formation deep in the intima. A lipid stain of the adjacent section shows evidence of lipid deposition, which is confirmed by PLM. At higher magnification it is difficult to be certain if the lipid is intraor extracellular, and it also appears to be in large droplets. However, when viewed by a PLM the lipid is obviously intracellular in small discrete droplets (Fig. 7). Thus the CE-filled cells correspond to the foam cells seen on HE.

If the unstained section is then heated and observed under the PLM, melting temperatures of the droplets within each cell (or extracellular droplets as well) can be easily determined and photographically recorded. In the absence of impurities in the droplets the melting temperature of cholesterol ester is a reflection of the fatty acid composition (6). Thus, biochemical differences in cells throughout the lesion can be observed histologically. Within the early rabbit lesions the lipid appeared to melt fairly uniformly. This uniform melting is not surprising since it is known that cholesterol is initially deposited in foam cells predominantly as cholesterol olate (18). However, as lesions progress to intermediate lesions and fatty plaques, the ester composition changes (5) and this should be observable within certain regions of the lesion. These biochemical differences may also be correlated with other histological changes that may be occurring in progressive and regressive lesions. For instance, when appropriate cellular markers are used, the changes in lipid biochemistry in smooth muscle cells and macrophages can be followed. Furthermore, changes in lipid biochemistry may be in certain cells of intermediate lesions which may be precursors to cellular necrosis and development of the plaques.

Preparation of the tissue in this way also allows detection of enzymatic activity, which can also be correlated with lipid deposition and its physical properties. For instance, using a substrate to detect β-galactosidase (19), a lysosomal enzyme, we can see slight activity in normal rabbit artery within smooth muscle cells. However, following production of atherosclerosis, activity is greatly increased but only in regions of cholesterol ester deposition. In areas of intimal thickening without CE deposition only minimal amounts of activity are present. It would appear that the enhanced lysosomal activity is associated with CE-filled cells.

At present we are using the technique to study the effects of various diets on the biochemistry of lesions in the atherosclerotic rabbit. Obviously the technique can be used to study lesions in any animal model, including man. However, we have found that it is necessary to use fresh, unfixed tissue to get the best results, which requires being present at autopsy. By concurrently determining biochemical, biophysical, and histological changes on the same histological section, a greater understanding of the mechanism of progression and regression should be possible.
Fig. 6 A: Adjacent sections of rabbit artery showing intimal thickening and foam cell formation deep in the intima. B: An oil-red-O stain confirms the presence of lipids. C: Photograph taken under polarized light the birefringent areas correspond exactly to the lipid seen on oil-red-O.

Fig. 7 At high magnification, (X400) photomicrographs of the same section before and after staining for lipid. Polarized light microscopy (A) gives better definition of cellular origin of the lipid than does oil-red-O (B).
REFERENCES

DISCUSSION: RICHARD W. ST. CLAIR

Drs. Small and Waugh have already described some of the physical biochemical changes that take place in the lipids of the atherosclerotic lesion. I would like to summarize for you in a very simplified way some of the other biochemical changes that take place in the atherosclerotic lesion and, finally, attempt to give you some perspective as to the role of the cellular components of the arterial wall responsible for some of these changes.

The following is a summary of the major biochemical changes that are found in atherosclerotic arteries:

Increased permeability to macromolecules and perhaps blood monocytes

Cholesterol and cholesteryl ester accumulation (intra- and extracellular)

Increased cholesterol esterification and fatty acid synthesis

Increased cell proliferation and cell death

Increased synthesis and accumulation of connective tissue matrix (collagen, elastin, proteoglycan)

Necrosis and mineralization.

These by no means represent a complete list, but instead are meant to include those processes that appear to be related to the basic pathogenesis of the lesion.

The first of these changes is an increase in the permeability of the arterial wall to macromolecules such as low-density lipoprotein, and other plasma proteins. In addition, if the endothelial surface is examined by scanning electron microscopy, one frequently sees blood cells that can be identified principally as monocytes adhering to the endothelium at the growing edge of the raised lesions. This may be the forerunner of the entry of blood monocytes into the arterial wall, where they ultimately are converted into macrophages. The hallmark of atherosclerosis is the accumulation of cholesterol and cholesteryl esters, both intra- and extracellularly. This does not represent a simple accumulation of cholesterol and cholesteryl esters from lipoproteins, but rather the arterial wall plays a significant role in remodeling the composition of the cholesteryl esters that accumulate. This is done by initial hydrolysis of lipoprotein cholesteryl esters, followed by reesterification to fatty acids either synthesized by the arterial wall or derived from the plasma. As a result, one of the earliest biochemical changes to occur in the developing lesion is an increase in cholesterol esterification. Increased cell proliferation is also a prominent early feature of the developing lesion. This is associated with an increased rate of cell death, but obviously since cells accumulate in the atherosclerotic intima cell proliferation must initially exceed cell death. As the lesion progresses there is an increased accumulation of connective tissue components, including collagen, elastin, and proteoglycans. This is then followed somewhat later in the progression of the disease by necrosis and mineralization and, ultimately, other events such as ulceration, hemorrhage within the plaque, and thrombosis. The major components of the atherosclerotic lesion that are responsible for the mass of the atheroma are the increased number of cells, connective tissue, and in particular, the massive accumulation of cholesterol and cholesteryl esters.

What cells are responsible for the above described metabolic changes? If one looks at an electron micrograph of an atherosclerotic lesion at least two types of fat-filled cells known as foam cells are seen. A number of these foam cells are clearly smooth muscle cells as evidenced by the presence of a prominent basement membrane, numerous pinocytic vesicles, and large numbers of myofilaments. On the other hand, there are clearly other foam cells that are more difficult to identify. They do not have the above described characteristics of smooth muscle cells and, at least by morphologic and some biochemical criteria, probably represent macrophages. The proportion of foam cells derived from smooth muscle cells and of macrophages within the lesion can vary considerably, and there is little information to explain the reasons for this distribution.
Let's consider the specific biochemical roles of each of these three cell types. First of all, the endothelium plays a major role as a permeability barrier for cells and macromolecules entering the arterial wall. It now seems clear that in the developing atherosclerotic lesion one does not necessarily have to have frankly denuded endothelium in order to have alterations in endothelial permeability. Consequently, more subtle metabolic changes in the endothelium would appear to play a critical role in determining the integrity of this endothelial barrier. In addition, the endothelium serves as an antithrombogenic surface by insulating the blood components from tissue constituents such as collagen and by the production of prostacyclin that acts to inhibit platelet aggregation. Smooth muscle cells represent the major cellular component of the arterial wall. They possess receptors for the uptake of normal LDL and this uptake of LDL can result in the accumulation of cholesteryl esters within the cell. Although it is difficult to envision how such a highly regulated process might result in the pathologic accumulation of cholesteryl esters, there must be such mechanisms since smooth muscle cells with abnormal accumulations of cholesteryl esters clearly are present in the atheroma. Another important process in which smooth muscle cells participate is in the proliferation of cells within the intima. Smooth muscle cell proliferation occurs early in the pathogenesis of the developing lesion and is thought to be stimulated by a variety of factors, including platelet-derived growth factor as well as growth factors produced by both macrophages and endothelial cells. Smooth muscle cells are also thought to be the major cell type responsible for the synthesis of the connective tissue matrix of the arterial wall, although again endothelial cells have also been shown to synthesize certain types of collagen. What stimulates connective tissue synthesis is unknown. Macrophages are not a major cellular component of the normal arterial wall. As a result, their appearance in the atherosclerotic lesion is thought to result from the migration of blood monocytes into the arterial wall in response to certain unknown stimuli. Macrophages have receptors for the uptake of abnormal lipoproteins. These abnormal lipoproteins are taken up by the cell in massive amounts, since this receptor is very poorly down-regulated. Consequently, the macrophage can accumulate large amounts of cholesteryl esters and show substantial increases in cholesterol esterification. As a result, it is not clear whether the early increase in cholesterol esterification in the developing lesion can be attributed to macrophages or to smooth muscle cells or both. As mentioned previously, macrophages have also been shown to produce growth factors that can stimulate proliferation of both smooth muscle cells and endothelial cells. As a result, the effect of one cell type on another may play an important role in the pathogenesis of atherosclerosis.

In conclusion, it seems clear that the components that accumulate in the atheroma are the result of a complex interaction of plasma constituents with the cellular elements of the arterial wall. As a result, both the plasma-derived and cell-mediated processes must be understood if the pathogenesis of the disease is to be fully appreciated.
Triolein–Cholesteryl Oleate–Cholesterol–Lecithin Emulsions: Structural Models of Triglyceride-Rich Lipoproteins

Kurt W. Miller† and Donald M. Small∗

ABSTRACT: The organization of lipids within emulsions composed of triolein (TO), cholesteryl oleate (CO), cholesterol (C), and egg yolk phosphatidylcholine (L) was examined. CO was substituted for TO in a series of emulsions to obtain TO:CO ratios comparable to the triglyceride:cholesterol ester ratios observed in subfractions of triglyceride-rich lipoproteins. The weight fraction of TO in the surface phase (0.02–0.05) was independent of the TO content of the emulsions. However, the weight fraction of CO in the surface phase depended upon the percentage of CO in the emulsions and was <0.004 even when 13.7% CO was present in the emulsion. When CO was substituted for TO, the percent of the total particle C which was carried in the droplet oil phase was increased. The interparticle equilibration of lipids was studied in subfractions of sonicated emulsions with particle sizes comparable to triglyceride-rich lipoproteins. The TO:CO ratios of the subfractions of a given emulsion were constant and independent of size, but the C:L ratio decreased in particles of smaller diameter. However, the surface C:L ratio was the same in all particles from a given emulsion. The size dependence of the C:L ratios was attributed to the partitioning of C into the oil cores of the emulsions. Because large droplets have the greatest core:surface mass ratios, more of their total particle C is carried in the core.

Polydisperse chylomicrons (Fraser, 1970; Yokoyama & Zilversmit, 1965; Lassov et al., 1969) and very low density lipoproteins (VLDL)† (Sata et al., 1972; Eisenberg et al., 1973) can be fractionated by size into chemically heterogeneous subfractions. The chemical composition of lipoproteins within the subfractions is related to their particle diameters. Large lipoproteins contain a greater percentage of nonpolar lipids, triglyceride and cholesteryl ester, and smaller percentages of polar lipids, cholesterol (C) and phospholipid, than small lipoproteins. The triglyceride:cholesterol ester ratios within VLDL subfractions are size dependent; the ratios decrease in particles with smaller diameters. Within the plasma, both the hydrolysis of triglyceride by lipoprotein lipase (Redgrave, 1970; Mjösg et al., 1975) and, in humans, the transfer of cholesteryl ester into VLDL by transfer factors (Nichols & Smith, 1965; Nestel et al., 1979; Marcel et al., 1980) may generate the observed triglyceride:cholesterol ester ratio–size relationship. Some of the small VLDL may have circulated longer than large VLDL and may be enriched with cholesteryl ester by both mechanisms (Chajek & Fielding, 1978).

Triglyceride-rich lipoproteins and triglyceride emulsions stabilized by phospholipid have common structural features. Thus, the phase behavior of lipids within lipoproteins can be predicted from the behavior of biological lipids within emulsions. Triolein (TO)–C–egg yolk phosphatidylcholine (L) emulsions of differing starting compositions contain a surface monolayer consisting of L, 0–33% C, and 2–4% TO, by weight (Miller & Small, 1982). The emulsion oil core contains TO and up to 2% C at 22–24°C. Since triglyceride-rich lipoproteins have similar lipid compositions to those of the emulsions, they should contain small amounts of triglyceride in their surface phases and minor amounts of C in their cores.

While cholesterol ester is soluble in phospholipid bilayers (Janiak et al., 1974, 1979) and may adopt a conformation in phospholipid vesicles in which its carbonyl group is hydrogen bonded with surface water molecules (Hamilton & Small, 1982), it is unknown whether cholesterol ester is also soluble in the surface monolayer of emulsions. All of the cholesterol ester may partition into the nonpolar core of triglyceride which is in contact with the phospholipid monolayer. Furthermore, it is not known if the incorporation of cholesterol ester into the particle alters the equilibrium distribution of lipids between the surface and core. For example, C is more soluble in liquid cholesterol ester than in triglyceride [when solubility data are normalized to the same number of degrees above the isotropic phase transition temperature of the lipid (Small, 1970; Janack et al., 1977),] and therefore C may be more soluble in triglyceride:cholesterol ester oil mixtures than in pure triglyceride oils. Since the triglyceride:cholesterol ester ratio varies in VLDL subfractions and may become ≤1 in β-VLDL isolated from C-fed animals (Shore et al., 1974; Mahley et al., 1976; Noel et al., 1979), and type III hyperlipoproteinemic patients (Havel & Kane, 1973), emulsions with a wide range of triglyceride:cholesterol ester ratios should be studied to best describe the phase behavior of chylomicrons and VLDL.

We have examined the effects of substituting cholesteryl oleate (CO) for TO in emulsions upon the phase behavior of the lipids. The emulsion phases have been isolated by high-speed centrifugation and the data analyzed with the aid of phase diagrams (Miller & Small, 1982). The data show that TO and CO may compete for surface orientation in emulsions and that the incorporation of CO into the TO oil phase increases the solubility of C in this phase. The phase diagrams were used to calculate particle diameters and the percents of the total particle lipids present in each phase of the emulsion droplet and to study the equilibration of lipids between particles.

Experimental Procedures

Materials. L was purchased from Lipid Products (Nutfield Ridge, England). The acyl chain composition of the L has been reported previously and contains 36% 16:0, 11% 18:0, 27% 18:1, and 17% 18:2 (Miller & Small, 1982). TO, CO, and C were obtained from Nu Chek Prep, Inc. (Elysian, MN). All lipids were confirmed to be >99% pure as monitored by

† Abbreviations: C, cholesterol; CO, cholesteryl oleate; L, egg yolk phosphatidylcholine; TO, triolein; VLDL, very low density lipoprotein; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.
thin-layer chromatography. Radiolabeled \([9,10^{-2}H]_{1,3,1,4,5,6,7,8,9,10} \)-trioleglycerol, \([\text{o}1-^{14}C]_{1,3,1,4,5,6,7,8,9,10} \)-cholesterol, \([4-^{14}C]_{1,3,1,4,5,6,7,8,9,10} \)-cholesterol, \([7-^{14}C]_{1,3,1,4,5,6,7,8,9,10} \)-tolaune, and Aquasol liquid scintillation fluid were purchased from New England Nuclear, Inc. (Boston, MA). The radiochemical purities of the lipids were maintained at >98\% by preparative thin-layer chromatography. Bio-Sil HA (minus 325 mesh) silica gel was purchased from Bio-Rad Laboratories (Richmond, CA). Silanized glass wool was obtained from Applied Science Laboratories (State College, PA). Glass capillary tubes (1.1–1.2 mm i.d. \(\times 75 \) mm) were purchased from Sherwood Medical Industries, Inc. (St. Louis, MO). Organic solvents were redistilled before use, and water was twice distilled and deionized before use. All other chemicals were at least of reagent grade quality.

**Preparation of the Lipid Mixtures.** The specific activities of the radiolabeled lipids were measured by weighing dried aliquots of the solutions with a Cahn automatic electrolab (Model 25), Cahn Instruments, Inc. (Cerritos, CA), and by counting other dried aliquots in Aquasol in a Beckman LS-250 liquid scintillation counter, Beckman Instruments, Inc. (Fullerton, CA). In all experiments the lipid specific activities were 1220 dpm/\(\mu g\) for TO, 1050 dpm/\(\mu g\) for CO, and 1370 dpm/\(\mu g\) for C. Six mixtures (A–F) were prepared with total lipid compositions similar to the triglyceride-rich lipoproteins and TO:CO ratios within the range of triglyceride:cholesterol ester ratios found for lipoprotein subfractions. The mixtures were stored at \(-40 \) °C and used to prepare emulsions as needed.

**Preparation of the Coarse Emulsions.** Aliquots of the mixtures containing 75 mg of lipid were dried under \(N\) atm in 15 \(\times 45 \) mm glass vials and vacuum desiccated 12–16 h at 4 °C. Water was brought to pH 7 by boiling and was gassed with \(N\) atm until cooled to room temperature: 0.67 mL of water was added per sample, to make mixtures composed of 10% lipid, by weight. Emulsions were prepared by continuously vortexing the samples under \(N\) atm for 24 h at room temperature (22–24 °C). No lipid decomposition occurred during agitation, as monitored by thin-layer chromatography.

**Centrifugation of the Coarse Emulsions.** Following agitation, the emulsions were sampled (\(n = 3\)) to determine their chemical compositions. Aliquots (\(n = 3\)) of 65 \(\mu L\) were centrifuged in glass capillary tubes inside plastic adaptors made for use in a Beckman SW 41 swinging bucket rotor, Beckman Instruments, Inc. (Palo Alto, CA). Centrifugations were performed for 10–16 h at 20 000 rpm and 24 °C by using a Beckman Model L5-75 ultracentrifuge, Beckman Instruments, Inc. (Palo Alto, CA). After the first centrifugation period, the separated emulsion phases were removed from the capillary tubes. The oils, located at the top of the samples (region I) (Miller & Small, 1982), were removed and dissolved in chloroform/methanol (2:1). The surface lipids at the bottom of the tubes (region V) were removed, and each was resuspended in 65 \(\mu L\) of water by vortexing. Aliquots of 65 \(\mu L\) were taken from the surface phase suspensions and were transferred to a second set of three capillary tubes. The tubes were recentrifuged 10–16 h at 20 000 rpm and 24 °C. The surface material (\(n = 3\)) which sedimented during the second centrifugation was analyzed.

**Preparation of the Sonicated Emulsions.** Two emulsions were prepared by sonicating 100 mg of lipids taken from mixture B and mixture E. These emulsions are designated B, and B, to distinguish them from the coarse emulsions. The samples were dried and desiccated as above in 28 \(\times 61 \) mm glass vials. The lipids were sonicated under \(N\) atm in 12 mL of 150 mM NaCl, 5 mM Tris-HCl, 0.02% sodium azide, and 0.01% Na,EDTA, pH 7.4, buffer using a Branson sonifier (Model W-350), Branson Sonic Power Co (Danbury, CT) set at 100–110 W continuous power. The sample temperature was controlled with an ice-water bath. For emulsion B, lipids were dispersed by a 10-min sonication period at 33 °C. For complete dispersion of the lipids in emulsion F, the sample was sonicated 10 min at 33 °C and then 4 min above the phase transition temperature of CO (51 °C) (Small, 1970). The emulsions were cooled to room temperature before proceeding to the centrifugation steps.

**Subfractionation of the Sonicated Emulsions.** Following sonication and cooling, 100 \(\mu L\) of each emulsion was extracted (Folch et al., 1957). The remainder of emulsions B and F, were transferred into cellulose nitrate tubes and centrifuged in the SW 41 rotor. Centrifugations were performed by using the \(\omega^2\) integrator to count the rad\(2\) s\(^{-2}\) accumulated during centrifugation. These values were used to calculate the \(g(\omega)\) (min) of each step. The run conditions are summarized here:

1. (1) 10 min at 24 000 rpm \(\{8.8 \times 10^{3}\}g(\omega)\) min; (2) 10 min at 25 500 rpm \(\{1.0 \times 10^{4}\}g(\omega)\) min; (3) 70 min at 28 500 rpm \(\{7.2 \times 10^{3}\}g(\omega)\) min; 19 h at 33 000 rpm \(\{1.6 \times 10^{5}\}g(\omega)\) min.

The values for the \(g(\omega)\) (min) represent total values accumulated during the sequential steps. Following the first three centrifugations, approximately 1 mL of the cream was removed (fractions 1–3), and an equal volume of distilled water was layered on top of the remaining samples. After run 4, 2.5 mL of cream (fraction 4) and the infranatant and pellets (fraction 5) were taken for analyses.

**Lipid Analyses.** Lipids were dissolved in benzene/hexane (1:1) to give concentrations in the range 2–3 \(\mu g/\mu L\). The mass of L was determined by chemical assay (Bartlett, 1959), and the masses of TO and C were determined by double-label liquid scintillation counting. Samples from emulsions B, C, E, and F, which contained CO, were chromatographed on silicic acid to separate CO from TO and C prior to performing liquid scintillation counting. Lipids were fractionated on 4–5-cm Bio-Sil HA columns packed on glass wool inside long disposable Pasteur pipets. The columns were equilibrated with 5–10 mL of benzene/hexane (1:1) prior to applying the samples. CO was eluted in fraction I (6 mL) with benzene/hexane (1:1). The columns were then washed free of TO and C by passing 8 mL of diethyl ether (fraction II) through the column. Samples were collected directly in vials which were dried before adding Aquasol and counting. Under the elution conditions, L did not elute from the column. The recovery of all lipid classes was complete. Provided \(\leq 25 \mu g\) each of CO and C was applied, \(<0.1\%\) of the C mass appeared in fraction I and \(<0.3\%\) of the CO mass appeared in fraction II. Chromatography was performed so that the columns were not overloaded with sample, and hence cross-contamination of CO and C fractions were avoided.

**Characterization of Sample Morphology.** Samples of the emulsions or their separated phases were examined by polarized-light microscopy with a Zeiss NL polarized light microscope, Carl Zeiss, Inc. (New York, NY). Samples were examined either in situ within capillary tubes or on slides.

**Statistical Analyses.** The nonpaired \(t\) test and analysis of variance tests were applied to analyze the data. For the analysis of variance tests, values which differed significantly from others within the groups were identified by using Scheffe's multiple comparisons procedure (Snedecor & Cochran, 1967).

**Results**

**Physical Characteristics of the Coarse Emulsions.** Substitution of CO for TO in the emulsions did not alter the gross
Table I: Chemical Compositions of Coarse Emulsions and Their Phases: Group I

<table>
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<th>TO</th>
<th>CO</th>
<th>C</th>
<th>L</th>
<th>TO</th>
<th>CO</th>
<th>C</th>
<th>L</th>
<th>TO</th>
<th>CO</th>
<th>C</th>
<th>L</th>
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<td>A</td>
<td>79.1</td>
<td>2.0</td>
<td>18.9</td>
<td></td>
<td>99.6</td>
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<td></td>
<td></td>
<td>9.4</td>
<td></td>
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<tr>
<td>B</td>
<td>76.8</td>
<td>2.3</td>
<td>21.7</td>
<td></td>
<td>96.3</td>
<td>3.1</td>
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<td></td>
<td>4.7</td>
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<tr>
<td>C</td>
<td>64.4</td>
<td>13.7</td>
<td>28.1</td>
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<td>1.2</td>
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<td></td>
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</tr>
</tbody>
</table>

*Values represent the percent, by weight, ± 1 SD from the mean (n = 3). The sum of TO + CO, the nonpolar lipids (N). The surface samples for emulsion C were pooled (n = 3) for chemical analyses.

Table II: Chemical Compositions of Coarse Emulsions and Their Phases: Group II

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<th>CO</th>
<th>C</th>
<th>L</th>
<th>TO</th>
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<td>0.4</td>
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<td>0.01</td>
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<td></td>
<td>0.2</td>
<td></td>
<td>0.3</td>
<td>0.2</td>
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<td>E</td>
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<td>17.5</td>
<td></td>
<td>95.8</td>
<td>3.2</td>
<td>0.94</td>
<td></td>
<td>1.8</td>
<td>0.05</td>
<td>24.4</td>
<td>73.8</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.1</td>
<td>0.5</td>
<td></td>
<td>0.05</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
<td>0.1</td>
<td>0.01</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>F</td>
<td>64.6</td>
<td>5.8</td>
<td>15.9</td>
<td></td>
<td>79.1</td>
<td>19.1</td>
<td>1.79</td>
<td></td>
<td>3.1</td>
<td>0.28</td>
<td>25.2</td>
<td>71.4</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
<td></td>
<td>0.06</td>
<td>0.05</td>
<td>0.01</td>
<td></td>
<td>0.1</td>
<td>0.01</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>78.3</td>
<td>0.2</td>
<td></td>
<td></td>
<td>98.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values represent the percent, by weight, ± 1 SD from the mean (n = 3). The sum of TO + CO, the nonpolar lipids (N).

morphological features of the samples from those observed in simpler TO–C–L–water emulsions (Miller & Small, 1982). CO needles, C monohydrate plates (Loonis et al., 1979), and myelinated figures (Small, 1967) were not observed by polarized-light microscopy after the samples had been agitated for 24 h at 22–24 °C. For emulsions C and F, which contained the most CO (13.7%, by weight) and had the lowest TO:CO ratios (4.7), the CO was dissolved in the TO oil and was not present in liquid-crystalline droplets (Small, 1970).

The constituent oil and surface phases of the emulsions were isolated by centrifugation. Samples formed five distinct regions within the capillary tubes as has previously been described (Miller & Small, 1982). Region I, the oil which floats to the top of the sample, was obtained in homogeneous state with one centrifugation. Region V, the surface phase, collected at the bottom of the centrifuge tube. This L-rich material was birefringent and has been shown to be composed of multilamellar bilayers which are formed from droplet surface monolayers after droplets coalesce (Miller & Small, 1982). The surface lipid fraction contained contaminating oil lipids after a single centrifugation step (data not shown). The oil was removed by resuspending the lipids recovered in region V in water and recentrifuging the suspension. The contaminating oil floated during the second centrifugation, and the purified surface phase sedimented and was recovered for analyses. This procedure has no effect upon the lipid composition of the surface phase (Miller & Small, 1982).

The chemical compositions of the emulsions and their oil and surface phases are presented in Tables I and II. Lipid ratios were calculated from the data and are presented in Table III. The general experimental design will now be summarized by using these tables for reference.

Two groups of emulsions with lipid compositions similar to those of nascent (Green et al., 1979; Swift et al., 1980), group

Table III: Lipid Ratios Calculated from the Data for Emulsions in Groups I and II

<table>
<thead>
<tr>
<th>sample</th>
<th>emulsion, TO:CO</th>
<th>oil, TO:CO</th>
<th>surface, TO:CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>group I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.051</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.055</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Values for the mean ± 1 SD (n = 3) lipid ratios. **TO:CO ratios are not significantly different (P > 0.05) according to the nonparametric test. *Surface TO:CO ratios differed (P < 0.05) from those of the emulsion and oil according to the multiple comparisons procedure.

I (Table I), and plasma (Deckelbaum et al., 1977b), group II (Table II), triglyceride-rich lipoproteins were prepared. Group I emulsions (A, B, and C) were relatively C poor (2-3%) C, whereas group II emulsions (D, E, and F) were C rich (5-6%) C. For each emulsion, the sum of the nonpolar lipids (N = TO + CO) was ~80% of the total lipid mass. CO was substituted for TO in emulsions B, C, E, and F to obtain a range of TO:CO ratios within both groups (Table III) which is comparable to the range of triglyceride:cholesterol ratios found in subfractions of human triglyceride-rich lipoproteins (Sata et al., 1972).

Tables I and II show that substitution of CO for TO increased the weight fraction of C in the oil phases. The increase
Table IV: Surface/Oil Phase Distribution Ratios and Standard Free Energies of Phase Transfer for the Coarse Emulsion Lipids

<table>
<thead>
<tr>
<th>Emulsion Group</th>
<th>(k_{TO})</th>
<th>(k_{CO})</th>
<th>(k_C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I A</td>
<td>0.044</td>
<td>0.0001</td>
<td>27.8</td>
</tr>
<tr>
<td>B</td>
<td>0.048</td>
<td>0.019 (-2.5)</td>
<td>18.2</td>
</tr>
<tr>
<td>C</td>
<td>0.065</td>
<td>0.018 (-2.5)</td>
<td>7.5</td>
</tr>
<tr>
<td>II D</td>
<td>0.019</td>
<td>0.002</td>
<td>38.5</td>
</tr>
<tr>
<td>E</td>
<td>0.018</td>
<td>0.015</td>
<td>26.1</td>
</tr>
<tr>
<td>F</td>
<td>0.039</td>
<td>0.015</td>
<td>14.1</td>
</tr>
</tbody>
</table>

\(k_{TO}, k_{CO}\), and \(k_C\) were calculated by using eq 1. \(\Delta G_{surf-oil}\) was calculated from their distribution ratios and are listed in Table IV. These values were obtained by using the equation

\[
RT \ln \left( \frac{\bar{x}_{i,surf}}{\bar{x}_{i,oil}} \right) = \mu_{i,surf} - \mu_{i,oil} = \Delta G_{surf-oil}^{\circ}
\]

where \(\bar{x}_{i,surf}/\bar{x}_{i,oil}\) is the ratio of the mole fractions of a lipid in the surface and oil phase, and \(\mu_{i,surf}\) and \(\mu_{i,oil}\) are the standard chemical potentials of the lipid in each phase (Glasestone & Lewis, 1963). Values for \(\Delta G_{surf-oil}^{\circ}\) were not calculated for TO since it is the oil phase solvent.

The signs of the free energy changes of phase transfer indicate that the emulsions are stabilized by the movement of C to the interface and CO to the core of the droplets. The magnitudes of the free energy changes for C transfer are less than that for the transfer of C between water and hydrocarbon solvents (Gilbert et al., 1975). If the orientation of C within the surface monolayer is similar to its orientation within phospholipid air-water monolayers (Shah & Schultian 1967) and phospholipid bilayers (Lecuyer & Dervichian, 1969), then the standard chemical potential of C within the surface monolayer would be expected to be less than that for C dissolved in water.

Characterization of the Sonicated Emulsion Subfractions. So that the effect of the buffer electrolytes on the solubility of the lipids in the emulsion phases could be studied, a series of coarse TO–C–L emulsions were prepared in Tris buffer, and their phases were isolated by centrifugation. The phase solubilities of the lipids were not changed by the presence of Tris (0.005 M) and NaCl (0.15 M) (data not shown). In agreement with these data, the solubility to TO in L vesicles is independent of the buffer salt concentration in the range 0.5–2.0% KCl (Hamilton & Small, 1981).

Two sonicated emulsions with compositions similar to lymph (emulsion B1) and plasma (emulsion F1) triglyceride-rich lipoproteins were prepared in order to study interparticle equilibration of lipids. Sonication of the lipids produced finer dispersions of droplets than were obtained by vortexing the samples. Possibly because inorganic salts were present in these samples or because smaller droplets are less likely to be unstable in concentrated creams (Vold & Groot, 1962, 1964), the emulsions did not coalesce during centrifugation.

The compositions of emulsions B1 and F1 and their subfractions isolated by sequential centrifugation steps are shown in Table V. The average size of droplets in the subfractions declines from a maximum in fraction 1 to a minimum in fraction 5. Therefore, the masses of the major core components, TO and CO, increase, and the masses of the major surface components, C and L, increase as the droplets become smaller. Because the compositions of the unfractionated emulsions are determined by the weighted average compositions of the particles present in their subfractions, the composition of the emulsion is intermediate to those of the extreme subfractions.

As shown in Table V, the TO:CO ratios are constant and independent of particle size. Therefore, the oil phases of all droplets in the emulsions appear to be the same. The study of these results using phase diagrams reveals why the C:L ratios of the subfractions decline from fraction 1 to fraction 5.

Study of Interparticle Lipid Equilibration Using Triangular Coordinate Diagrams. It is first necessary to discuss how the sample compositions which contain four lipid components can be plotted on triangular coordinate diagrams. TO and CO behave similarly in the emulsions; they constitute only a small weight fraction of the surface phase and are predominantly carried in the oil phase. Therefore, the major oil phase lipids,
Table V: Sonicated Emulsion B₁ and F₁ Subfraction Compositions

<table>
<thead>
<tr>
<th>subfraction</th>
<th>TO</th>
<th>CO</th>
<th>N</th>
<th>C</th>
<th>L</th>
<th>TO:CO</th>
<th>C:L</th>
</tr>
</thead>
<tbody>
<tr>
<td>B₁</td>
<td>63.5</td>
<td>13.8</td>
<td>77.3</td>
<td>5.7</td>
<td>17.0</td>
<td>4.6</td>
<td>0.34</td>
</tr>
<tr>
<td>1</td>
<td>68.3</td>
<td>14.7</td>
<td>83.0</td>
<td>4.8</td>
<td>12.2</td>
<td>4.6</td>
<td>0.39</td>
</tr>
<tr>
<td>2</td>
<td>64.5</td>
<td>13.8</td>
<td>78.3</td>
<td>5.7</td>
<td>16.0</td>
<td>4.7</td>
<td>0.36</td>
</tr>
<tr>
<td>3</td>
<td>55.0</td>
<td>11.4</td>
<td>66.4</td>
<td>7.5</td>
<td>26.2</td>
<td>4.8</td>
<td>0.29</td>
</tr>
<tr>
<td>4</td>
<td>13.5</td>
<td>2.9</td>
<td>16.4</td>
<td>16.9</td>
<td>66.7</td>
<td>4.7</td>
<td>0.25</td>
</tr>
</tbody>
</table>

a Weight percent values are listed. b Nonpolar lipids, N = TO + CO. c Lipid weight ratios. d Unfractionated emulsion compositions.
Subfractions 1–4, isolated by sequential ultracentrifugation steps. f The combined infranatant and pelleted vesicle fraction obtained after the final centrifugation.

TO and CO, will be combined and plotted as the nonpolar lipid component, N, which is assigned to the lower left apex of the triangular coordinate diagrams. C and L will remain at the upper and lower right apices, respectively, as in the phase diagram of TO–C–L–water (Miller & Small, 1982). Combining TO and CO and treating them as one component is reasonable because the oil phase TO:CO ratios are the same in all droplets in a given emulsion (Table V).

The data for the coarse emulsions (Tables I and II) are plotted in Figures 1 and 2, respectively. The oil (O), emulsion (E), and surface (S) compositions determine tie lines (OES) in the two-phase region of the diagrams. Only one tie line per group of emulsions is shown, but as revealed by the figure insets, the oil phase compositions vary, and a distinct tie line can be drawn for each emulsion. The slopes of these tie lines relative to the N–L base of the diagram decrease as the weight fractions of CO in the emulsions (and therefore, the % C in the oil phases) increase. The figures show that the surface compositions (S) are similar within each group, and that by fixing N ≈ 80%, the emulsion compositions (E) plot close to one another.

The compositions of the sonicated emulsion subfractions are plotted in Figure 3. The data points delineate a tie line for each emulsion. Since the compositions of the emulsion fractions fall on a single tie line, we conclude that C establishes interparticle and particle surface-to-core equilibrium in these sonicated emulsions as in coarse TO–C–L–water emulsions described earlier (Miller & Small, 1982). Furthermore, TO and CO are in equilibrium between particles because the TO:CO ratios of the subfractions (Table V) are constant.

FIGURE 1: Plot of the compositions of the emulsions in group I. The compositions of TO and CO have been combined and plotted as a single component, the nonpolar lipids (N), which is located at the lower left apex of the diagram. (Inset) The oil phase compositions. (•) Emulsion A, (●) emulsion B, and (○) emulsion C; O = oil phase compositions, E = emulsion compositions, S = surface compositions, L = lecithin, and C = cholesterol.

FIGURE 2: Plot of the compositions of the emulsions in group II. (Inset) the oil phase compositions. (A) Emulsion D, (O) emulsion E, and (D) emulsion F.

FIGURE 3: Plot of the compositions of subfractions isolated from sonicated emulsions B₁ (●) and F₁ (○). Fractions 1–4 are the creams isolated by sequential centrifugation steps. Fraction 5 is the remaining infranatant and resuspended pelleted vesicle fraction. (Inset) Oil phase compositions (O) (obtained by extrapolation). (Symbols) N = TO + CO, L = lecithin, C = cholesterol, E = emulsion compositions, and S = surface phase compositions (obtained by extrapolation).

The tie lines in Figure 3 were extrapolated to intersect the oil phase boundary on the N–C edge of the figure and the surface phase boundary, established by the study of the six coarse emulsions as a line drawn with N:L = 0.05. The oil and surface TO:CO ratios of emulsions B and F were used to calculate the weight fractions of TO and CO found in the sonicated emulsion phases.

For emulsion B₁, the oil phase contains 99.4% N (96.3% TO and 3.1% CO) and 0.6% C. The surface phase of B₁ contains 4.5% N (4.4% TO and 0.06% CO), 7.5% C, and 88% L. For emulsion F₁, the oil phase contains 98.2% N (79.1% TO and 19.1% CO) and 1.8% C. The surface phase of F₁ contains 3.0% N (2.75% TO and 0.25% CO), 19.5% C, and 77.5% L.

The percent distribution of the lipids between the surface and oil phases can be calculated by using the value of K, eq...
For each lipid and the ratio of the surface-oil masses, \( M_s/M_o \), in the emulsion. Values of \( M_s/M_o \) can be determined for an emulsion of known composition. For determination of \( M_s/M_o \), the lengths of the tie line segments of E and ES on the phase diagram (Figure 3) are measured. According to the inverse lever law (Findlay, 1951), the ratio of \( M_s/M_o \) is
\[
M_s/M_o = \text{OE:ES} \quad (3)
\]
Then for C, the ratio of the amount of total particle C carried in the surface phase to that carried in the oil phase is
\[
K_C(M_s/M_o) = X_C / X_C \quad (4)
\]
where \( X_C \) and \( X_C \) designate the fractions of the total emulsion C carried in the surface and oil phases, respectively. Finally, the percent of the total emulsion C carried in the surface phase is given by
\[
\% C_s = \frac{X_C X_C}{1 + X_C X_C} \times 100 \quad (5)
\]
and the percent of the total particle C carried in the oil phase is given by
\[
\% C_o = 100 - \% C_s \quad (6)
\]
Note that to perform these calculations, particle diameters need not be measured.

Emulsion droplet diameters can also be calculated by using the values of \( M_s/M_o \) measured on the phase diagram. Three assumptions must be made to perform these calculations. First, the densities of the lipids in the phases are equal to the densities of the bulk lipids. Second, the lipid densities are constant even upon mixing, so that the weight fraction, or average, densities of the phases can be calculated. Third, the emulsion droplets are composed of two distinct phases—an oil core and a surface monolayer of 20 Å thickness.\(^2\)

The calculation of particle diameters using values for \( M_s/M_o \) will now be described. The weight fraction densities of the phases, \( P_o \) and \( P_s \), are calculated from the relations
\[
P_o = x_{TO} \rho_{TO} + x_{CO} \rho_{CO} + x_{CS} \rho_{CS} \quad (7a)
\]
\[
P_s = x_{TO} \rho_{TO} + x_{CO} \rho_{CO} + x_{CS} \rho_{CS} + x_{L} \rho_{L} \quad (7b)
\]
where \( x_{TO} \) etc. are the weight fractions of the lipids found in the phases, and \( \rho_{TO} \) etc. are the densities of the lipids.\(^1\) When the \( M_s/M_o \) values are multiplied by the ratios of the oil/surface phase lipid densities, \( P_o/P_s \), the surface-oil volume ratios, \( V_o/V_s \), are obtained:
\[
V_o/V_s = (M_s/M_o)(P_o/P_s) \quad (8)
\]
For determination of the radius of a particle with the calculated phase volume ratio, the equation
\[
V_o/V_s = (r_o^3) - (r_o^3) \quad (9)
\]
is solved for values of \( r_o \), the radius measured from the center of mass of the spherical particle to the surface–water interface, and \( r_c \), the radius measured from the center to the oil–surface boundary. The third assumption fixes \( r_o = r_c = 20 \) Å.

The weight-average particle diameters (D) of the emulsion droplets in the subfractions listed in Table VI were determined by measurement of their \( M_s/M_o \) ratios from Figure 3 and solution of eq 7–9. The values of D (Table VI) decrease as the \( M_s/M_o \) ratios increase. The results indicate that lipoprotein-sized emulsion droplets were produced by the brief (<15 min) sonication periods. However, some vesicles were produced by sonication, and they were included in the infranatant fraction (fraction 5). Therefore, fraction 5 contains excess surface phase material in addition to a population of microemulsion droplets. Consequently, the calculated diameters of emulsion droplets in fractions 5 were too small (<100 Å). For calculation of the mean diameter of the microemulsion particles from the compositions of fraction 5, the contribution of the vesicle lipids in fraction 5 would have to be determined and subtracted.

Values for \( M_s/M_o \) for the subfractions and \( K_C \) for the lipids were substituted into eq 4–6 to determine the percent phase distributions of lipids in emulsions B\(_s\) and F\(_s\) (Table VII). The results show the >97.8% of the TO and CO were carried in the oil phases of droplets floated in fractions 1–4 (S\(_f\) > 20) (Dole & Hamlin, 1962). The high % TO\(_s\) and % CO\(_s\) values for fraction 5 are due to the large mass of vesicles in these fractions. Substantial (>20%) amounts of the total droplet C were carried in the oil phases of droplets with D > 530 Å in fractions 1–3 (Tables VI and VII).

The molecular compositions of the weight-average diameter particles in fractions 1–4 (Table VIII) were calculated from the particle diameters (Table VI) and surface and core chemical compositions (Figure 3). Lipid compositions of the

---

\(^2\) If the molecular volume of L is 1267 Å\(^3\)/molecule (Small, 1967), then a 20 Å thick monolayer would give a mean area per L molecule of 63 Å\(^2\). This is similar to the surface area per L molecule in L bilayers (Small, 1967), and in condensed L monolayers (Shah & Schulman, 1967).

\(^1\) Lipid densities are listed for 23 °C and 1 atm of pressure from data derived from the following sources: \( \rho_{TO} = 0.913 \) g/mL (Singleton, 1963); \( \rho_{CO} = 0.96 \) g/mL (Dyer & Edmonds, 1974); \( \rho_{CS} = 1.045 \) g/mL (Craven, 1976); and \( \rho_{L} = 1.016 \) g/mL (Small, 1967).
particle surface and core regions were converted from weight fraction to volume fraction values. They were multiplied times the respective volumes of the surface and core regions to obtain the volumes \( (\text{Å}^3) \) occupied by each class of molecule. Then these were divided by the molecular volumes to obtain the number of molecules per region of the droplet. While the total number of molecules representing a lipid class is greater in both phases of large compared to small particles, the surface and core chemical compositions of all particles within the emulsions are invariant. Only the percent of the total particle lipid classes carried in each phase is size dependent.

By use of the graph presented in Figure 3, the relationship between particle diameter and C:L ratio (Table V) can be explained. At equilibrium all particles in an emulsion have the same surface composition (given by point S, Figure 3) and thus the same surface C:L ratio. These have been calculated from the data in Figure 4 and are 0.08 = C:L (emulsion B), and 0.25 = C:L (emulsion F). Therefore, the size dependence of the particle C:L ratios can be attributed to the partitioning of C into the oil phase. Because large droplets have the greatest core:surface mass ratios, more of their total particle C is carried in their cores, and thus their particle C:L ratios are higher than those of smaller droplets.

The percent phase distributions of lipids in emulsions A–F were calculated over the range of diameters of 100 Å to 1 cm (Figure 4). The shape of these curves is dependent upon the value of \( k^3 \), the cubed particle radius. The substitution of CO for TO in the emulsions shifted the position of the curves to higher C\(_0\) values for a given particle diameter. Droplets with \( D = 1000 \text{ Å} \) (emulsion C) carry 50% of their total particle C in the oil phase. Plots of the values for the calculated droplet diameters vs. \% TO\(_0\) and \% CO\(_0\) for emulsions B and F are presented in Figure 5. Droplets with \( D > 200 \text{ Å} \) \((S > 20)\) have <5% of their total TO and <2% of their total CO molecules in their surface phases.

Discussion

When CO is substituted for TO in the emulsions, the value of the surface:oil C distribution ratios (Table IV) were decreased. Therefore, the incorporation of CO into the oil phase increased the affinity of C for the oil. When the percentage of CO in the oil is raised from 0 to 19%, an additional 15–30% of the total particle C is shifted into the oil phase of droplets with diameters of 0.1–1.0 μm (Figure 4). On the basis of the data in Tables I and II, incorporation of >19% CO into the oil should further increase the solubility of C in the oil. The
oil phase solubility of C should increase up to the maximum solubility of CO in TO (23%, by weight) at 22–24 °C (Jandacek et al., 1977). It is not known if the observed increase in the solubility of C in the oil phase is specifically dependent upon the presence of TO and CO, or whether other triglyceride–cholesterol ester–C combinations behave similarly.

Because polyunsaturated cholesterol esters are more soluble in TO at 22–24 °C, the maximum solubility of C in TO–cholesterol ester oils might be increased by substituting lower melting cholesterol esters for CO.

The molecular basis for the dependence of the C content of the oil (Tables I and II) and the free energy of transfer of C between phases (Table IV) upon the oil CO content is not known, but we shall speculate. Oils which contain CO may act as better solvents if their short-range structure allows for more favorable interactions between the solvent molecules and C. The solute–solvent interaction energies may be lower in oils containing C, and the chemical potential of C monomers dissolved in these oils would decrease relative to the chemical potential of C in the surface. Thus, a greater proportion of the emulsion droplet C molecules would partition into the oil.

Alternatively, the substitution of CO for TO may promote the formation of C–C association complexes within the oil phase. We have predicted that C molecules dimerize when the concentration of C in pure TO oil phases is above 1%, by weight (Miler & Small, 1982). Higher order complexes are formed between C molecules in simple organic solvents, and the size and stability of the complexes depend upon the solvent polarity (Parker & Bhaskar, 1968; Fehér et al., 1974; Foster et al., 1981). Presumably, the complexes are stabilized by hydrogen bonds between the 3-hydroxy groups of linked molecules. It should be noted that, if C molecules form complexes in the oil phases, then the free energy of transfer of C between phases cannot be calculated by using eq 2. Instead, a form of eq 2 modified to account for the size of the association complexes would be required (Glastone & Lewis, 1963).

As shown in Tables I and II, a small percentage of CO was soluble in the emulsion surface phases. The amount present in the surface phase appears to be strictly dependent on the percentage of CO in the emulsion, because values for \( k_{CO} \) were the same whether there was 3% CO or 13% CO in the emulsions (Table IV). Cholesterol esters may adopt several possible conformations in phospholipid bilayers (Janiak et al., 1979). Cholesterol esters may interdigitate between phospholipid acyl chains or may fold to allow their carbonyl groups to hydrogen bond with surface water molecules. In the emulsion surface, cholesterol esters may adopt the latter conformation.

The solubility of CO in the surface phases of emulsions C and F (≈0.4%) is only 10–20% of that reported for the solubility of cholesteryl linoleate and cholesteryl myristate in phospholipid multilamellar bilayers (Janiak et al., 1974, 1979) and 25% of the solubility of CO in L vesicles (Hamilton & Small, 1982). Because the solubility of TO in the surface (2–5%) (Tables I and II) was much greater, the results suggest that TO adopts a lower energy conformation than CO in the interface. The acyl chains of TO could be oriented parallel to the phospholipid acyl chains, while the carbonyl region remains hydrogen bonded with the surface water molecules (Hamilton & Small, 1981).

A method has been presented for the calculation of droplet diameters from chemical compositions by using the triangular coordinate diagram and eq 7–9. This method takes into account that lipids distribute between the emulsion phases and thus is an improvement on the method of calculation which assumes that TO and CO partition exclusively into the oil and C and L partition exclusively into the surface phase regions. For large droplets, \( D > 1000 \) Å, the diameters calculated by using the latter assumptions are considerably smaller (<10%) than those calculated by using eq 7–9. Because the oil contains a significant number of molecules of C (Table VIII), the ratio of oil/surface masses for the droplet is increased, and therefore, particle diameters are larger. Of course the absolute accuracy of either method of calculating diameters is dependent upon the assumptions concerning the lipid densities and the thickness of the surface phase.

Since CO was incorporated into the surface phase of the emulsions, we predict that a small mass of cholesterol ester will partition into the surface monolayer of triglyceride-rich lipoproteins. Surface cholesterol ester molecules may be the preferred substrates of cholesterol ester transfer proteins. Because the surface CO concentration was related to the weight fraction of cholesterol ester in the emulsion (Tables I and II), in triglyceride-rich lipoprotein subfractions, greater surface concentrations of cholesterol ester may be found in the smaller particles because these contain higher proportions of cholesterol ester (Sata et al., 1972; Eisenberg et al., 1973). If cholesterol esters equilibrate between subfractions of plasma VLDL, then the higher surface concentration of cholesterol ester in the small fractions may direct the transfer of cholesterol ester from small to large particles. Furthermore, reciprocal transfer of cholesterol ester and triglyceride between

\[ p > 1.006 \text{ g/mL lipoproteins and VLDL} \] (Nichols & Smith,
TRIOLEIN-CHOLESTERYL OLEATE EMULSIONS

1965; Chajek & Fielding, 1978) may occur because the surface concentration of cholesterol ester in the higher density cholesterol ester rich lipoproteins (2% by weight; Deckelbaum et al., 1977a,b) is greater than in LDL. The transfer protein would encounter more triglyceride than cholesterol ester molecules per unit area of the LDL surface than in the higher density lipoprotein surface.

Some of the above hypotheses may be tested by using these model emulation systems and purified transfer proteins. We have shown that emulsions with accurately defined surface and core compositions can be prepared to represent nascent, plasma, remnant, and β-migrating triglyceride-rich lipoproteins. Cholesterol ester rich microemulsion particles with physical properties resembling the smaller cholesterol ester rich lipoproteins can also be prepared (Ginsburg et al., 1982). The transfer of individual lipids between mixtures of these different types of apoprotein-free particles can be used to test the importance of both transfer proteins and lipoprotein apoproteins.

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Registry No. TO, 122-32-7; CO, 303-43-5; C, 57-88-5.

References

Fraser, R. (1973) J. Lipid Res. 11, 60–65.

Reassembled Plasma Low Density Lipoproteins
PHOSPHOLIPID-CHOLESTEROL ESTER-APPROTEIN B COMPLEXES

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Reassembled low density lipoprotein (LDL) complexes have been prepared by the interaction of lipoprotein B (apoB) of native human LDL with preformed, 200 Å in diameter, microemulsions of cholesteryl oleate (CO), surface-stabilized by either egg yolk phosphatidylcholine (EYPC) or dimyristoyl phosphatidylcholine (DMPC). Gel chromatography of PC/CO/apoB complexes shows co-elution of the complex at 43% PC, 43% CO, and 14% apoB. Negative stain electron microscopy shows the particles to be circular, homogenous, and approximately 200 Å in diameter. PC/CO/apoB complexes exhibit β-migration on agarose gels and show one high molecular weight protein band on 3.0% sodium dodecyl sulfate-polyacrylamide gels.

Differential scanning calorimetry and x-ray scattering show the lipids in the complexes to undergo at least two specific thermal transitions depending on lipid composition, one associated with the core-located cholesterol esters similar to LDL and the protein-free microemulsions and the other from the phospholipid forming the surface monolayer. In addition, particle disruption-protein unfolding/denaturation occur reversibly at 80–85 °C.

At 4 °C, the secondary structure of apoB on complexes of EYPC/CO/apoB is similar to that of native LDL. For complexes of DMPC/CO/apoB, the secondary structure shows less α-helix which correlates with the difference in surface lipid environment.

The reassembled complexes of PC/CO/apoB provide a defined system in which the components may be varied systematically in order to study the molecular organization, molecular interactions, and metabolism of LDL.

Low density lipoprotein, the primary cholesterol transport vehicle in the blood, has been clearly implicated in the etiology of atherosclerosis. Physical studies on LDL1 are consistent with a model for the lipoprotein as being a microemulsion, ~220 Å in diameter, consisting of a neutral lipid core (cholesterol ester (45 weight %), triglycerides (3 weight %)) surface-stabilized by a monolayer of polar phospholipids (22 weight %) and cholesterol (10 weight %) and a single protein (apoB (20 weight %)). The core-located lipids have been shown to undergo a liquid crystal—liquid transition near body temperature, the actual transition temperature being determined by both the diet-dependent fatty acyl chain composition of the cholesterol esters in monkeys and the cholesterol ester/triglyceride ratio in humans and monkeys (1–3). In animal models, atherogenesis is positively correlated with an ordered physical state of the core lipids at body temperature (4–6).

Essential to understanding the atherogenicity of LDL is the elucidation of the precise lipid-lipid and lipid-protein interactions which determine the physical properties and structure of the particle, influence its uptake by cellular receptors, and direct its intracellular catabolism. To this end, Kreiger et al. (7) have reported the reconstitution of LDL and replacement of endogenous core lipids with retention of full biological activity (in vitro). However, to fully understand the role of each molecular component in LDL metabolism, a model system is needed in which not only can the cholesterol esters be systematically varied (with respect to degree of unsaturation and chain length) but also the phospholipid and apoB complement.

We have recently described a method for the complete solubilization of apoB from native human LDL and the recombination of apoB with phospholipid using sodium deoxycholate (8). We have also described the development of a protein-free microemulsion system with the size and lipid organization of LDL in which the phospholipid components and cholesterol ester components can be systematically varied (9). We now report the recombination of solubilized LDL apoB with phospholipid/cholesterol ester microemulsions to yield reassembled LDL particles which exhibit many of the structural properties of native LDL.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were standard reagent grade unless otherwise indicated. Sodium ["C]deoxycholate was purchased from Amersham (Arlington Heights, IL). Sepharose CL-4B and all chromatographic materials and columns were products of Pharmacia Fine Chemicals (Uppsala, Sweden). High molecular weight protein standards and reagents for polyacrylamide gel electrophoresis were electrophoresis purity grade and purchased from Bio-Rad (Richmond, CA).

Methods

Lipoprotein Isolation—Plasma was obtained from freshly drawn blood from normal human volunteers after an overnight fast. 0.01% disodium EDTA and 0.02% sodium azide were added to plasma. LDL
with the diameter calculated from the partial specific volumes (21, 22) and mass fractions of the protein and lipid components.

As shown in Fig. 2, 3.0% NaDodSO₄-polyacrylamide gels of LDL (Fig. 2A), LDL dialyzed against standard buffer for 36 h as for the complexes (Fig. 2B), and both DMPC/CO/apoB and EYPC/CO/apoB complexes (Fig. 2, C and D) showed one co-migrating high molecular weight band (8). ApoB of native LDL and apoB of both DMPC/CO/apoB and EYPC/CO/apoB complexes migrate identically when they were mixed and co-electrophoresed on the same gel (Fig. 2, E and F). NaDodSO₄-polyacrylamide gels (7.5%) of similar column fractions performed up to 2 weeks after complex formation show no degradation of apoB (i.e., all the protein that is applied to the gel remains at the top of the gel with no smaller molecular weight bands appearing over the time course of the
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36 Å⁻¹ which is absent from the scattering of LDL at 45 °C (data not shown). These data, together with differential scanning calorimetry and x-ray studies on neat cholesterol esters and the extracted cholesterol esters of LDL (1-3), provide evidence that the thermal transition of LDL is a smectic-like to disordered transition of the core-located cholesterol esters.

Similar to LDL, differential scanning and x-ray scattering studies have shown that a broad low enthalpy transition at 42 °C in EYPC/CO microemulsions (ΔH = 0.6 cal/g of CO) (Fig. 3B) and at 46 °C in DMPC/CO microemulsions (ΔH = 0.7 cal/g of CO) (Fig. 3D) is due to the order-disorder transition of the core-located cholesterol esters. Additionally, a high enthalpy transition (ΔH = 4.9 cal/g of DMPC) at 25 °C observed in DMPC/CO microemulsions (Fig. 3D) has been shown to be associated with an order-disorder transition of the fatty acyl chains of the surface monolayer of DMPC (9).

EYPC/CO/apoB complexes (Fig. 3C) undergo a single fully reversible low enthalpy transition between 32 and 47 °C with a peak transition temperature at 36 °C and an enthalpy of 0.30 ± 0.03 cal/g of CO. Similar to native LDL, particle disruption occurs irreversibly at 85 °C. DMPC/CO/apoB complexes (Fig. 3E) exhibit a similar low enthalpy transition between 33 and 48 °C, with a peak temperature of 42 °C and a low enthalpy of 0.30 ± 0.05 cal/g of CO. An additional endotherm occurs between 3 and 35 °C with a peak temperature of 22 °C and an enthalpy of 2.6 ± 0.1 cal/g of DMPC. Particle disruption of DMPC/CO/apoB complexes occurs irreversibly at about 80 °C.

Similar to both LDL and the protein-free microemulsions, x-ray scattering and diffraction profiles at 10 °C on DMPC/CO/apoB complexes (Fig. 3) and EYPC/CO/apoB complexes (not shown) show an intense scattering maximum at 1/36 Å⁻¹ (Fig. 3). At 45 °C, above the temperature of the thermal transition of DMPC/CO/apoB complexes, the scattering maximum corresponding to 1/36 Å⁻¹ is absent while other subsidiary maxima remain (data not shown). However, in contrast to the scattering profile for LDL which shows five subsidiary maxima, and similar to the scattering pattern of DMPC/CO microemulsions, DMPC/CO/apoB complexes at

Fig. 2. Three per cent NaDodSO₄-polyacrylamide gels. A, LDL; B, LDL dialyzed for 36 h against standard buffer simultaneously with preparation of PC/CO/apoB complexes; C, DMPC/CO/apoB; D, EYPC/CO/apoB. For both C and D, fractions from the central region of the co-eluting DMPC or EYPC, CO, and apoB peaks from the Sepharose CL-4B column were pooled, dialyzed against buffer containing 0.01% disodium EDTA for 24 h at 4 °C, lyophilized, and delipidated with ethanol/diethyl ether (31). Thirty μg of protein were applied to the gels for A-D. E, LDL plus DMPC/CO/apoB; F, LDL plus EYPC/CO/apoB. For E and F, 15 μg of LDL and 15 μg of the complex were mixed and applied to the gel.

study). Similar to native LDL, the complexes exhibit β-migration by agarose gel electrophoresis (11).

Differential Scanning Calorimetry and X-ray Scattering of Microemulsion-ApoB Complexes—Differential scanning calorimetry of LDL (Fig. 3A) has shown a broad reversible thermal transition over the temperature range of 20-40 °C (Tm = 28 °C, ΔH = 0.7 cal/g of cholesterol ester). Particle disruption occurs irreversibly at 85 °C. X-ray scattering measurements of LDL (1-3) have demonstrated that LDL is a quasimembrane particle of ~220 Å. At 10 °C (Fig. 3), the scattering profile shows a relatively intense maximum at 1/36 Å⁻¹ which is absent from the scattering of LDL at 45 °C (data not shown). These data, together with differential scanning calorimetry and x-ray studies on neat cholesterol esters and the extracted cholesterol esters of LDL (1-3), provide evidence that the thermal transition of LDL is a smectic-like to disordered transition of the core-located cholesterol esters.

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10 °C exhibit four maxima at similarly larger angular spacings, consistent with a particle size slightly smaller than LDL (4-6, 23). These results indicate that the structure of LDL and DMPC/CO/apoB complexes are similar having a spherical morphology and a radially organized "smectic-like" arrangement of the core lipids.

**Studies on the Secondary Structure of ApoB**—The secondary structure of apoB in the microemulsion-apoB complexes was investigated by circular dichroic spectroscopy and compared to apoB of LDL. Fig. 4A shows CD spectra of LDL, complexes of EYPC/CO/apoB and DMPC/CO/apoB in the far UV region which were recorded at 4 °C, a temperature at which the fatty acyl chains of the surface lipids of both LDL and EYPC/CO/apoB are in a disordered state and the fatty acyl chains of the surface lipid of DMPC/CO/apoB are in an ordered state. At 4 °C, the core cholesterol esters of all the particles are in a smectic-like ordered state. Fig. 4B shows CD spectra at 50 °C, a temperature at which all the lipids of all the particles are above their order-disorder transitions.

The CD spectrum of LDL at 4 °C (Fig. 4A) is characterized by a high overall ellipticity with negative minima at 217 nm (\(\theta_{217} = -12.20\)) and 222 nm (\(\theta_{222} = -11.99\)) suggesting large amounts of \(\alpha\)-helix and \(\beta\)-structure. The spectrum of EYPC/CO/apoB is characterized by negative minima at 217 nm (\(\theta_{217} = -8.30\)) and 222 nm (\(\theta_{222} = -8.68\)) suggesting that these particles contain less \(\alpha\)-helix and \(\beta\)-structure than LDL. The spectrum of DMPC/CO/apoB is characterized by a negative minimum at 222 nm (\(\theta_{222} = -4.94\)) suggesting a moderate amount of \(\alpha\)-helix with little \(\beta\)-structure.

The CD spectrum of LDL at 50 °C (Fig. 4B) is again characterized by a high overall ellipticity with negative minima at 217 nm (\(\theta_{217} = -11.48\)) and 222 nm (\(\theta_{222} = -11.55\)) suggesting again both large amounts of \(\alpha\)-helix and \(\beta\)-structure. The spectrum of EYPC/CO/apoB complexes is similar to that of LDL, with negative minima at 217 nm (\(\theta_{217} = -8.70\)) and 222 nm (\(\theta_{222} = -11.79\)), suggesting that these particles contain a similar amount of \(\alpha\)-helix as LDL. However, the lower value of the ellipticity at 217 nm suggests that complexes of EYPC/CO/apoB have less \(\beta\)-structure than LDL. The spectrum of DMPC/CO/apoB complexes is characterized by a very low overall ellipticity with a negative minimum at 228 nm (\(\theta_{228} = -5.20\)), suggesting a small amount of \(\alpha\)-helix and a large amount of "unordered" structure.

The possibility of a contribution to the CD spectrum from the lipids of EYPC/CO or DMPC/CO microemulsions was examined by recording spectra of the EYPC/CO or DMPC/CO microemulsions alone over the wavelength region 200 to 250 nm at the appropriate temperatures and at lipid concentrations identical with those used for the microemulsion/apoB measurements. For both EYPC/CO and DMPC/CO microemulsions, no contribution to the CD spectrum is observed at the concentrations of lipid used for the study over the wavelength region examined at these temperatures.

**DISCUSSION**

Reassembled LDL particles have been prepared by the interaction of lipid-free solubilized apoB of native human LDL with model phospholipid/cholesterol ester microemulsion systems (24, 25) at room temperature. Both the surface phospholipid and core cholesterol ester components may be varied (24, 25), which has allowed the systematic investigation of a number of the chemical and physical properties of the complexes.

As mentioned under "Results," the pH at which solubilized apoB is interacted with the microemulsions is critical. As shown by other investigators (26, 27), apoB in a neutral detergent environment undergoes a pH-dependent aggregation which may be prevented by titration to a higher pH. The pK, of 10 mM NaDC is approximately 6 and precipitation of the insoluble acid commences at pH 6.9 (20). Thus, in order
to favor the pH requirements of NaDC and the above cited observations about pH-dependent aggregation of apoB, either pH 9 or 10 was used for the reassembly. The complex, once formed and isolated, may be dialyzed to pH 7.4 or 8 without any apparent changes in chemistry or physical properties.

The co-elution of PC, CO, and apoB on gel filtration chromatography is indicative of a stable PC/CO/apoB complex of stoichiometry: 43% PC, 43% CO, and 14% apoB. Negative stain electron microscopy shows the particles to be circular (spherical), homogeneous, and approximately 200 Å in diameter. The complexes exhibit β-migration on agarose gels and one high molecular weight band on 3.0% NaDodeSO4-polyacrylamide gel electrophoresis which co-migrates with apoB of LDL on similar gels. A range of 3.9–4.6 × 10^6 for the apparent molecular weight of the complex was determined by agarose gel column chromatography at 4 °C (28) and high performance gel filtration chromatography (29) using an 125I-labeled LDL molecular weight standard. Thus, the PC/CO/apoB complexes are stable and exhibit many of the chemical, compositional, and morphological properties of native LDL.

Differential scanning calorimetry and x-ray scattering show the lipids in the complexes to undergo at least two specific thermal transitions depending on composition, one associated with the core-located cholesterol esters (ΔH = 0.3 cal/g of cholesterol ester), similar to LDL and the protein-free microemulsions, and the other in complexes with DMPC, from the phospholipid forming the surface monolayer (ΔH = 2.8 cal/g of phospholipid). In addition, particle disruption-protein unfolding-denaturation occur irreversibly at 80–85 °C.

For EYPC/CO/apoB complexes, the transition of the core-located cholesterol esters occurs with a peak temperature of 38 °C and an enthalpy of 0.3 cal/g of CO. Similarly, for DMPC/CO/apoB complexes, the transition of the core-located cholesterol ester occurs at a peak temperature of 42 °C with an enthalpy of 0.3 cal/g of CO. In addition, the disorder transition of the surface phospholipid occurs at a peak temperature of 22 °C and an enthalpy of 2.6 cal/g of DMPC. In both EYPC/CO/apoB and DMPC/CO/apoB complexes, the thermal transitions are similar to those observed in their precursor protein-free microemulsion particles; however, the transition temperatures and enthalpies suggest that apoB either directly or indirectly influences the physical properties and order-disorder transitions of both the core and surface components.

Thus, these studies suggest that direct interactions between the core and surface components generally occur in native lipoproteins. The protein at the surface of native lipoproteins is thus likely to play a role in the interactions between the core and surface while providing additional stability to the "microemulsion."

As shown in Fig. 4c, the secondary structure of apoB of native LDL is the same at 4 and 50 °C (30). The secondary structure of apoB on EYPC/CO/apoB complexes, however, does vary in response to temperature. At 4 °C, the percentage of α-helix (27.17 ± 1.27) is significantly different than that at 50 °C (37.93 ± 2.70% α-helix).

The secondary structure of apoB on complexes of DMPC/CO/apoB complexes at either 4 °C (20.35 ± 0.74% α-helix, 0 ± 5% β-sheet) or 50 °C (9.78 ± 2.32% α-helix, 11 ± 5% β-sheet).

For either LDL or EYPC/CO/apoB at 4 and 50 °C (Fig. 4, A and B), the CD spectra exhibit their minima at identical wavelengths with the only variation being the magnitude of the minima. For both LDL and EYPC/CO/apoB, the physical state of both the surface and core lipids are the same at each temperature (i.e., at 4 °C, the surface lipids are disordered and the core lipids are smectic-like; at 50 °C, all the lipids are disordered). For DMPC/CO/apoB, on the other hand, the CD spectrum at 4 °C is very different from the spectrum at 50 °C. The minima are shifted and their magnitudes are different. At 4 °C, the surface lipid of DMPC/CO/apoB is ordered and the core is smectic-like; at 50 °C, all the lipids are disordered. These variations in the CD spectra and the secondary structure of apoB on DMPC/CO/apoB complexes may be a reflection of the physical state of the lipids and may suggest that apoB is responsive to the physical state of its lipid environment.

Reassembled LDL complexes of microemulsions and apoB provide a well defined model system in which to study the molecular interactions and structural organization of LDL, including the lipid-lipid interactions in the particle core, the lipid-lipid and lipid-protein interactions which determine the surface organization and protein conformation, and the interactions between the core and surface components. Preliminary experiments have shown the ability of microemulsion/apoB complexes to compete with human 125I-LDL for binding to the apoB/E receptor of cultured human fibroblasts. These reassembled LDL complexes should serve as an important model to study the delivery of isotopically labeled lipids with differing physical properties to cells in order to investigate the metabolic complexity of intracellular LDL catabolism and its relationship to positive cholesterol balance and atherogenesis.

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REFERENCES

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LIPID DIGESTION AND ABSORPTION

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INTRODUCTION

Gastrointestinal lipid digestion consists of three sequential steps: (a) the dispersion of bulk fat globules into finely divided emulsion particles, (b) the enzymatic hydrolysis of fatty acid esters at the emulsion-water interface, and (c) the desorption and dispersion of insoluble lipid products into an absorbable form. Here we emphasize several newer aspects of the biochemistry and biophysics of lipid digestion and absorption. We review the physical-chemical behavior of dietary lipids in model systems and correlate this information with their physiological behavior throughout the alimentary canal.

Dietary Phospholipids, Triglycerides, and Cholesterol

In both animals and plants long-chain triglycerides constitute the major biological form of storage lipids. These stores occur intracellularly as liquid oil droplets stabilized by a layer of phospholipid and protein (1). Cells also contain a variety of membranes whose lipids are largely phospholipids. The average western adult consumes about 150 g of triglyceride and 4-8 g of
phospholipids (predominantly lecithin) each day, of which two thirds is of animal origin (2). Quantitatively more important is endogenous phospholipid (essentially pure lecithin) of hepatic origin (7–22 g/day) which is secreted into the intestinal lumen via the bile (3–6).

In the solid crystalline state, a triglyceride molecule (Figure 1) adopts a tuning-fork conformation (7–9) with the sn-2 fatty acids pointing in the opposite direction to the sn-1 and sn-3 fatty acids. Solid triglycerides are polymorphic; and by means of X-ray diffraction, three distinct chain crystallization patterns (β, β' and α) have been identified (10). In the liquid state, atoms rotate around all single-bonded aliphatic chain carbons. However, the individual molecules are probably not oriented randomly in space (Figure 1); Larson's (10) X-ray scattering studies suggest short-range order in liquid triglyceride with close-packed swarms of molecules, and Calfayan's 13C spin-relaxation NMR measurements (11) suggest nearest neighbor interactions of up to ≈ 200Å in liquid tristearin. Pure synthetic triglyceride mixtures exhibit complex composition- and temperature-dependent physical-chemical behavior (12, 13).

Because natural fats are complex blends of triglycerides, melting occurs over a wide temperature range. Human fat melts a few degrees below the ambient temperature: 30–35°C for visceral fat, and 0–10°C for peripheral subcutaneous fat (14). Lard and tallow do not melt completely until 50–60°C (9) and hence are either a mixture of liquid and solid fats or supercooled melts at 37°C. A variety of triglyceride-rich lipoproteins are, in fact, metastable emulsions at 37°C in which the triglyceride core is undercooled several degrees below its true melting point (15–18).

Long-chain triglycerides and lecithins are essentially insoluble in water, (molecular solubility is < 10–20 and < 10–10 M, respectively). A useful classification based on bulk and surface aqueous behavior is that long-chain triglycerides are insoluble nonswelling amphiphiles; lecithins are insoluble monomolecular surface films.

The interfacial orientation of triglycerides is shown in Figure 1. Medium-chain triglycerides form more expanded surface films (≈ 100Å/molecule) than long-chain saturated triglycerides which form condensed or solid monomolecular films (≈ 60Å/molecule) (20). By both (Figure 1), the chains of the three fatty acids in parallel toward the hydrophobic side of the interface (20, 21). Synthetic short-chain triglycerides (triacetin and tripalmitin) give the smallest interfacial areas (≈ 40–50Å2/molecule) (21) and adopt a tuning-fork arrangement at interfaces with one fatty acid that contain one short chain and two long chains probably oriented with the

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**Figure 1** Above: Chemical structures, short-hand representations, and physical states of a typical long-chain lecithin (3-sn-phosphatidylycholine) and triglyceride (triacylglycerol) molecules. The two (R₁, R₂) long-chain fatty acids of lecithin (wriggly lines) are esterified to the sn-1 and sn-2 hydroxyl groups of glycerol (sausage-shaped symbol); the sn-3 group of glycerol is esterified to the zwitterionic phosphonyl choline (head) group. In all physical states, the glycerol moiety lies in line with the sn-1 fatty acid and both are perpendicular to the interface. Natural triglyceride has three (often different) (R₁, R₂, R₃) fatty acids esterified to the hydroxyl groups of the glycerol molecule.

Below: Dispersed states of the major dietary lipids in water. (a) Lecithin, showing configuration of the molecules in a typical unilamellar vesicle (or liposome). The molecules form a complete and continuous bilayer enclosing a water core. The phosphonylcholine head-groups, the glycerol moieties, and the ester linkages of the sn-2 fatty acids are all hydrated and have the configuration shown above. (b) Triglyceride forming an unstable emulsion particle in water. All 3 fatty acid chains of the surface monolayer of molecules are directed toward the core in a configuration typical of triglyceride at an oil-water interface (above). The glycerol moiety lies normal to the interface and is hydrated. The triglyceride molecules in the core adopt a tuning-fork arrangement typical of that in the crystal or isotropic melt (above). (c) Triglyceride droplet emulsified with a monomolecular layer of lecithin molecules: The core triglyceride molecules are identical in configuration to that described under (b), and the surface layer of lecithin molecules is equivalent to that in the outer monolayer of a vesicle. The surface monolayer of lecithin contains a few triglyceride molecules (≈ 3%) that are available for direct hydrolysis by lipases (38).
short chains projecting into the aqueous side of the interface (D. Kodali, T. Redgrave, J. Hamilton, D. Small, unpublished observations).

In the solid state, lecithins form bilayer lamellae, tilted with respect to the interface to allow close packing between their two long fatty acid chains. Line lecithins have been described (22–28). The average orientation of the monolayers of natural and synthetic lecithins (29–31) is approximately in the bilayer plane, i.e., parallel to the interface (Figure 1). Cholesterol can be incorporated in equimolar ratios into lamellar liquid crystalline phases (32) and forces the hydrocarbon chains to adopt an “intermediate fluid” nature; trans conformations are increased in the proximal parts of the chains, and gauche conformations at the terminal ends; however, the head-group conformation is unaltered (33–35). Saturated lecithin monolayers when fully expanded occupy an interfacial area of 60–70 Å²/molecule, condensed films ≈ 46–50 corresponding areas for long-chain triglyceride molecules (20, 31, 36). Lecithins with unsaturated chains don’t form solid films above 0°C, and their areas at an interface are several Å² larger than saturated lecithins (36).

When shaken in excess water, triglyceride forms crude unstable emulsion droplets and lecithins disperse to form relatively stable concentric lamellar structures called myelin figures or liposomes. Liposomes can be further dispersed as unilamellar liposomes by ultrasonication (Figure 1). When 20:1 they form stable emulsions with the phospholipid acting as the emulsifier by forming a stable monolayer enveloping the triglyceride droplets (Figure 1). Emulsification with phospholipids increases the oil-water interfacial area (i.e., surface-volume ratio) by the dispersion of large oil masses into fine oil/water emulsion particles. The interfacial tension between pure triglyceride and water is ≈ 15–20 mN/m whereas that between phospholipids and water is ≈ 1–5 mN/m. Therefore, phospholipids lessen the energy requirements for emulsification by significantly lowering the lipid-water interfacial tension. They also stabilize the emulsion droplet; they prevent coalescence and breakage of the emulsion by forming close-packed monolayers. By this means the interfacial “concentration” of molecules is increased, thus promoting hydrolysis of both phospholipid and triglyceride by various lipases (37).

Recent studies using phase equilibria techniques (38) and 13C NMR spectroscopy (39) define the phase diagram of egg lecithin, triolein, cholesterol, and water (Figure 2). The surface monolayer of lecithin on an emulsion particle was found to contain about 3% triglyceride by weight. As free cholesterol was increased in the emulsions, it entered both the surface and core of the emulsion and slightly decreased the amount of triglyceride in the surface to approximately 2% at maximum saturation and equilibrium of the surface; that is, one molecule of cholesterol for one molecule of lecithin. The conformation of triglyceride in the interface indicates that all three chains lie parallel to the chains of the lecithin molecules, and the glycerol end of the molecule is directed toward the water (Figure 1). The 13C NMR studies indicated that the sn-1 and sn-3 positions were more highly hydrated than the sn-2 positions; further, the sn-1 and 3 positions appear to lie slightly below the level of the sn-2 carbonyl of the lecithin. Finally, the rate of exchange between the core and surface was estimated to be rapid, and therefore the interfacially oriented triolein could be a substrate for lipases. First, it is surface oriented where the water molecules and enzyme could reach it easily. Second, the 1 and 3 positions are more conveniently located for hydrolysis than the 2 position. And finally, rapid movement of molecules from core to surface should occur, thus replenishing the surface concentration.

More complex systems containing small proportions of fatty acids, cholesterol esters, and partial glycerides as well as triglyceride, phospholipid, and cholesterol do not greatly change the surface composition relative to triglyceride (K. Miller, D. M. Small, unpublished observations) (Figure 2). Similar surface compositions appear to be present in triglyceride-rich lipoproteins and presumably in emulsion droplets occurring in the intestinal lumen during fat absorption. Studies on short-chain lecithins and triglycerides show a similar picture with perhaps somewhat more triglyceride located in the surface (40). The low concentrations of triglyceride in the surface layer may be related to the lag phase and low hydrolytic rates noted when pure pancreatic lipase is added to phospholipid emulsified triglycerides (37, 41, 42).

**ROLE OF THE STOMACH AS A DIGESTIVE ORGAN**

Muscle contractions of the stomach—particularly peristalsis against a closed pylorus and the squirting of fat through a partially opened pyloric canal—produce the shear forces sufficient for emulsification. Potential emulsifiers that can function in the acid milieu of the stomach include peptic digests of dietary proteins, complex polysaccharides, and membrane-derived phospholipids. Enzymatic hydrolysis of triglycerides also begins in the stomach (43–45), and since triglyceride is stored for 2–4 hours, 30% of the total dietary triglyceride may be digested. However, quantitative hy-
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drolysis and absorption, especially of the long-chain fatty acids, require less acidic conditions, appropriate lipases and detergents (bile salts), and specialized absorptive cells. This environment is provided by the lumen and mucosa of the upper small intestine.

Lingual Lipase Hydrolysis

The major source of gastric lipolytic activity originates in a group of serous glands (von Ebner) beneath the circumvallate papillae of the tongue (46). The acini of the glands drain through short ducts into troughs that surround the papillae. The fine structure of these lingual glands is reminiscent of pancreatic acinar cells. Basal secretion apparently occurs continuously but can be stimulated by neural (sympathetic agonists), dietary (high fat), and mechanical (suckling and deglutition) factors (47). Although unrecognized as such, the calf, kid goat, and lamb lipases are important components of rennet paste and have been used by Mediterranean cheesemakers for centuries to curdle milk (48). Thus lingual lipases (usually called pre-gastric esterases or pharyngeal or salivary lipases) have received much attention in the dairy and veterinary literature (49). Despite their discovery in 1924 (49), no lingual lipase has yet been purified to homogeneity. The partially purified rat enzyme (50) is a sparingly soluble hydrophobic glycoprotein (Mₖ ≈ 50,000) specific for triglycerides. It is neither inhibited by, nor demonstrates a lag response with, lecithin-coated emulsions or short-chain lecithin-triglyceride mixed micelles. It is inhibited ≈ 20% by bile salts above their critical micellar concentration (in contrast to 100% for the pancreatic enzyme). With trypsin and trilin, the pH optimum is 4–4.5, but activity begins at pH 2 and is still detectable at pH 7.5.

Both lingual and pancreatic lipases are true lipases (in contrast to esterases) and by definition act only on insoluble aggregated substrates. They are specific for the primary (sn-1 and sn-3) ester bonds of triglycerides; and fatty acid ester linkages of 2-monoglycerides, phospholipids, and cholesterol esters are resistant (5). Both enzymes are much more active on triglycerides with short-chain than on those with long-chain fatty acids (50–53). However, in contrast to pancreatic lipase, lingual lipase cleaves the fatty acid at the sn-3 ester linkage in preference to the sn-1 position (52–54). This appears to be a stereo-specific preference independent of the sn-3 fatty acid chain length (53, 54). In addition, product inhibition apparently occurs since the major products in vitro and in vivo are diglycerides and fatty acids (51–53, 55). In contrast, sn-2 monoglycerides and fatty acids are the final products of pancreatic lipase hydrolysis.
Physical State and Fate of Hydrolytic Products

In milk triglycerides short- and medium-chain fatty acids are esterified principally at the sn-3 position (51), whereas long-chain fatty acids occupy all three ester positions of common animal and vegetable triglycerides (49). With the former, lingual lipase hydrolysis produces short- or medium-chain free fatty acids and sn-1,2 diacylglycerols containing the long-chain fatty acids. Short- and medium-chain fatty acids in water exhibit pKa values in the vicinity of 4.8 (56), but because they are hydrophilic they are fairly soluble in both ionized and un-ionized states. As indicated in Figure 3, these fatty acids leave the surface of the fat droplets and are absorbed passively by the stomach mucosa, to be transported to the liver bound to albumin in the portal vein (52, 53, 57). The aqueous pKa values of long-chain fatty acids released by lingual lipase hydrolysis of common triglycerides are at or above neutrality (58-60); thus they will be predominantly protonated at stomach pH values and form liquid fatty acid oils. In micelles, oleic acid is 50% ionized at pH 7, in phospholipid bilayers at pH 8, and in triolein emulsions at pH 9 (D. P. Cistola, J. A. Hamilton, D. M. Small, unpublished observations). In both their crystalline structures (61) and in the liquid state, protonated fatty acids are extensively hydrogen bonded intermolecularly via their carboxyl groups (58, 60). Because they are hydrophobic, their solubility in triglyceride/diglyceride droplets is high (62), and in the stomach at low pH they probably partition mainly within the core of fat droplets with only a small fraction in the interface (Figure 3).

DIGESTION IN THE UPPER SMALL INTESTINE

Lingual lipase hydrolysis of dietary fat in the stomach facilitates duodenal jejunal hydrolysis in a number of ways. (a) Long-chain fatty acids dissolved with the fat droplets become partly ionized and promote fine emulsification in the duodenum (63-66). (b) Small amounts of partially ionized long-chain fatty acids increase the binding of colipase (an essential cofactor for pancreatic lipase action) to the emulsion interface (67). (c) As a result of b the binding of lipase to colipase (67-69) is promoted. (d) Diglycerides being more surface active may be preferentially located at the emulsion-water interface; they are hydrolyzed faster by pancreatic lipase (70). (e) Absorption of long-chain fatty acids (and not triglyceride) in the duodenum accelerates the release of CCK, which induces gallbladder contraction, relaxation of Oddi's sphincter, and pancreatic enzyme secretion (71, 72). (f) Being unaffected by luminal amphiphiles, including bile salts (50), lingual lipase continues to hydrolyze tri- and diglycerides in the upper small intestine at pH 5-7 (73).

Figure 3 Hypothetical depiction of the physical states and fate of fatty acids released by lingual lipase hydrolysis of triglyceride molecules in the stomach. Only the substrate and product molecules are shown. Most of the surface is actually covered by phospholipid. The sn-3 fatty acid ester linkage is preferentially hydrolyzed, releasing medium- and short-chain fatty acids in the case of milk fat (a), and long-chain fatty acids in the case of common dietary fats (b). Fatty acids (FA), irrespective of chain length, are predominantly un-ionized (i.e., un-ionized) at the low stomach pH. In this state, the short- and medium-chain species have appreciable aqueous solubility (a); thus they leave the emulsion and diffuse as monomers to the mucosa, where they are absorbed by passive diffusion mechanisms to enter tributaries of the portal vein. From here they are transported to the liver, bound to albumin, and provide an immediate source of metabolizable energy (ruminants obtain the majority of their energy sources from the production and absorption of volatile FA in the rumen). Protonated long-chain fatty acids (b) are hydrogen bonded crystals or oils at 37°C and are mainly solubilized within the core of the triglyceride (+ diglyceride) droplets, although some partition into the surface coat. Lingual lipase also catalyzes resynthesis of some triglycerides from these long-chain fatty acids and diglycerides (c) [from data in (51-55, 57)].

Fine Emulsification

The emulsion particles in the upper small intestine are generally less than 0.5 μm in diameter and are extremely stable (74-76). When stomach chyme is propelled through the small opening of the pyloric canal into the duodenum, the strong shear forces (analogous to those produced in a commercial "colloid mill") tear the liquid interfaces apart (77, 78). The oil solubilized
molecules at the interface, which induces surface shearing forces, turbulence, and interfacial agitation (78).

As bile enters the duodenum it mixes with these particles. While pure bile salts are extremely poor emulsifiers of fat (64, 65, 88), bile salts in dilute combinations with triglyceride digestive products and biliary lipids aid in fat emulsification (Figure 4). Recent work on the phase equilibria and structure of biliary mixed micelles (89, 90) and upper intestinal contents after a fatty meal (91; R. J. Stafford, M. C. Carey, unpublished observations) suggests that the biliary micellar phase becomes saturated with lecithin when diluted within the intestinal lumen. Similarly, the micellar phase during established fatty digestion is saturated with the products of lipolysis (91; R. J. Stafford, M. C. Carey, unpublished observations). Figure 4 displays how the emulsion droplets within the upper small intestine could be enveloped with a monolayer of biliary lipids mixed with the products of hydrolysis. Lairon et al (92) demonstrated that the composition of an adsorbed monomolecular film of porcine biliary lipids around siliconized glass beads was 1: cholesterol : 2 phospholipid : 3 bile salt molecules; Nalbone et al (93) found most of the biliary phospholipid associated with the emulsion particles during fat digestion in the rat. Other studies (64, 65) indicate that binary or ternary combinations of bile salts, lecithin, “acid-soap,” and monoglycerides in appropriate physiologic ratios and concentrations produce triglyceride emulsion particles that are extremely small and stable at pH 6.5 and require low shear forces or even emulsify “spontaneously” (Figure 4).

**Pancreatic Lipase-Colipase and Phospholipase A2 Hydrolysis**

In humans and most animals there appear(s) to be one (or at most two) form(s) of the classic pancreatic lipase, and the enzyme(s) is are secreted in an active form. Both pancreatic colipase and phospholipase A2 are secreted in a proenzyme and proenzymic form, respectively, and require activation by trypsin hydrolysis of an Arg-Gly-Arg-Ala bond in their N-terminal chains, respectively (94, 95). Pancreatic phospholipase A2 catalyzes the hydrolysis of fatty acids esterified at the sn-2 position of a variety of phosphoglycerides (phosphatidycholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidyserine, phosphatidylinositol, cardiolipin) but is without effect on sphingolipids (sphingomyelin, cerebrosides, and gangliosides) (96). The enzyme has an absolute requirement for Ca²⁺ ions which bind in a 1 : 1 stoichiometry to substrate and enzyme (97). It has been suggested that this ensures fixation and stabilization of the enzyme-substrate complex. Aggregation of the substrate is necessary, and this appears to facilitate the nonequivalent interaction of the sn-2 fatty acid ester with the catalytic site of the enzyme (98). Hydrolytic rates also depend on the
type of aggregation (liposomes, mixed micelles, emulsions), and the presence of other lipids which presumably influences the packing of the fatty acid chains and head-group area (99).

In the absence of bile salts and phospholipids, lipase can readily bind by hydrophobic interactions to the triglyceride-water interface; the enzyme, in model systems, bile salts, like other detergent lipase) from triglyceride-water interfaces (100). Collapsing a bile salt-covered triglyceride-water interface and provides a high-affinity "anchor" site for lipase. With physiological triglyceride emulsions carrying an envelope of dietary phospholipids, biliary bile salts, lecithin and cholesterol, and partially ionized fatty acids from lipase lipase hydrolysis (Figure 4), the interactions of pancreatic lipase, phospholipase A₂, collapse, and bile salts are highly complex (Figure 5). The "physico-chemical" interpretation of these data is that pancreatic lipase alone (in contrast to lingual lipase) is inhibited by emulsifying amphiphiles, presumably because the interface is not recognized, since only 2–3% of triglyceride is solubilized without the emulsifying monolayer at any time. Thus, depending on the physical form of the substrates, fatty acids are released slowly from mixed micelles (Figure 5A) or demonstrate a "lag inhibition" from emulsions (Figure 5B) (37, 41, 42, 101). The "lag-inhibition" (Figure 5) can be counteracted by colipase (Figure 5B, D) (37, 41, 42), which is known to penetrate and perturb lecithin bilayers (102). The cofactor attaches to the ester bond region of triglyceride by hydrogen bonding and possibly electrostatic interactions; lipase tightly anchors to colipase by electrostatic bonding and is then in a suitable configuration to hydrolyze the substrate (103). Bile salts may enhance (a) the binding of colipase (Figure 5B, D) by forming colipase–bile salt aggregates (104), and (b) the binding of phospholipase A₂. Phospholipase A₂ hydrolysis of the emulsifier (Figure 5C) may be a mechanism for facilitating triglyceride hydrolysis in vivo (37, 41, 42). Phospholipase A₂ prepared according to standard procedures contains 0.1–0.2% colipase; it has been shown that pure phospholipase A₂–treated milk fat globules require colipase to be hydrolyzed by lipase in the presence of bile salts (105).

In addition to the promoting phospholipase A₂ hydrolysis, calcium ions (Figure 5D) may facilitate the desorption of the lipolytic products from the interfaces (106). Thus pancreatic lipase, colipase, phospholipase A₂, calcium, and bile salts all act synergistically in the lipid hydrolytic reactions of the upper small intestine.

**Pancreatic Nonspecific Lipase, Cholesterol Esterase, and Human Milk Lipase Hydrolysis**

Pancreatic nonspecific lipase (carboxyl ester hydrolase), a "minor" lipase of pancreatic juice, and pancreatic cholesterol esterase are apparently the same enzyme (5). The rat enzyme (107) is secreted in an inactive monomeric form \(M_r \approx 65,000 \) and only becomes active when polymerized by trihydroxy bile salts to a higher molecular weight form \(M_r \approx 400,000 \). The human enzyme has also been purified and characterized (108–111) and has been shown to be a glycoprotein with a broad substrate specificity. It catalyzes the hydrolysis of water-soluble carboxyl esters (triacetin, tripropionin, methylbutyrate, lysolecithin) and insoluble esters (of cholesterol and lipoproteins A, D, and E) dispersed in bile salt micelles (110). Emulsified substrates appear to be hydrolyzed poorly. The monomeric form of the enzyme \(M_r \approx 100,000 \) dimerizes to the active form in the presence of 3α, 7α-hydroxylated primary bile salts (cholate, chenodeoxycholate, and their conjugates) in concentrations well below their critical micellar concentrations. As with lingual lipase, the interfacial adsorption of the active dimeric enzyme is not affected by micellar concentrations of bile salts (111).
While the milk of all mammals contains a lipoprotein lipase derived from serum, only the milks of humans (112) and gorillas (113) are known to contain in addition a nonspecific true lipase that is activated by bile salts. It is identical to the pancreatic nonspecific lipase-cholesterol esterase just described (115–117). It is a glycoprotein (M, ≈ 125,000) that is stable at pH 3.5 for 1 hr at 37°C; it requires activation by primary bile salts in concentrations below their CMC values. The enzyme hydrolyzes dispersed water-insoluble substrates (triglycerides, lipoproteins, and cholesterol esters) and water-soluble substrates (short- and medium-chain mono-glycerides, etc). Activation by taurocholate is due to enhancement of the binding of the bile salt–induced dimerization has not yet been investigated. In view of its properties to those of pancreatic nonspecific lipase (cholesterol esterase), and since it is immunologically identical with a pancreatic enzyme (118), its origin in the pancreas is virtually certain. It is possible that lipase moves from blood to human milk via a receptor-mediated transfer in the lactating mammary gland. The enzyme may play an important role in the intestinal digestion of milk triglycerides and other esters in the neonate (119–122), at a time when exocrine pancreatic function and bile salt secretion are suboptimal (119–22).

**Chemical Concentrations of Substrates and Enzymes**

The physical-chemical environment during lipid digestion and absorption is remarkably constant. Between 1 and 1.5 hr after the ingestion of a meal, the mean pH of the upper intestinal contents falls from a pH ≈ 6.8 to about pH 5.3 (123, 124) as stomach acid briefly overwhelms the buffering capacity of the duodenum. Owing to the buffering capacity of food, the pH of which is ≈ 6.0, this pH value is typical of that in the human stomach about 30 min after the ingestion of a meal (123). While osmolality of stomach contents generally reflects that of the diet (> 600 mOsm/kg), osmotic equilibration occurs in the duodenum (but not in the stomach) and lowers the osmolality of gastric chyme to 300–350 mOsm/kg (123). This value is only slightly higher than that of plasma. Due to continuous duodenal secretion of hepatic bile, fasting bile salt levels are in the range of 3–7 mM (125). Postprandial gallbladder emptying causes a transient elevation in total bile acid concentration to 13–46 mM (125). During established lipid digestion (i.e. > 30 min after a meal), the bile salt concentration decreases to 2.5–10 mM and remains constant despite 2–3 enterohepatic cycles of the bile salt pool (4, 125). The triglyceride concentration is also fairly constant, ≈ 10–40 mM (37, 126), but the total phospholipid concentration (127–131)

is somewhat more variable (0.3–5.5 mM). Owing to biliary lecithin secretion, the molar ratio of lecithin to triglyceride in duodenal contents is about ≈ 1:10, in contrast to ≈ 1:40 in the diet. Calcium concentrations vary from 5 to 15 mM (129); sodium and potassium concentrations are ≈ 100mM and ≈ 25mM (129), respectively. Lipase and colipase are secreted in equimolar proportions, giving duodenal concentrations of ~ 1 → 2 × 10⁻⁷ M (132). These concentrations can release 150–300 μmoles of sn-1 and sn-3 long-chain fatty acids/min/ml of intestinal fluid (5) (Figure 6). The corresponding activity of intestinal phospholipase A₂ is much lower, releasing 0.5–1.5 μmoles of sn-2 long-chain fatty acids/min/ml of intestinal fluid (127) (Figure 6). The fatty acids become partially ionized and immediately form “acid soaps” within the surface coat. Together with 2-monoglycerides and 1-lysophospholipids, they greatly expand the surface, reduce the size of and stabilize the emulsions, and eventually desorb from the surface with bile salts into dispersed physical states (Figure 7). Pancreatic lipase has a remarkably high turnover number: 250,000 to 500,000 long-chain triglyceride molecules/min. Its activity in intestinal content is 100–1000-fold in excess of that needed for complete hydrolysis of triglycerides in the upper small intestine. Phospholipase A₂ hydrolysis is less efficient, and dietary and

![LUMEN pH 6-7](image)

**Figure 6** Hydrolytic products of pancreatic lipase and phospholipase A₂ in the duodenum. Fatty acids, 2-monoglycerides (MG), and 1-lysophosphatidic acid (1-Lyso) are dispersed in liquid crystalline liposomes or bile salt micelles for absorption by enterocytes. Within the mucosal cell, chylomicrons (CM) containing a triglyceride core and a phospholipid and apoprotein surface are synthesized, secreted into lymph, and transported into blood via the thoracic duct.
endogenous phospholipids undergo continued hydrolysis in the jejunum and possibly in the ileum (127).

PHYSICAL-CHEMICAL STATE OF LIPIDS DURING DIGESTION

Hofmann & Borgström's work (133, 134) and subsequent studies (128–131, 135–145) concluded that the postprandial human intestine contained a "two-phase" lipid system composed of oil and aqueous micellar phases. To isolate the phases these authors relied on either prolonged ultracentrifuga-

solution (130, 131, 133–145) after lipase inactivation at 70°C, or multiple ultracentrifugations after mild centrifugation (128, 129). Heat treatment, filtration, and centrifugation induced demulsification (38, 78). Further, by initially stimulating lipase activity, heating increases fatty acid production, alters phospholipid composition (128) and perturbs phase boundaries (89). Several workers (128, 129, 146) have suggested that the Hofmann-Borgström hypothesis may be an oversimplification. Ultracentrifugation produces a marked aqueous bile salt–fatty acid gradient and only a small portion of duodenal fluid becomes optically clear (128). Other authors have noted that an insoluble pellet phase is invariably present (146) and that ultrafiltration generally results in a slightly turbid aqueous filtrate (128, 129).

Puttnam & Carey (126) examined the digestion of stomach emulsions and purified olive oil by light microscopy in vitro and observed the sequential appearance of a birefringent calcium soap phase and a nontriglyceride 1:1 monoglyceride–fatty acid phase (viscous isotropic phase). The remaining oil phase was largely triglyceride and diglyceride. In an attempt to reconcile these findings with the Hofmann-Borgström hypothesis, Stafford & Carey (91, 147), and unpublished observations] defined the phase equilibria of simplified model systems of the aqueous intestinal lipids for typical physiologic conditions (Figure 7) and also elucidated the compositions of the aqueous phases of postprandial distal-duodenal content after porcine acid—inhibition of pancreatic lipase activity. After low-speed centrifugation, the total aspirates separated into an oily emulsion portion and a turbid triglyceride-free aqueous portion. Upon ultrafiltration of the turbid portion a bile salt micellar phase, saturated with the products of lipolysis, and a liquid-crystalline phase, saturated with bile salts, were obtained (Figure 7).

Since liquid crystalline vesicles and micelles coexist during human fat digestion, the aqueous "phase" analyses in earlier studies probably represent a mixture of both particles. Laser light scattering reveals hydrodynamic radii of about 200Å for the micelles in the model micellar phase at the mixed lipid/bile salt phase boundary (91, 147). Similarly sized particles are found in separated aspirates of the human postprandial duodenum whose relative lipid composition plots on the micellar phase boundary (Figure 7). The structure of these large mixed micelles may be similar to, but larger than, that of the "mixed disc" model of bile salt–lecithin micelles (90). The aqueous lipids dispersed as liquid crystals—i.e., nonmicellar particles—have hydrodynamic radii of 400–600Å. These are probably unilamellar—i.e., single-shelled liposomes (R. J. Stafford, M. C. Carey, unpublished observations) (see Figure 7).

This discovery may have important pathophysiological consequences. It is well known that in many patients with low intraluminal bile salt concentrations, fat malabsorption may be minimal (148). Even in patients with
are unique (156), and odd-numbered and branched-chain fatty acids have been identified in feces of conventional but not germ-free animals (157). About half of fecal lipid is endogenous, the remainder of dietary origin (158, 159). There is no evidence for absorption of long-chained lipids in the colon. During transit, several bacterial modifications occur. These include hydrolysis of glycerides, phospholipids, wax esters, and cholesterol esters by various bacterial lipases (160, 161), hydroxylation of double-bonds of fatty acids (160), and dehydrogenation, epimerization, and deconjugation of bile salts (162, 163).

**Fecal Lipids**

Some attempts have been made using conventional methods (acid, alkali, organic solvents, heat treatment) to measure separate classes of fecal lipids (164–170). In all of these methods, however, the physical-chemical state of the excreted lipid is altered during the extraction process. Thus while total lipids can be measured, these methods do not determine the physical state of lipids as they exist in feces at the time of excretion.

We have devised a method to separate fecal lipids according to their different physical states before chemical extraction and quantitation (161, 171, 172). Oil, water, and solid fractions were mechanically separated by a series of low and high speed centrifugations. Each fraction was then separately extracted using methods designed to alter the lipid as little as possible. The lipids were then quantified chromatographically (173). Figure 8 shows the chemical species, physical states, and relative amounts of the 4–6 g of fat that are normally excreted in the daily stool of healthy people. The major fractions are oil- or water-soluble fatty acids; soaps of divalent ions, sterols, and other lipids make up minor fractions. No triglyceride is found in normal feces. In pancreatic insufficiency, large amounts of triglycerides may be present (161) while in mucosal disease, divalent soaps may predominate (171).

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**Literature Cited**

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**Literature Cited**

strify of cholic acids. See Ref. 84, 1249–356


LIPID DIGESTION & ABSORPTION


125. Sjövall, J. 1959. On the concentration of


Methods in Laboratory Investigation
Identification and Detection of in Situ Cellular and Regional Differences of Lipid Composition and Class in Lipid-Rich Tissue using Hot Stage Polarizing Light Microscopy

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To determine whether in situ tissue lipid characterization is possible, we examined carefully prepared frozen sections from a variety of lipid-rich tissues of the cholesterol-fed rabbit by hot stage polarizing light microscopy and conventional histologic staining. Heating of frozen sections to less than 60° C did not affect tissue architecture or staining characteristics making pathologic and physical chemical correlations possible. The melting temperatures of cholesterol ester inclusions in individual foam cells in rabbit atherosclerotic lesions and adrenal gland could be determined as well as the melting characteristics of crystals and triglyceride in these and other tissues. Differential scanning calorimetry and polarized light transmittance were used to confirm melting temperatures determined by microscopy. Combining data from histologic staining, polarizing light microscopy, and the thermal characteristics of lipid enables the various lipid classes to be identified within individual cells. Differences in melting temperatures between lipids of the same class give indications of the degree of saturation of the lipids.

Regional differences of cholesterol ester-melting temperatures in the chow-fed rabbit adrenal cortex were detected which implied differences in chemical composition. Cholesterol feeding raised the melting temperature and tended to abolish the marked regional differences in melting temperature of the cholesterol esters in the adrenal cortex. Rabbit atherosclerotic lesions, induced by balloon deendothelialization and cholesterol feeding, revealed differences in foam cell-melting temperatures within the same lesion. Melting temperatures of cholesterol ester deposited in the liver were more uniform. Each tissue studied revealed distinctly different cholesterol ester-melting characteristics.

Additional key words: Atherosclerosis, Adrenal gland, Lipid histochemistry, Cholesterol ester, Triglyceride, Cholesterol, Phospholipid.

Many of the physical properties of the major lipids of biologic importance have been well characterized (20–22, 24–26). In biologic specimens it is possible to use polarizing light microscopy, and a variety of other physical techniques, to identify not only the class of lipid but also to estimate the degree of saturation of their fatty acid components. Thus, crystals of cholesterol monohydrate are rhomboid with characteristic edge angles and melt at high temperatures (>85° C) (17). Phospholipid-rich liquid crystals have a smectic birefringence but do not melt until high temperatures (20). Cholesterol ester smectic liquid crystals melt at temperatures between 0° and 85° C, depending primarily on the degree of unsaturation. Saturated esters melt at high temperatures (>60° C), whereas polyunsaturated esters melt at lower temperatures (10 to 35° C) (22). Monounsaturated esters melt at approximately 40 to 50° C (22). Cholesterol ester crystals are needles and, although not usually seen in tissue, could be artfactually produced by cooling tissue to less than body temperature. They can be recognized by a needle shape, by their melting point of 40 to 60° C, and, importantly, by the fact that after melting to an isotropic liquid they form a characteristic smectic state on cooling (22). Finally, triglycerides do not form liquid crystals but rather short needle-like crystals which melt directly to an isotropic liquid. On cooling there is marked undercooling of 20 to 30° C before recrystallization occurs (22).

Polarizing light microscopy has already been used to study the lipids in a variety of lipid-rich tissue, especially the lesions of atherosclerosis (6, 7, 9–12, 14, 16, 27), the foam cells of Tangiers disease (13), cholesterol ester storage disease (23), tendon xanthomata (29), and lipoproteins (4). In most of these studies techniques were used that did not maintain tissue architecture, and information about the histologic sites of lipid or in situ lipid chemical heterogeneity was not available. Hillman and Engleman (7) used frozen sections of human fatty streaks taken at postmortem and showed that clusters of
lipid droplets within lesions appeared to melt at a similar temperature but that differences between droplet clusters were apparent. These clusterings of similar melting temperature droplets were assumed to be within the same cell, but actual demonstration of this was not undertaken nor were comparisons made with histology.

The purpose of this study was to determine whether (a) frozen sections of lipid-rich tissue could be used to determine the physical properties of the lipid and (b) stained such that the characterized lipid could be located histologically within the tissue microanatomy. We found that rapidly freezing tissue immediately upon removal from the animal enabled good quality frozen histologic sections to be obtained that preserved both the lipid organization and physical characteristics. By subsequently staining the same and adjacent sections with standard staining techniques correlation between histology and lipid class and composition was possible.

Using such techniques we have identified regional cellular lipid compositional differences in a variety of tissues that may be of biologic significance.

MATERIALS AND METHODS

Male New England white rabbits weighing 2.5 to 3.5 kg were initially fed a chow diet ad libitum. Atherosclerosis was induced by balloon deendothelialization of the left iliac and aorta as described by Baumgartner (1), combined with a 1.5% cholesterol, 5% corn oil diet. The high cholesterol diet was commenced after balloon deendothelialization, and rabbits were sacrificed at various time periods up to 16 weeks.

Tissue Handling

At sacrifice the aorta, left and right iliac arteries, adipose tissue, liver, and adrenal glands were removed for histologic and physical studies. Loose connective tissue was carefully stripped from around the arteries, and 2- to 3-mm rings were cut from balloon-deendothelialized and control arteries. Tissue taken for histology was rapidly frozen in a beaker containing 10 ml of hexane kept at −70°C in a slurry of dry ice and acetone. Following freezing, the tissue was placed in vials kept on dry ice and stored at −40°C until sectioned. To prevent any thawing of tissues, vials were always transported on dry ice and the tissue was handled with forceps also kept on dry ice. Adipose tissue was not frozen, but small pieces were compressed between a microscope slide and cover slip for microscopic examination.

To assess whether lipid was removed by 10 ml of hexane, after tissue had been frozen the hexane was spotted onto silica G thin layer plates and developed in neutral and polar lipid solvent systems. No lipid was seen. Based on the lipid content of the frozen tissue, if greater than 1/1000 of the lipid was extracted into the hexane, it would have been detected by thin layer chromatography. Thus, hexane extracted little or no lipid from tissue during freezing.

Histology

Unfixed frozen blocks were mounted on a Damon cryostat (IEC model) chuck and sections cut at 8 to 10 μm. Individual sections were picked up on microscope slides and were examined and photographed under polarized light within 2 hours. Sections examined by hot stage polarizing light microscopy were stained after heating so that correlations between lipid melting and histology could be made. Sections were stained with hematoxylin and eosin and neutral lipid stains (Sudan black or oil red O) by standard procedures (8). In some cases sections were reacted with 5-bromo-4-chloro-3-indolyl-b-galactoside to assess activity of the lysosomal enzyme β-galactosidase (15). Microscopy was performed with a Leitz Dialux microscope fitted with polarizer, analyzer, and hot stage (22). A Nikon microflex UFX camera (Nikon Inc., Garden City, New Jersey) was fitted to the microscope for photomicrography and for use in the melting studies.

MELTING STUDIES

Hot Stage Microscopy. Sections used for melting studies were not fixed or covered with a cover slip since it was found that lipid, in sections over which a cover slip had been placed, tended to leak out of cells and coalesce, thus losing much of the cellular definition seen under the polarizing light microscope. The melting temperature of lipid was determined by heating the slides at 1 to 2°C/minute while observing the tissue for changes in birefringence. Photographs of the tissue were taken at appropriate temperatures as a permanent record. Onset of melting was taken as the intensity of the birefringence diminished and completion when birefringence had disappeared indicating the liquid-crystal to isotropic liquid transition. The melting temperature of lipid was unaltered by leaving sections uncovered.

At the completion of the heating run the slide was stained with either hematoxylin and eosin or a neutral lipid stain, then realigned exactly as it had been on the microscope stage, and photographed under bright field. In this way, differences in lipid melting temperatures throughout the tissue could be correlated with histology. Heating of tissue to 60°C or less did not cause any detectable alteration in tissue architecture, and the tissue was able to be stained using conventional staining techniques. Adjacent unheated sections were stained and compared as controls.

Differential Scanning Calorimetry. Differential scanning calorimetry was performed essentially as described before (4, 13) in a Perkin-Elmer DSC-II using 75-μl stainless steel sample pans (Perkin-Elmer Corporation, Norwalk, Connecticut). Whole pieces of lipid-rich tissues (adrenal, liver, arterial intima, adipose tissue) were loaded into individual pans, hermetically sealed, weighed, and balanced against an equivalent mass of normal saline in a reference pan. The tissue was then heated and cooled at 5°C/minute from −10 to 60°C, and thermograms were recorded. The thermograms indicate the transitions occurring in the predominant lipid of the specimen.

Optical Methods. Objective measurements of lipid melting using the polarizing light microscope were obtained by plotting the inverse of the exposure time of the camera light meter as a function of temperature. The area of the section to be examined was centered within
In these tissues both collagen and certain lipids are birefringent, but the melting temperatures of collagen (\( \sim 70^\circ C \)) (29) is well above the range of heating used, whereas that of the lipid is usually well within the range. Thus, changes in light transmittance accurately reflect changes in the state of the lipids.

RESULTS

HISTOLOGY

Arterial Wall. In control arteries no intimal thickening was seen in the first few weeks of cholesterol feeding. Under polarized light with crossed analyzer, the internal elastic lamina (IEL) and other connective tissue components (elastin and collagen) were seen in the media and adventitia particularly of the aorta. However, no birefringence due to lipid was detected in the intima or media as determined by loss of birefringence on melting (see later) or lipid staining with oil red O.

In contrast to control arteries, sections from balloon-deendothelialized arteries showed marked intimal thickening due to cellular proliferation (Fig. 1). These proliferating cells could be separated into foam cells, usually seen deeper in the intima, and nonfoam cells, seen nearer the lumen. The foam cells often appeared elongated with their long axes radially aligned on the IEL. The more superficial nonfoam cells did not appear to be organized in any definite arrangement and resembled the medial cells in staining characteristics. The IEL was commonly split, reduplicated, or disrupted. The media on the whole was unchanged. At times foam cells were present near the IEL, and occasionally necrotic material was seen just deep to the IEL, presumably the result of balloon injury. Staining with Sudan black showed that the foam cells were filled with neutral fat. However, small amounts of sudanophilic staining was often present in more superficial intimal cells as well as in the media adjacent to the IEL. Unstained sections viewed under crossed polarizer and analyzer revealed strong birefringence in a distribution similar to the foam cells seen in hematoxylin-stained sections. The birefringence was organized within cells as masses of small birefringent “droplets.” The IEL could also be easily seen and revealed a brighter birefringence than other connective tissue elements, suggesting possible lipid in association. As well as these strongly birefringent regions, faint birefringence could be seen in the more superficial layers of the intima and in the media adjacent to the IEL, similar in distribution to the sudanophilic droplets seen with the neutral fat stain. In sections reacted with the enzyme marker, ß-galactosidase, activity was most prominent in foam cells, although lesser activity was also present in regions of the intima in which definite foam cells could not be identified.

Figure 2 shows a high-power photomicrograph of the same section before and after staining. Individual foam cells seen with hematoxylin and eosin staining (B) can be easily identified in the unstained section viewed under polarized light (A) and allow correlations between cell morphology and the melting characteristics of the cellular lipid to be made. The birefringence within cells is composed of rounded droplets, and no definite sharp-angled crystals were seen.

Adrenal Gland. Adrenal glands from rabbits fed a chow diet weighed between 100 and 150 mg. The cortex was composed of cells arranged in cords which were aligned perpendicular to the capsule. The cells were all of similar size, vacuolated when stained with hematoxylin and eosin (Fig. 3), and sudanophilic on staining with Sudan black. No definite demarcation of the cortical zone (glomerulosa, fasciculata, and reticularis) was obvious with these two stains.

In animals fed the high cholesterol diet for any period longer than 2 weeks a striking difference in the glands was noted. They appeared larger and generally weighed two to three times more than those from the chow-fed animals. In the first few weeks two populations of cortical cells were seen. The first resembled those of the normal gland, being smaller and less vacuolated than a second population of cells which were greatly increased in size and had a foamy appearance on staining with hematoxylin and eosin (Fig. 3). With time the whole adrenal cortex was replaced by these large foam cells, and it appeared that this process proceeded from outer to inner cortex (Fig. 3).

These large foam cells stained heavily with Sudan black. In some instances vacuolated cells also appeared within the capsules, and these two were sudanophilic. Although the inner cortex was still clearly demarcated from the medulla, some sudanophilic cells were present within the medulla (Fig. 4).

Differences between glands from chow-fed rabbits and cholesterol-fed rabbits were also noted when unstained sections were viewed by polarizing light microscopy. Normal adrenal gland showed marked regional differences in birefringence within the cortex when observed at room temperature. There was no birefringence of the outer cortex but there was strong birefringence of the inner cortex (Fig. 3). Adipose tissue that surrounded the gland was also birefringent but was distinguished from the
Fig. 3. Comparison of sections of adrenal cortex from chow-fed and cholesterol-fed rabbits. A. Outer cortex from chow-fed rabbits shows cords of vacuolated cells with no readily discernable zones. Outside the cortex adipose tissue is present. B. Under crossed polarizers an adjacent section examined at 22°C reveals birefringent material in the inner cortical region and adipose tissue but very little in the outer cortex. Characteristics of the birefringence seen in the cortex is different from that of the adipose tissue. C. Cholesterol feeding resulted in formation of large foam cells, particularly of the outer cortex, and increased vacuolation of cells in the inner cortex. D. At 22°C there was no regional difference in birefringence between inner and outer cortex. Figure 3A and C, Hematoxylin and eosin; ×160.

Fig. 4. Sections of adrenal cortex and medulla demonstrating the differences in lipid composition of each region. A. Cortex composed of masses of foam cells, whereas none are seen in the medulla. B. Vivid demonstration of the differences in neutral lipid content of cells in each region. However, there are some sudanophilic cells seen in the medulla. C. Strong birefringence within cortical cells is seen under polarized light. However, at room temperature (22°C) not all of the foam cells are birefringent due to a lower melting temperature of the cholesterol esters of the adrenal gland than those in foam cells in atherosclerotic lesions (see Table 2). Outlines of “melted” foam cells can still be discerned in the unstained section (arrows). Medulla contains a few small foam cells which contain birefringent lipid at room temperature, Figure 4A, Hematoxylin and eosin; B, Sudan black B, ×200.

Liver. Liver sections from rabbits fed a high cholesterol diet for short periods of time did not show much birefringent lipid or sudanophilia on staining. However, with increasing duration of feeding the number of sudanophilic cells, foam cells, and birefringent cells increases.
such that by 16 weeks the livers were packed full of birefringent, neutral lipid.

Adipose Tissue. Microscopic adipose tissue studies were performed on adipose tissue taken directly from the animal and compressed between a microscope slide and cover slip (unfrozen and unstained). Large adipocytes could be seen under polarized light but they contained no birefringent material under crossed polarized light at room temperature and thus the lipid was in the liquid state.

THERMAL BEHAVIOR

Artery. Unstained frozen sections of lipid-rich atherosclerotic artery were observed under polarized light while being heated at 1 to 2°C/minute. No change was seen in birefringence until about 30°C when there appeared to be a slight diminution in the intensity of birefringence. At about 40°C the birefringence appeared to fade and was completely gone by 50 to 51°C. On cooling the tissue, birefringence returned a few degrees lower than the disappearance temperature observed on heating. Note, in Figure 5, that not all cells melt at the same temperature, but rather there is a range of melting between 35° and 52°C. Although in some cases the larger lipid deposits appeared to melt at higher temperatures, this was not a universal finding. Nor did the melting temperature change when lipid droplet size was artifically increased by repeated heating and cooling. This suggests that there were differences in either the cholesterol ester makeup or triglyceride content of different cells. On repeated heatings and cooling the lipid appeared to coalesce into larger droplets, and even leak out of cells, but the melting temperature did not change. As the lipid coalesced into larger droplets definite metastatic crosses characteristic of liquid crystalline phase were observed. When viewed under the quarter wave plate these droplets were positively birefringent, indicating a smectic organization (22, 23). The leakage of the lipid from the cells

Fig. 5. Melting sequence of foam cells in atherosclerotic lesion viewed under polarized light. At 22°C birefringent foam cells can be seen aligned along the IEL similar to Figure 2. However, foam cells are also present in the media and intima nearer to the lumen. In addition crystals can be seen in the media (arrow). Birefringent material in the adventitia is connective tissue (collagen and elastin). B. At 30°C the birefringence is beginning to fade, consistent with liquid crystal melting, but connective tissue birefringence is unchanged. Crystals in the media are also melting. C. By 42°C most of the cells and crystals have melted, but there is differential melting of the cells; that is, some cells appear to melt before others. Since the crystals have all melted by 42°C they cannot be cholesterol monohydrate. On cooling they form liquid crystals and, therefore, are cholesterol esters. The connective tissue birefringence remains unchanged. D. Same section stained after heating. Figure 5D, Hematoxylin and eosin; ×160.
was accentuated by covering the tissue with a cover slip (Fig. 6).

When crystals were present within these tissues, they melted first at approximately 41 to 43° C into birefringent liquid crystals and then into isotropic liquid. On cooling the section, the crystals did not reform but rather formed liquid crystalline birefringent lipid droplets. These melting characteristics and the reforming of liquid crystals were characteristic of cholesterol ester crystals (22) but not cholesterol monohydrate crystals (17) or triglycerides (25).

Plots of this melting profile were made by using the light meter readings obtained during heating, as described in "Materials and Methods" and as illustrated for an adrenal gland in Figure 7. A sigmoidal curve resulted, from which a range of melting, and a temperature at which 50% of birefringence remained, could be obtained. On repeated heating and cooling similar curves were generated with a coefficient of variation of endpoint and midpoint melting <10%.

Differential scanning calorimetry thermograms of arterial intima and other tissues were also obtained (Table 1). Normal intima showed no transition between 0 and 50° C. However, in lipid-rich arterial intima a broad transition over the temperature range 28 to 50° C was obtained. Melting temperatures of arterial intima from lipid-rich aorta and iliac arteries of rabbits from all three periods after institution of the cholesterol-rich diet were similar, suggesting a similar chemical composition. With the final melting temperatures of tissue cholesterol esters obtained by the three techniques (differential scanning calorimetry, microscopy, and light transmittance) were compared and found to be similar (Table 2).

Adrenal Gland. When frozen sections of adrenal cortex from chow-fed rabbits were examined by hot stage polarizing light microscopy, differences in lipid melting were readily apparent for different regions (Fig. 8). At room temperature only the inner cortical region was birefringent. However, as tissues were cooled, birefringence developed in a progressive manner from inner to outer cortex, until the entire cortex was birefringent. Birefringence was that of optically positive mica cross which indicates liquid crystalline structures. On reheating, the cortex lost its birefringence in reverse sequence, and all lipid had melted by 35° C.

Differential scanning calorimetry of adrenal co
from chow-fed rabbits showed peak melting temperatures of melting at 16° and 24° C with a range of 5° to 33° C (Table 1). Since differential scanning calorimetry gives an average melting of the predominant lipid, the regional differences observed by microscopy were not indicated by the thermograms of whole gland melting.

Melting of adrenal cortical lipid from cholesterol-fed rabbits was strikingly different. Not only was the melting temperature over a much higher range, but the regional difference so characteristic of the chow-fed rabbit gland was obliterated. Differential scanning calorimetry revealed a melting range of 19° to 43° C with peaks at 29° and 37° C (Table 1). A plot of cholesterol-fed rabbit adrenal cortical cholesterol ester melting, as determined by light transmittance, is displayed in Figure 7. This was taken from the central 1% of the field of view at a magnification of ×250 and included five to six cells. Melting occurred over a range of 24° to 38° C with 50% reduction of light transmittance at approximately 30° C.

Liver. Although livers from cholesterol-fed rabbits became rich in cholesterol ester after prolonged cholesterol feeding, there was often not sufficient ester present in early periods for melting studies to be performed by light transmittance or differential scanning calorimetry. However, direct observation of birefringence droplets during heating allowed their melting temperature to be determined. In liver tissue that was sufficiently lipid-rich to perform differential scanning calorimetry (Table 1), melting temperatures obtained by differential scanning calorimetry, direct observation, and light transmittance were similar (Table 2). In no cases were crystals seen in liver sections.

Adipose Tissue. Observation of adipose tissue melting over the temperature range of −5° through 50° C revealed different behavior than the cholesterol ester-rich tissues (Fig. 9). At −2° C the adipocytes were composed of a mass of small needle-like crystals whose morphology could not be perturbed when the cover slip was depressed (Fig. 9a). At approximately 0° C a change in the intensity of the birefringence was noted, and by 10° C small oil droplets were present on the surface of the large mass of crystals (Fig. 9b). Between 12° (b) and 19° C (c) some areas became less birefringent (upper right) and others became more birefringent (middle), indicating different polymorphism in these two regions. Between 22° and 25° C a further change in the character of birefringence of the crystals was noted, and then at 36° C a definite melting of the crystals took place (Fig. 9d), and this was complete by 48° C.

The differential scanning calorimetry tracing obtained from adipose tissue was also complex (Fig. 10) but correlated with what was seen by microscopy. Two definite endothermic reactions occurred with peak temperatures of 4° and 40° C which correspond to the change in

![Fig. 7. Plot of the light transmittance (% birefringence) through birefringent cells from rabbit adrenal gland cortex as they were heated from 20° through 50° C, a range of lipid melting as well as the temperature at which 50% of birefringence remaining could be determined. Quantitation of light transmittance was made by taking the inverse of the exposure time from the camera light meter and normalizing by the following formula: % of light transmittance at temperature X° = \[\frac{(E_r - E_i)}{(E_s - E_i)}\] × 100, where \(E_i\) = initial exposure time before heating, \(E_r\) = exposure time at completion of heating, and \(E_s\) = exposure time at temperatures between \(T_i\) and \(T_r\).]

### Table 1. Melting Temperatures of Cholesterol Ester-Rich Tissues from Cholesterol-Fed and Chow-Fed Animals as Determined by Differential Scanning Calorimetry

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Onset</th>
<th>Peak(s)</th>
<th>Complete</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial intima (5)</td>
<td>28.8 ± 5.9</td>
<td>41.4 ± 2.7</td>
<td>49.8 ± 1.9</td>
</tr>
<tr>
<td>Adrenal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol fed animal (9)</td>
<td>18.7 ± 3.6</td>
<td>29.3 ± 2.1</td>
<td>43.3 ± 2.4</td>
</tr>
<tr>
<td>Cholesteryl ester</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (3)</td>
<td>17.6 ± 4.0</td>
<td>36.6 ± 1.8</td>
<td>42.3 ± 3.5</td>
</tr>
</tbody>
</table>

*Results are means ± SD. Numbers in parentheses are numbers of animals. Adrenal gland lipids had a second small peak (values in brackets) due to cholesteryl-liquid transitions.
intensity and melting, respectively. Another possible endothermic peak was observed at 20°C. These temperatures appeared close to visual melting in some of the tissue crystals as they were heated. The undulating base-

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Rabbit no.</th>
<th>Differential scanning calorimetry</th>
<th>Microscopy</th>
<th>Light transmittance</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>°C</td>
<td>°C</td>
<td>°C</td>
</tr>
<tr>
<td>Intima</td>
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<td>49</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>51</td>
<td>49.5</td>
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<td></td>
<td>3</td>
<td>50</td>
<td>48.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>42</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>44.5</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td></td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4</td>
<td>46</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>42</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>39</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Adrenal</td>
<td>1</td>
<td>40</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>41</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>48</td>
<td>43</td>
<td></td>
</tr>
</tbody>
</table>

*The differential scanning calorimetry and light transmittance data are not different as judged by paired t-test.

DISCUSSION

Several studies in the past have shown the value of polarizing light microscopy in the study of lipid-rich tissue since it enables information about lipid composition and lipid-lipid interaction to be obtained. We have extended this concept by showing that carefully prepared frozen sections of lipid-rich tissue are suitable for correlative studies between the physical properties of lipid and tissue histology. The two major prerequisites for this technique are that frozen sections of sufficient quality can be obtained for adequate histologic study and that

![A, Chow-fed rabbit adrenal cortex examined at 20°C under polarized light with analyzer and polarizer at 45°C (half-crossed). Birefringence is only present in the inner cortex. B, With analyzer and polarizer fully crossed, at 5°C birefringence extends almost to the capsule. C, Boxed area from A enlarged 2.5 times shown. D, At -5°C birefringent droplets are seen all the way to the capsule. Figure 8A, ×160.](image-url)
the lipid properties are not irretrievably altered during the preparation of the tissue.

An important question concerns the possible alteration of lipid transitions by the hexane used in the freezing process. If hexane were to penetrate tissue and dissolve in the lipid, it should depress the temperature of the lipid transitions. The reason that hexane does not penetrate the tissue blocks is related to the temperature of freezing. The tissue surface is covered with a continuous thin sheet of water in continuity with the extracellular water. As soon as the tissue is dropped into the hexane at about −70°C, the water at the surface freezes and creates an effective solid barrier to the penetration of hexane.

The fact that the lipid transition temperatures are not altered by hexane freezing is partially indicated in Table 2. The differential scanning calorimetry lipid transition temperatures were obtained without hexane freezing, whereas the microscopy and light transmittance data were carried out on hexane-frozen tissue. The paired t-test between differential scanning calorimetry and light transmittance data shows that there is no significant difference between the end points of melting.

Because differential scanning calorimetry and light transmittance are different techniques used to measure melting, we applied the same technique on hexane-frozen and unfrozen arterial intima and compared the results. Unfrozen intima was dissected from an iliac artery and examined directly without freezing. Adjacent intima was hexane frozen, sectioned, and examined as described in "Materials and Methods." Three separate heating runs were performed on each tissue, and the results for midpoint and end of melting are given in Table 3. Differential scanning calorimetry was also performed on the adjacent section of intima. Neither the midpoint nor end of the transition are effected by freezing, nor are they different from the differential scanning calorimetry results.

Rapid freezing of tissue in some cases did change the physical state of the lipids. For example, triglyceride in adipose tissue exists as a liquid in vivo. Freezing the tissue crystallizes part of the triglyceride so that crystals are observed in frozen sections reheated to room temperature. However, the in vivo physical state and character of the triglyceride could be retrieved by reheating the tissue above the triglyceride-melting point and returning the temperature to body temperature. In tissue in which lipid existed predominantly in cholesterol ester-rich foam cells, polarizing light microscopy revealed rounded intracellular positively birefringent droplets which melted at the same temperature on the second heating.
TABLE 3. EFFECT OF RAPID FREEZING IN COLD HEXANE ON MIDPOINT OF CHOLESTEROL ESTER TRANSITIONS FROM CONTIGUOUS REGIONS OF AN Atherosclerotic Rabbit Iliac Arterial Intima

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Run</th>
<th>Transition temperature (°C)</th>
<th>Midpoint</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen</td>
<td>1</td>
<td>44.5</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>43.5</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>43</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Unfrozen</td>
<td>1</td>
<td>44</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>44</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>44</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>Unfrozen (Differential scanning calorimetry)</td>
<td></td>
<td>44.5</td>
<td>51</td>
<td></td>
</tr>
</tbody>
</table>

run as the first, indicating that the liquid crystalline state had been "preserved" during the rapid cooling stage.

We used three methods to determine the melting temperatures of tissue lipid. The reliability of the melting temperatures observed by direct observation was tested by comparing them with determinations made by differential scanning calorimetry and/or polarized light transmittance. In tissues rich in cholesterol ester, such as adrenal glands and lipid-rich arterial intima, the melting temperatures were not different among the different methods (Table 2). Triglyceride melting was complex but both polymorphic changes and melting were observed by differential scanning calorimetry and microscopy, and the correlation of the final melting by the two methods was good. However, information gained from each method is not the same. Microscopy allows individual droplets and cell melting to be obtained but has the disadvantage of some subjectivity. The technique measuring polarized light transmittance is objective and can be used to measure regional differences in melting. Finally, differential scanning calorimetry, although accurate and reproducible, requires relatively large amounts of lipid for scans and, thus, an average melting profile of tissue lipid is obtained. However, important thermodynamic information can be obtained which helps in lipid characterization.

The ability to detect lipid heterogeneity within tissue was one of the major purposes of this study. This includes not only detection of differences in lipid classes but also possibly different cholesterol esters or triglycerides within the same tissue. Since more unsaturated lipids have lower melting temperatures, differences in lipid melting would indicate a differing chemical makeup. Cholesterol ester-melting temperature is also a function of the triglyceride content within the same droplet; the higher the triglyceride content the lower the melting temperature (4, 22).

We have clearly shown that chemical heterogeneity of lipid within lipid-rich tissues does exist, at least in the rabbit, and that this can be relatively easily detected by hot stage polarizing microscopy. The most striking example of this was in the adrenal glands taken from chow-fed rabbits in which large variation in lipid melting exist. Since the normal rabbit adrenal gland contains no tri-glyceride, differences in the cholesterol ester melting in different regions imply that different esters are segregated within the cortex with more unsaturated (and perhaps shorter chain) esters, more peripherally situated. The outer cortex contains the zona glomerulosa and outer zona fasciculata which in the rabbit cannot be readily distinguished by hematoxylin and eosin or Sudan black stains; thus, it is not clear at this stage whether the different zones have specific esters. However, we did note a sequential melting of esters rather than distinct bands of melting. The biologic significance of these ester differences is not immediately obvious. Nor is it known whether these differences are brought about by stress. For example, if saturated esters are more easily mobilized in the outer cortex, this may have been preferentially depleted of the higher melting esters resulting in a decrease in the melting temperature of the remaining esters during the stress of sacrifice.

In the arterial lesions produced by balloon deendothelialization and cholesterol feeding some foam cells melted abruptly at 35°C while others did not melt until much higher temperatures. Similar differences in cells melting within the same tissue have been noted before in the foam cells from Tangier spleen (13) and human fatty streaks (7, 12). Thus, it seems likely that within these arterial sections there were foam cells of differing chemical makeup. These lesions in rabbit are similar to the fatty streak in man in histologic and chemical makeup, and this observation may have clinical relevance. Katz, Shiple, and Small (10) have observed that not all fatty streaks in man are identical; some have a higher free cholesterol content, such that they are super-saturated with cholesterol, and these same lesions are richer in cholesterol linoleate and the cholesterol ester droplets melt at lower temperatures. In many respects these lesions were between fatty streaks and gruel plaques in composition and Katz et al. (10) termed them intermediate lesions (10). This observation suggested that indeed some fatty streaks are precursors of gruel plaques. Intermediate lesions are defined by physicochemical characteristics, and there are no histologic equivalents. However, now that we are able to correlate histology with physicochemical changes, perhaps these intermediate lesions can be further characterized and aid in understanding the pathogenesis of this disease. Other evidence that heterogeneity of lipid composition of more advanced atherosclerosis lesions comes from the careful microdissection studies of Smith and Slater (28).

The cholesterol ester melting of the deposits within the liver appeared to be relatively uniform in those animals studied. However, the melting temperature of the esters was different again from the other tissues (Table 1). The rabbit is very sensitive to cholesterol feeding, developing marked hypercholesterolemia and cholesterol esters being deposited in a wide variety of tissues. Despite this, each organ stores esters with different melting temperatures which do not appear to be altered by the vehicle used for cholesterol administration, i.e., safflower, corn, or coconut oil (D. A. Waugh and D. M. Small, unpublished observations).

By combining information gained from conventional
<table>
<thead>
<tr>
<th>Lipid</th>
<th>State(s) at room temperature</th>
<th>Crystal/morphology</th>
<th>Thermal behavior</th>
<th>Neutral lipid staining</th>
</tr>
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<tbody>
<tr>
<td>Cholesterol monohydrate</td>
<td>Crystalline</td>
<td>Plates edge angle 79° Needles</td>
<td>Melt &gt; 85°C Melt to liquid crystal, then isotopic liquid. On cooling form liquid crystals just before melting temperature.</td>
<td>No</td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>Crystals* (tend to be satu- rated or monounsaturated esters)</td>
<td>Maltese cross (smectic = positive birefringence, cholesterol = negative birefringence)</td>
<td>Melt to isotropic liquid. Reform on cooling without undercooling.</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Liquid crystals (tend to be rich in monounsaturated esters)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>Crystals* (tend to be more saturated)</td>
<td>Short needles</td>
<td>Form liquid crystals on cooling to low temperatures. Crystals melt over a broad temperature range directly to isotropic liquid. On cooling crystallization occurs 20–30° below melting temperature.</td>
<td>Yes</td>
</tr>
<tr>
<td>Complex lipids (phospholipids)</td>
<td>Oils (tend to be more unsaturated)</td>
<td>Liquid crystals (membranes or vesicles)</td>
<td>Myelin figures, vesicles* (smectic liquid crystals)</td>
<td>Melt at &gt; 90° C No</td>
</tr>
</tbody>
</table>

* Usually crystals are seen only if tissue is frozen first or left at low temperature (0 to 10°C) for several hours.
* Stain if solvent for dye dissolves crystals. If not, crystals do not stain.
* Membranes or unilamellar vesicle cannot be seen by polarizing microscope, therefore, aggregates must be multilamellar (myelin figures).

Histologic stains, polarizing light microscopy, and the thermal behavior of the lipid, we can identify the class of tissue lipid and estimate their fatty acid components where relevant (Table 4). For example, if crystals are seen in a section, knowledge of the thermal history (frozen or unfrozen sections and previous heating), the crystal morphology, and the thermal properties should allow cholesterol ester, cholesterol monohydrate, and triglyceride crystals to be easily distinguished. Similarly, the two lipids taking up neutral lipid stains (cholesterol ester and triglyceride) can be differentiated by the ability of cholesterol esters to form liquid crystals and exhibit characteristic smectic birefringence under crossed polarized light. Finally, large multilamellar aggregates of phospholipids also form smectic liquid crystals (20, 26) that are indistinguishable from cholesterol esters at room temperature. However, on heating they retain their liquid crystalline character at high temperature (~90° C), whereas cholesterol esters melt (22).

Correlations between histology and lipid deposits are not the only potential uses of this technique. As demonstrated enzyme activity and lipid deposition can be studied by studying serial sections. Also, recent advances in fluorescence technology have allowed microscopic detection of various lipoprotein receptors on lipid-rich cells grown in cell culture (19). If such cells could be labeled in vivo, then the possibility of identifying cell types within lesions and determination of their lipid-melting characteristics might indicate cellular differences in the uptake, handling, and storage of lipid.

Acknowledgments: The authors would like to thank Anne Gibbons and Irene Miller for secretarial assistance and Cynthia McCormack for excellent technical help.

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REFERENCES
6. Hata Y, Hower J, Insull W: Cholesterol ester-rich inclusions from
28. Smith EB, Slater RS: The microdissection of large atherosclerotic plaques to give morphologically and topographically defined fractions for analysis. I. The lipids in the isolated fractions. Atherosclerosis 15:37, 1972
Effects of Polyunsaturated Lecithin on Plasma and Lipoprotein Cholesterol and Fatty Acids in Normal Men

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Dept. of Medicine and Biophysics Institute
Boston University Medical School
Boston, MA

INTRODUCTION

It is well established that high total cholesterol values are associated with increased risk for atherosclerotic cardiovascular disease. Furthermore, there is abundant evidence that high density lipoprotein cholesterol (HDL-CH) has a protective effect and is associated with decreased risk; therefore, it is conceivable that lowering total cholesterol or raising HDL-CH may alter the progression or contribute to regression of the atherosclerotic process.

Polyunsaturated lecithin has been reported to induce lipid and fatty acid changes which would be favorable to regression of atherosclerosis (1-3). The combination of intravenous and oral polyunsaturated lecithin administered to Type II hyperlipemic patients appeared to result in a relative decrease of total cholesterol and a relative increase in total plasma phospholipids(s). A decrease in low density lipoprotein cholesterol (LDL-CH) and a decrease in the oleic/linoleic acid ratio in cholesteryl esters was also noted (2). In normal volunteers, crude, mixed phospholipids containing less than 30% phosphatidylcholine (PC) increased HDL-CH levels, but corn oil did not. However, both fats decreased LDL-CH (3).

As lipoprotein composition is strongly dependent on the degree of saturation of the diet, the aim of this study was to document plasma and lipoprotein cholesterol, phospholipid and fatty acid changes in normal, young, male volunteers during oral supplementation with moderate amounts of pure, unsaturated PC or sunflower seed oil (SO) having a similar fatty acid composition. Further, it is known that human LDL undergo a core-cholesteryl ester transition around 30°C (range 26-38°C) (4,5).
Normal subhuman primates also have LDL transition above body temperature and the higher LDL transition temperature is correlated with development of atherosclerosis (6). Because unsaturated PC or SO might increase the unsaturation of LDL core cholesteryl esters and thus lower the LDL transition temperature, we have also measured LDL transitions before and after PC and SO ingestion.

METHOD

SUBJECTS
Nine healthy, normal weight, male volunteers (ages 24-40), previously screened for normal baseline lipid levels, were recruited from Medical Center personnel. Volunteers were randomly assigned to one of two groups after giving informed consent. Both groups were studied over three periods (Fig. 1) consisting of two six-week periods of dietary supplementation with pure PC (Nattmann, polyenylphosphatidycholine) or SO (Sunlite, Hunt-Wesson Foods) interposed with a six-week period of unsupplemented dietary intake. Volunteers were instructed to take 6.3 g PC (14 capsules) or 10 mL (8.8 g) of SO (calibrated spoon) daily. The fatty acid compositions of the two lipid supplements are given in Table 1.

Group I received PC during the first period and SO supplementation during the third period, whereas group II received SO first and then PC (Fig. 1). One volunteer in group II did not complete the final period of study. Only data from subjects who completed the series were used in calculating statistics. All subjects were instructed in keeping three-day diet records at the beginning of each period to monitor whether PC or SO supplements altered dietary habits.

SAMPLE PREPARATION
Venous blood was collected in vacuum packs containing 15 mg NaEDTA. Plasma was separated from cells by centrifugation in an IEC moGel PR-2 centrifuge at 1,000 rpm for 20 min at 4°C. Fifty-mL samples taken at weeks 0, 6, 12 and 18 were separated into component lipoprotein fractions by sequential ultracentrifugation using density solutions of NaCl and KBr as described elsewhere (7). Densities of the lipoprotein fractions were measured with an Abbe refractometer (American Optical). Lack of sample contamination with other lipoproteins was assessed by electrophoresis on 5% agarose slab gels. Ten-mL samples were obtained on days 14 and 21 of each ingestion period for plasma total lipid fatty acid analysis (Fig. 1).

LIPID ANALYSIS
Plasma and HDL cholesterol, triglycerides and HDL cholesterol were measured by standard enzymatic methods.
FIG. 1. Dietary oil supplementation schedule. Abbreviations are described in text. (1) Large arrow indicates time points where complete lipoprotein profiles were prepared. (2) Small arrow (weeks 2, 4, 14, 16) indicate time points where only samples for plasma-CH and HDL-CH and plasma fatty acids were prepared.

| TABLE 1 |
| Fatty Acid Composition of Supplement Oils (% Total Fatty Acid) |
| Fatty acid | Lecithin | Sunflower seed |
| 14:0 | 10.7 | 2.27 |
| 16:0 | 4.1 | 5.35 |
| 18:0 | 4.1 | 5.21 |
| 18:1 | 13.8 | 17.9 |
| 18:2 | 64.1 | 69.3 |
| 18:1/18:2 | .226 | .258 |
| P/S | 4.79 | 5.41 |

reagent set (Dow Diagnostics, Indianapolis, IN). Lipids were extracted with chloroform/methanol following the procedure described by Polich et al. (8). In lipoprotein fractions, cholesterol was measured by the method of Rudel and Morris (9) and lipid phosphorus was measured by the Bartlett method (10). Fatty acid methyl esters, from total plasma lipids, and LDL and HDL cholesteryl esters were prepared as described by Morrison and Smith (11). Cholesteryl esters were isolated by preparative thin layer chromatography (TLC) on Silica Gel H plates (Applied Science) in hexane/diethyl ether/acetic acid, 78:22:1. Lipids were eluted from the silica with 10 mL chloroform. Fatty acid methyl ester composition of samples was evaluated on a Hewlett Packard Model 5710A gas chromatograph with integrating recorder (Model 3385). Separations were performed.
times were standardized using reference mixtures of known methyl ester composition (Supelco).

DIFFERENTIAL SCANNING CALORIMETRY (DSC)

The LDL transition was measured using the simplified, rapid procedure of Waugh and Small (12). One or two mL plasma was taken from each subject at four time periods (0, 6, 12, 18). The apoB- containing lipoproteins were precipitated using dextran and magnesium sulfate and then the precipitate was transferred to 75-μL sample pans (Perkin Elmer, Norwalk, CT), hermetically sealed, and calorimetry was performed in a Perkin Elmer DSC-2 differential scanning calorimeter as reported previously (4,5) to determine the LDL cholesteryl ester transition temperature. Peak transition temperatures are reported.

STATISTICAL ANALYSIS

Data were evaluated by paired t-test and two-way analysis of variance (ANOVA) of repeated measures with trend analysis (13).

RESULTS AND DISCUSSION

All subjects tolerated both PC and SO supplements well and no side-effects were noted.

CHOLESTEROL AND PHOSPHOLIPIDS

There was no significant change in total phospholipids in HDL or LDL during either ingestion period, despite an increased dietary intake during PC ingestion (Table 2). No significant change in plasma total cholesterol (plasma-CH) values was detected by ANOVA, although group I showed a trend toward increased levels. HDL-CH values showed significant increases by ANOVA over the time course of the study (Table 3). There was no evidence of a difference by ANOVA between the two supplementation regimens in altering HDL-CH values. In both groups, the change in this parameter was more pronounced during the second ingestion period.

### TABLE 2

<p>| HDL and LDL Phospholipid (mg/100 mL, X ± SEM) |
|-----------------|----------|----------|----------|----------|
|                 | 0        | 6        | 12       | 18       |
| <strong>Group I</strong>     |          |          |          |          |
| HDL phospholipid | 38.4 ± 1 | 60.6 ± 1 | 69.4 ± 1 | 66.4 ± 1 |
| LDL phospholipid | 49.2 ± 2 | 56.8 ± 2 | 68.0 ± 1 | 50.4 ± 1 |
|                 |          |          |          |          |
| <strong>Group II</strong>    |          |          |          |          |
| HDL phospholipid | 61.0 ± 3 | 58.7 ± 1 | 70.9 ± 2 | 63.8 ± 1 |
| LDL phospholipid | 80.5 ± 5 | 60.0 ± 4 | 44.9 ± 3 | 55.8 ± 3 |</p>
<table>
<thead>
<tr>
<th>Weeks of study</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>plasma-CH</td>
<td>148 ± 3.4</td>
<td>169 ± 6.8</td>
<td>164 ± 5.0</td>
<td>167 ± 6.4</td>
<td>180 ± 7.0</td>
<td>168 ± 2.8</td>
<td>180 ± 8.6</td>
<td>185 ± 8.0</td>
</tr>
<tr>
<td>HDL-CH</td>
<td>43 ± 1.4</td>
<td>50 ± 1.4</td>
<td>43 ± 1.4</td>
<td>40 ± 1.8</td>
<td>44 ± 1.6</td>
<td>49 ± 1.0</td>
<td>44 ± 0.6</td>
<td>51 ± 1.4</td>
</tr>
<tr>
<td>HDL-plasma CH</td>
<td>295 ± 0.2</td>
<td>308 ± 0.2</td>
<td>314 ± 0.2</td>
<td>269 ± 0.1</td>
<td>282 ± 0.1</td>
<td>260 ± 0.1</td>
<td>303 ± 0.2</td>
<td>307 ± 0.2</td>
</tr>
<tr>
<td>plasma-CH</td>
<td>198 ± 11.0</td>
<td>195 ± 10.4</td>
<td>165 ± 10.6</td>
<td>176 ± 10.6</td>
<td>171 ± 4.2</td>
<td>169 ± 4.8</td>
<td>190 ± 10.4</td>
<td>192 ± 11.2</td>
</tr>
<tr>
<td>HDL-CH</td>
<td>44 ± 1.4</td>
<td>49 ± 1.0</td>
<td>46 ± 1.4</td>
<td>44 ± 1.4</td>
<td>48 ± 2.0</td>
<td>48 ± 0.6</td>
<td>53 ± 1.6</td>
<td>57 ± 1.2</td>
</tr>
<tr>
<td>HDL-plasma CH</td>
<td>276 ± 0.2</td>
<td>298 ± 0.2</td>
<td>296 ± 0.2</td>
<td>268 ± 0.2</td>
<td>273 ± 0.2</td>
<td>270 ± 0.2</td>
<td>295 ± 0.2</td>
<td>312 ± 0.2</td>
</tr>
</tbody>
</table>

*aSignificant by two-way analysis of variance, no difference between groups I and II.

*bSignificantly different by paired t-test (p < .025) from weeks 0 and 12.
PLASMA AND LIPOPROTEIN FATTY ACIDS

A comparison of the fatty acid composition of plasma total lipids is given in Table 4. The major components are linoleic, oleic and palmitic acids. During the course of PC or SO ingestion, there was a significant increase in the relative amount of linoleic acid. No differences were detected between the treatment regimes by ANOVA. The ratio of oleic/linoleic acid at 18 weeks was significantly decreased compared to 0 and 12 weeks as evaluated by paired t-test.

In HDL and LDL cholesteryl esters (Tables 5 and 6), there was no consistent trend to increase or decrease the relative amount of any fatty acid.

LDL CHOLESTERYL ESTER TRANSITIONS

The peak transition temperatures (Tm) from the four study periods are summarized in Table 7. The mean melting temperature of all samples studied was 28.1 ± 3.3 C (range 22-32.5) which is about 2.2 C lower than the mean values reported by us for an older, more heterogeneous group of subjects (5).

TABLE 4

Plasma Total Lipid Fatty Acid Methyl Esters (% Composition ± SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks of study</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PC</td>
<td>SO</td>
<td>PC</td>
<td>SO</td>
</tr>
<tr>
<td>14:0</td>
<td>2.16 ± .63</td>
<td>1.28 ± .09</td>
<td>1.23 ± .05</td>
<td>1.29 ± .03</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>2.14 ± .09</td>
<td>2.44 ± .14</td>
<td>2.33 ± .14</td>
<td>2.44 ± .07</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>9.49 ± .11</td>
<td>9.24 ± .12</td>
<td>8.88 ± .07</td>
<td>7.09 ± .07</td>
<td></td>
</tr>
<tr>
<td>18:1</td>
<td>8.23 ± .36</td>
<td>6.13 ± .34</td>
<td>5.59 ± .36</td>
<td>5.39 ± .34</td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>8.23 ± .36</td>
<td>6.13 ± .34</td>
<td>5.59 ± .36</td>
<td>5.39 ± .34</td>
<td></td>
</tr>
<tr>
<td>18:3</td>
<td>7.27 ± .22</td>
<td>9.27 ± .53</td>
<td>7.67 ± .21</td>
<td>9.17 ± .35</td>
<td></td>
</tr>
<tr>
<td>18:4</td>
<td>0.56 ± .02</td>
<td>0.52 ± .01</td>
<td>0.60 ± .02</td>
<td>0.52 ± .05</td>
<td></td>
</tr>
<tr>
<td>18:1/18:2</td>
<td>0.636 ± .02</td>
<td>0.522 ± .01</td>
<td>0.600 ± .02</td>
<td>0.520 ± .05</td>
<td></td>
</tr>
<tr>
<td>P/S</td>
<td>1.92 ± .03</td>
<td>2.04 ± .16</td>
<td>2.26 ± .03</td>
<td>2.47 ± .08</td>
<td></td>
</tr>
</tbody>
</table>

Group II

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
<td>SO</td>
<td>PC</td>
<td>SO</td>
</tr>
<tr>
<td>14:0</td>
<td>2.16 ± .63</td>
<td>1.28 ± .09</td>
<td>1.23 ± .05</td>
<td>1.28 ± .09</td>
</tr>
<tr>
<td>16:0</td>
<td>21.43 ± .44</td>
<td>22.42 ± .14</td>
<td>21.91 ± .09</td>
<td>19.96 ± .63</td>
</tr>
<tr>
<td>18:0</td>
<td>8.23 ± .36</td>
<td>6.13 ± .34</td>
<td>5.59 ± .36</td>
<td>5.39 ± .34</td>
</tr>
<tr>
<td>18:1</td>
<td>6.36 ± .02</td>
<td>0.522 ± .01</td>
<td>0.600 ± .02</td>
<td>0.520 ± .05</td>
</tr>
<tr>
<td>18:2</td>
<td>27.86 ± 1.46</td>
<td>31.35 ± 0.88</td>
<td>33.81 ± 1.71</td>
<td>37.89 ± 1.77</td>
</tr>
<tr>
<td>18:3</td>
<td>0.56 ± .02</td>
<td>0.522 ± .01</td>
<td>0.600 ± .02</td>
<td>0.520 ± .05</td>
</tr>
<tr>
<td>18:4</td>
<td>8.50 ± .69</td>
<td>8.72 ± .39</td>
<td>7.91 ± .41</td>
<td>8.96 ± .33</td>
</tr>
<tr>
<td>18:1/18:2</td>
<td>0.636 ± .02</td>
<td>0.522 ± .01</td>
<td>0.600 ± .02</td>
<td>0.520 ± .05</td>
</tr>
<tr>
<td>P/S</td>
<td>1.89 ± .07</td>
<td>1.85 ± .01</td>
<td>2.32 ± .04</td>
<td>2.69 ± .10</td>
</tr>
</tbody>
</table>

aSignificant by two-way analysis of variance, no difference between groups I and II.
bSignificantly different by paired t-test (p < .025) from weeks 0 and 12.
cP = polyunsaturated fatty acids; S = saturated fatty acids.
The mean melting temperatures were not significantly different from each other at any time, by ANOVA. No differences were observed when transition temperatures were compared across diet regimes (group I + group II PC ingestion vs group I + group II SO ingestion).

One of the aims of this study was to determine the response of plasma lipoproteins to dietary polyunsaturated PC and to evaluate whether this response varied from that induced by SO, a polyunsaturated triglyceride. No significant difference between the two fat supplements was observed. Supplement sequence did not appear to modify the results. In both dietary regimes, the effects were more pronounced in the second ingestion period.

A consistent increase in HDL-CH is evident throughout the study in both groups. This is in contrast to a previous report that a mixed phosphatides preparation, but not corn oil, raised HDL-CH values (3). Similarly, changes in plasma fatty acid profiles do not indicate any difference between treatment groups.
# TABLE 6

**LDL Cholesteryl Ester Fatty Acids**

(\% Composition $\pm$ SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>Weeks of study</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>14:0</td>
<td>0.83 $\pm$ 0.04</td>
<td>2.02 $\pm$ 0.15</td>
<td>2.14 $\pm$ 0.15</td>
<td>0.98 $\pm$ 0.04</td>
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<tr>
<td></td>
<td>16:0</td>
<td>16.69 $\pm$ 0.54</td>
<td>17.12 $\pm$ 0.18</td>
<td>18.52 $\pm$ 0.05</td>
<td>16.35 $\pm$ 0.36</td>
</tr>
<tr>
<td></td>
<td>18:1</td>
<td>2.26 $\pm$ 0.05</td>
<td>3.42 $\pm$ 0.07</td>
<td>3.11 $\pm$ 0.03</td>
<td>2.77 $\pm$ 0.02</td>
</tr>
<tr>
<td></td>
<td>18:2</td>
<td>3.73 $\pm$ 0.44</td>
<td>3.77 $\pm$ 0.23</td>
<td>4.71 $\pm$ 0.06</td>
<td>2.67 $\pm$ 0.08</td>
</tr>
<tr>
<td></td>
<td>18:4</td>
<td>18.98 $\pm$ 0.66</td>
<td>19.30 $\pm$ 0.08</td>
<td>19.53 $\pm$ 0.05</td>
<td>17.68 $\pm$ 0.47</td>
</tr>
<tr>
<td></td>
<td>18:5</td>
<td>53.65 $\pm$ 1.23</td>
<td>50.10 $\pm$ 0.28</td>
<td>49.13 $\pm$ 0.07</td>
<td>53.15 $\pm$ 0.39</td>
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<tr>
<td></td>
<td>18:6</td>
<td>5.31 $\pm$ 0.21</td>
<td>5.81 $\pm$ 1.31</td>
<td>6.00 $\pm$ 0.05</td>
<td>5.17 $\pm$ 0.38</td>
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<tr>
<td></td>
<td>18:7</td>
<td>.379 $\pm$ .02</td>
<td>.385 $\pm$ .01</td>
<td>.401 $\pm$ .01</td>
<td>.340 $\pm$ .05</td>
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<tr>
<td></td>
<td>F/S</td>
<td>3.64 $\pm$ .17</td>
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<td>3.92 $\pm$ .10</td>
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</table>

**Group II**

<table>
<thead>
<tr>
<th>14:0</th>
<th>.531 $\pm$ .14</th>
<th>2.09 $\pm$ .79</th>
<th>3.30 $\pm$ .16</th>
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<tr>
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<td>14.45 $\pm$ 6.0</td>
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<td>19.84 $\pm$ .36</td>
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<tr>
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<td>3.67 $\pm$ .40</td>
<td>4.56 $\pm$ .44</td>
<td>3.69 $\pm$ .38</td>
<td>4.3 $\pm$ .41</td>
</tr>
<tr>
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<td>2.33 $\pm$ .19</td>
<td>5.05 $\pm$ .67</td>
<td>3.24 $\pm$ .09</td>
<td>11.00 $\pm$ 2.31</td>
</tr>
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<td>4.09 $\pm$ 2.26</td>
<td>2.13 $\pm$ 1.75</td>
<td>18.96 $\pm$ 1.10</td>
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<tr>
<td>18:5</td>
<td>2.31 $\pm$ .78</td>
<td>3.14 $\pm$ .24</td>
<td>2.38 $\pm$ .26</td>
<td>2.53 $\pm$ .80</td>
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<tr>
<td>18:6</td>
<td>.399 $\pm$ .22</td>
<td>.704 $\pm$ .14</td>
<td>.552 $\pm$ .09</td>
<td>.465 $\pm$ .04</td>
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<tr>
<td>18:7</td>
<td>4.59 $\pm$ .20</td>
<td>2.51 $\pm$ .20</td>
<td>3.28 $\pm$ .90</td>
<td>2.522 $\pm$ .42</td>
</tr>
</tbody>
</table>

$^a$P = polyunsaturated fatty acids; S = saturated fatty acids.

# TABLE 7

**Mean LDL Transition Temperature (C) (x ± SEM)**

<table>
<thead>
<tr>
<th>Weeks of study</th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>18</th>
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<tbody>
<tr>
<td></td>
<td>PC</td>
<td>SO</td>
<td>PC</td>
<td>SO</td>
</tr>
<tr>
<td>Group I</td>
<td>28.3 $\pm$ 1.5</td>
<td>28.7 $\pm$ .58</td>
<td>29.5 $\pm$ .80</td>
<td>30.2 $\pm$ .1</td>
</tr>
<tr>
<td>Group II</td>
<td>26.3 $\pm$ 1.1</td>
<td>28.5 $\pm$ 1.36</td>
<td>28.8 $\pm$ 2.1</td>
<td>28.8 $\pm$ 2.0</td>
</tr>
</tbody>
</table>

These data suggest that, in normal men, the effect of oral PC is related to its fatty acid content as has been previously reported in rat studies (14).

ACKNOWLEDGMENTS

We wish to thank J. Steiner, E. Freiberg, R. Corey and A. Pathak for technical assistance, and I. Miller for expert preparation of this manuscript. This work was supported in part by NIH Research Grant Number RR00220 of the National Institutes of Health, Bethesda, Maryland.
REFERENCES

Epidemiological Evidence Associating Lipids with Cancer Causation
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Denmark

INTRODUCTION

The idea that fats and oils may play a role in cancer causation is not a new one, as Stern (1) has questioned "the reason for which the number of cancers is so much higher in nuns than in other women? Should one seek it in the food they eat and more particularly in the excessive use of fish and oil or in the long and repeated fasts?" These ideas emerged from observations of cancer in humans which, in recent decades, have again drawn attention to the possible role of our lifestyles in the causation of cancer (2). Particular interest has focused on our refined diet which is rich in protein and fatty foods. Rigoni Stern, more than 100 years ago, had to infer solely from the distribution of cancers in various populations, whereas, modern epidemiology provides opportunities to penetrate further into the hypotheses emerging from human observations. In attempts to relate fats to cancer occurrence, the combined efforts of epidemiology and biochemistry have proven of particular importance, so that metabolic epidemiology has almost reached the status of the subspecialty.

This chapter describes human observations associating fat with a variety of cancers, and particular attention will be given to cancer of the large bowel. Aspects of metabolic epidemiology will only be mentioned briefly, as these subjects are covered in greater detail elsewhere in the monograph.

EVIDENCE OF CARCINOGENICITY FROM HUMAN STUDIES

Chemical carcinogenicity has been increasingly recognized as important in cancer causation. The criteria for judging evidence of chemical carcinogenicity from human studies also apply to the possible role of fat as a risk factor.
Transluminal Angioplasty in Experimental Atherosclerosis
Analysis for Embolization Using an In Vivo Perfusion System

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SUMMARY We used polarized light microscopy and thin-layer chromatography to determine whether embolization of atherosclerotic material occurs after transluminal angioplasty. The experimental model consisted of an in vivo perfusion system of the atherosclerotic rabbit left iliac artery. Of eight rabbits that underwent successful angioplasty, four had angiographic evidence of dissection and three showed aneurysm formation. Histologic studies demonstrated fracture of the intimal plaque, dissection, and stretching of the noninvolved portion of the vessel. Perfusion analysis revealed no detectable cholesterol by thin-layer chromatography in six of eight rabbits. In two rabbits, a very small amount of cholesterol was measured, which was totally accounted for by hemorrhage into the perfusate rather than from cholesterol in the plaque. No evidence of arterial wall embolic debris could be detected by polarized light microscopy in seven rabbits, but lipid debris from the plaque was found in the perfusate of one rabbit that had excessive arterial trauma.

We conclude that the major mechanism of successful transluminal angioplasty in this experimental model is intimal fracture combined with stretching of a noninvolved portion of the vessel. Furthermore, embolization of atheromatous lipid debris was an uncommon event related to arterial trauma during catheter placement rather than transluminal angioplasty itself.

TRANSLUMINAL ANGIOPLASTY is being used increasingly as a nonsurgical alternative in the management of obstructive atherosclerotic disease. Using a balloon-tipped catheter developed by Gruentzig,1 successful reduction of stenosis has been reported in the peripheral,2 renal,3 and coronary arteries.4 How this technique works, however, is incompletely understood. Only two prior studies of the histologic effects of transluminal angioplasty using an experimental atherosclerosis model have been reported.5,6 Both studies demonstrated evidence of intimal plaque fracture, with dissection extending between the intima and media after transluminal angioplasty. Histologic evidence of stretching of the intima and media was also reported.

Angioplasty involves the enlargement of a narrowed arterial lumen by an intraluminal balloon catheter. Such a procedure could redistribute plaque contents throughout the arterial wall or actually cause embolization of arterial wall lipid debris. Reports of rare emboli after angioplasty in peripheral arteries1,7 raise concern about this possibility. Distal embolization has not been reported in clinical coronary angioplasty. Furthermore, Gruentzig et al.8 reported that when transluminal angioplasty was performed during coronary artery bypass surgery in three patients, no evidence of embolic debris was found on millipore filters.8 However, detailed lipid analyses were not performed. Accordingly, we used the sensitive methods of polarized light microscopy and thin-layer chromatography to determine if there is any evidence of atheromatous lipid embolization after transluminal angioplasty in experimental atherosclerosis in an in vivo perfusion system.

Methods

Experimental Model and Design

Atherosclerosis was induced in the aorta and left iliac artery of 14 3-kg male New Zealand white rabbits (eight experimental and six control) using the Baumgartner technique,9 followed by feeding an atherogenic diet. A #3F Fogarty catheter was passed retrogradely through the left femoral artery to 20 cm, inflated to occlude the vessel, and slowly removed. Denudation of the aorta and left iliac endothelium was assured by passing the Fogarty balloon twice to 20 cm. The rabbits were then placed on a 2% cholesterol diet composed of rabbit chow supplemented with 2% cholesterol mixed with 10% peanut oil for 6 weeks. Previous studies have shown the development of significant atherosclerosis in 68% of animals placed on this atherogenic diet for this time period. At the end of 6 weeks, the eight experimental rabbits were reoperated under pentothal anesthesia to expose the aortic bifurcation and iliac arteries. Ligatures were placed in the upper aorta, right iliac artery, and side branches of the left iliac artery (fig. 1). A #5F Cordis sheath was inserted through an arteriotomy into the aorta, while a #3F Goode-Lebun catheter was advanced retrogradely through the left femoral artery. Both catheters were secured with silk ties. The #3F catheter closely approximated the internal circumference of the femoral...
artery and served to prevent collection of debris between the catheter and the vessel wall. The aortoiliac system was then perfused at 90 mm Hg with heparinized Ringer's lactate solution (2000 U heparin/l).

Cineangiography was performed through the Cordis sheath to visualize the arterial lesions and confirm complete ligation of all side branches. This control ensured that all perfusate would be collected completely. Three milliliters of Renografin 76 were injected with a hand syringe over 3 seconds. Angiographic images recorded on 35-mm film using a Philips 6-inch image intensifier with a resolution of 3.8 line pairs/mm. Since the rabbit iliac artery approximates the size of the human coronary artery, a 2.5-cm Gruntzig intraoperative transluminal angioplasty catheter was found suitable and was advanced through the Cordis sheath to the site of greater iliac stenosis under fluoroscopic guidance. This position was confirmed by comparison with video recordings of the angiogram. Aliquots of the perfusate were collected before and after catheter insertion as a baseline determination before balloon dilation. Subsequently, the dilation catheter was inflated to 5 atmospheres for 30 seconds, then deflated and removed. The perfusate was again collected in three 1-minute intervals after transluminal angioplasty. Flow rates for all sampling were 2–5 ml/min and were a function of the resistance of the #3F catheter. Repeat angiography was then performed to document the results of angioplasty. Care was taken to position the image intensifier at the same height for both angiograms. A 1-cm grid was positioned on the film to permit calculation of the actual luminal diameter and to provide correction for any magnification errors between films. Immediately after angioplasty and the final perfusate collection, the aorta and iliac vessels were perfused with formalin at 80 mm Hg as previously described. The rabbits were then killed and the vessels were surgically removed and placed in formalin.

Angiographic, Histologic and Biochemical Analysis

Comparison of the pre- and postdilation cineangiograms were made on a Vanguard projector to determine change in luminal diameter. A change of 20% (0.4 mm when corrected for magnification) could easily be resolved using this technique and was considered significant. Angiographic dissection was defined as a linear density of contrast material extending beyond the luminal outline. Anomalous formation was defined as a luminal diameter greater than that in proximal nondilated segments. Each angiogram was read independently by two angiographers and discrepancies were resolved by a subsequent simultaneous reading.

The surgically removed iliac vessels were examined histologically by preparing serial 1-cm segments of the left iliac artery and allowing for at least two segments through the dilated areas and the nondilated proximal segment. Sections stained with hematoxylin-cosin and Verhoff's van Gieson-elastin were reviewed by at least two investigators and a consensus reading was made of the histologic findings. Intimal fracture was defined as a radial tear through the intima, dissection was defined as a circumferential tear along the internal elastic membrane, and stretching was defined as a thinning of the wall with loss of nuclear staining.

Both unspun and centrifuged (2000 rpm × 20 minute) samples of the five aliquots collected from each rabbit were examined by polarized light microscopy to determine birefringence, as previously described. Plasma cholesterol and lipid content on the perfusate samples were extracted overnight in 10 ml of chloroform:methanol (2:1 vol/vol), after which a Folch procedure was performed. Quantitative thin-layer chromatography was used to measure free cholesterol ester, and phospholipid in duplicate as previously described. Red cell counts were also performed on the perfusate.

To document that the experimental model was highly atheromatous, and thus suitable for study of possible lipid embolization, we determined the lipid concentration in the arterial wall. Therefore, mean left iliac artery lipid composition was measured on a group of six control rabbits that underwent endothealization and cholesterol feeding as in the experimental group but not angioplasty and formalin fixation. After 6 weeks on the atherogenic diet, the rabbits were killed under pentothal anesthesia and the left iliac artery was removed, opened longitudinally, and rinsed free of any residual blood with cold saline. The intimal-medial portion of the vessel was then carefully stripped away from the adventitial layer according to the method of Wolinsky and Daily. The samples were minced with
Results

Experimental Atherosclerosis

After 6 weeks on the atherogenic diet, all eight experimental rabbits had angiographic and histologic evidence of marked atherosclerotic disease. The mean serum cholesterol was 1476 ± 467 mg/dl. An example of the type of lesion created is shown in figure 2, which is a cross section of the left iliac artery. It demonstrates marked intimal thickening that is highly cellular and contains accumulations of foam cells. The atheromatous nature of this lesion is dramatically visualized under polarized light microscopy. A considerable amount of lipid was deposited in the thickened intima and indicates the suitability of this experimental lesion for investigating the possibility of lipid embolization after balloon dilation.

The mean lipid composition (mg/g wet weight) of the left iliac artery of the six control animals that did not undergo angioplasty and formalin fixation was: cholesterol, 15.01 ± 11.1; cholesterol ester, 57.4 ± 38.2; and phospholipid, 7.13 ± 3.7. The mean value of four normal left iliac artery segments that did not have cholesterol feeding or endothelial denudation was: cholesterol, 0.8 ± 0.3; cholesterol ester, 1.23 ± 0.8; and phospholipid, 3.41 ± 0.8.

Angiography

Table 1 is a summary of the angiographic results before and after transluminal angioplasty. All eight rabbits showed an increase in luminal diameter of greater than 20%. Proximal segments that did not undergo angioplasty showed no change in luminal diameter. Dissection, defined as an angiographic linear density, was seen in four of eight animals. Three rabbits showed aneurysm formation.

No evidence of extravasation of dye, catheter perforation, or total occlusion of a vessel after angioplasty was noted. Positioning of the catheter at the site of greatest iliac stenosis was performed successfully and with little difficulty in all rabbits except one (no. 7), in which the angle between the Cordis sheath and the left iliac artery as well as the presence of a long concentric lesion made catheter placement difficult. Figure 3 is an example of the angiographic results of transluminal angioplasty.

Pathology

Three types of histologic results were seen in the dilated segments (table 2). In five of eight rabbits, there was fracture through the neoointima, and a flap of

Figure 2. A balloon/cholesterol fed lesion viewed with polarized light microscopy. The atheromatous lipid is seen as brightly illuminated birefringent material, accumulated primarily in the intima (I) and also in the media (M). Magnification × 200.
Table 1. Angiographic Iliac Artery Diameters Before and After Angioplasty

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Proximal segment (mm)*</th>
<th>Angioplasty segment</th>
<th>Difference</th>
<th>Dissection</th>
<th>Aneurysm</th>
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<tr>
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<tr>
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<td>2.2</td>
<td>1.5</td>
<td>2.5</td>
<td>+1.0</td>
<td>+</td>
</tr>
</tbody>
</table>

*Segment diameters were determined by correction for magnification using a 1-cm grid.

Abbreviations: + = present; 0 = absent.

Table 2. Histologic Results

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Intimal fracture</th>
<th>Dissection</th>
<th>Stretching</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
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<td>++</td>
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<td>8</td>
<td>++</td>
<td>++</td>
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| 5/8    | 6/8              | 2/8        |

Abbreviations: + = local fracture, dissection or stretching; ++ = marked changes; 0 = absence of changes.

The thickened intima often folded back into the lumen. In addition, there was dissection along the internal elastic membrane, at times extending into the media, in six of eight rabbits (fig. 4). Two of eight rabbits showed an eccentric foam cell lesion. Angioplasty in these vessels resulted in thinning of the noninvolved portion of the vessel wall without intimal fracture. The stretched portion of the wall showed loss of nuclear staining and densely packed layers of extracellular matrix.

**Perfusate Analysis**

Despite the marked degree of fracture, dissection, and stretching of the vessel wall, no evidence of red cells, cholesterol, or debris was detected in the perfusate in six of eight rabbits that had successful angioplasty (table 3). As the perfusion system was maintained in a continuous infusion of heparinized Ringer’s lactate solution, no debris was found adherent to the arterial or catheter wall. As thin-layer chromatography can measure 1–2 μg of cholesterol, and lipid analysis of similar iliac vessels documented a great amount of cholesterol in the vessel wall, embolization of small fragments of arterial debris could be easily detected by this technique. In rabbits 1 and 7, minimal bleeding was detected in the perfusate. Since the aorta and iliac branches were ligated as confirmed by angiography, this bleeding was probably derived from capillaries that penetrated the diseased vascular wall. Since the ratio of perfusate red blood cells to whole blood and perfusate cholesterol to plasma cholesterol were both 1:100, this lipid in the perfusate could be totally accounted for by bleeding from the neovascularization rather than from the atheromatous arterial wall itself.

In rabbit 7, angioplasty was very difficult. Visually, the catheter penetrated out of the lumen and into the adventitia. Angioplasty was nevertheless performed, and in the first aliquot after dilation, a 5-mm piece of atheromatous debris was noted in the collection (fig. 5). The detection of cholesterol by thin-layer chromatography and debris by polarized light microscopy indicates that the perfusion system can detect lipid material when present.

**Discussion**

Since transluminal angioplasty caused intimal plaque fraction and dissection in experimental studies,™ embolization of atherosclerotic debris could be an undesirable consequence of successful transluminal angioplasty. Using an experimental model with significant lipid deposition in the neointima, the present study provides evidence that embolization of plaque material is rare and unlikely. With the highly sensitive

![Figure 3](image_url)  
**Figure 3.** The angiographic results of transluminal angioplasty. Arrows indicate two lesions of approximately 50% stenosis before transluminal angioplasty. They showed total resolution of luminal narrowing after dilation.
technique of thin-layer chromatography, no cholesterol from the atheromatous plaque was found in the perfusate. Polarized light microscopy is useful for visualizing the atheromatous lipid (fig. 2). With this technique, subcellular lipid droplets as small as 0.5 μ have been identified, and as little as 10 ng of cholesterol were detected in the present study. Therefore, if embolization of arterial wall lipid debris were to occur after transluminal angioplasty, polarized light microscopy would be another sensitive method for detecting its presence. In the present study, no evidence of embolic atheromatous debris was noted in seven of eight rabbits. Rabbit 7 had marked arterial trauma due to difficult catheter placement in a diffusely diseased vessel. Therefore, the lipid material collected in the perfusate of this rabbit is attributed to the damage to the arterial wall during catheter placement rather than to angioplasty itself.

There have been a few clinical reports of rare embolization after transluminal angioplasty of peripheral arteries. Gruentzig found evidence of embolization in 3% of patients undergoing angioplasty of peripheral arterial stenosis; Zeitler et al. reported three of 161 clinically detected cases of emboli after peripheral angioplasty in which minor local ischemia was noted, which cleared spontaneously. In the only prior attempt to collect blood distal to the site of angioplasty, Gruentzig et al. found no evidence of embolic debris on millipore filters collected after transluminal angioplasty performed during coronary artery bypass surgery. Endothelial denudation was reported after transluminal angioplasty in normal canine coronary arteries. The fate of these superficial non-lipid-laden cells is unknown. No endothelial cells were noted in centrifuged samples of the perfusate examined by bright field or polarized light microscopy; however, staining was not performed. Certainly, there is no evidence that the majority of the atheromatous lesions embolized in the present study. We emphasize that care must be taken in catheter placement to prevent significant arterial trauma.

We used an experimental model of atherosclerosis to investigate the effects of transluminal angioplasty. Marked intimal thickening and highly cellular foam

Table 3. Perfusion Results

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Blood</th>
<th>Debris</th>
<th>Cholesterol (mg/dl)*</th>
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Abbreviations: + or 0 = presence or absence, respectively, of red cells detected with light microscopy or lipid debris seen by polarized light microscopy.

*The cholesterol reported is the total cholesterol (free cholesterol and cholesterol ester).
technique of thin-layer chromatography, no cholesterol from the atheromatous plaque was found in the perfusate. Polarized light microscopy is useful for visualizing the atheromatous lipid (fig. 2). With this technique, subcellular lipid droplets as small as 0.5 μ have been identified, and as little as 10 ng of cholesterol were detected in the present study. Therefore, if embolization of arterial wall lipid debris were to occur after transluminal angioplasty, polarized light microscopy would be another sensitive method for detecting its presence. In the present study, no evidence of embolic atheromatous debris was noted in seven of eight rabbits. Rabbit 7 had marked arterial trauma due to difficult catheter placement in a diffusely diseased vessel. Therefore, the lipid material collected in the perfusate of this rabbit is attributed to the damage to the arterial wall during catheter placement rather than to angioplasty itself.

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We used an experimental model of atherosclerosis to investigate the effects of transluminal angioplasty. Marked intimal thickening and highly cellular foam

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<th>Debris</th>
<th>Cholesterol (mg/dl)*</th>
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Abbreviations: + or 0 = presence or absence, respectively, of red cells detected with light microscopy or lipid debris seen by polarized light microscopy.

*The cholesterol reported is the total cholesterol (free cholesterol and cholesterol ester).
cell lesions predominate in this model (fig. 2). In addition, the lesion created is highly vascular, with media and adventitia neovascularization. Thus, the model is not entirely analogous to advanced human atherosclerosis, which includes cell necrosis and calcification. However, Block et al. found histologic findings in three patients who died within 5 days of transluminal angioplasty that were similar to previous experimental findings. This is also found in the perfusion system used in the present study, as well as in prior in vivo rabbit experimental studies. It is thus likely that the findings of the present study reflect transluminal angioplasty in human atherosclerotic disease.

Experimental angioplasty studies have documented endothelial denudation and platelet adhesion, fracture of the intimal plaque with or without dissection along the elastic membrane, and stretching of the noninvolved portion of an eccentric lesion after transluminal angioplasty. Studies in coronary arteries of human cadaver hearts have shown endothelial disruption, and compression of atheroma has been suggested as a mechanism of dilation, although detailed histologic analysis was not performed. Morphometric studies measuring luminal and arterial wall size, and intimal and medial cross-sectioned areas are necessary to confirm compression and also to determine if aneurysm formation is a mechanism of successful angioplasty. Furthermore, although angioplasty is often successful in reopening a stenotic vessel, it still produces considerable arterial wall trauma. Thus, with respect to the response to injury hypothesis of atherogenesis, the long-term consequences of angioplasty remain to be determined.

References

PHYSICOCHEMICAL CHARACTERIZATION OF THE URINARY LIPID FROM HUMANS WITH NEPHROTIC SYNDROME

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Physicochemical characterization of the urinary lipid from humans with nephrotic syndrome

ROBERT S. MARTIN* and DONALD M. SMALL Boston, Mass.

The aim of this study was to investigate the nature of urinary lipid in humans with nephrotic syndrome. Fresh urine specimens were fractionated by centrifugation into a lower cellular fraction and an upper noncellular fraction. Of the 11 urine specimens examined, six cellular fractions contained cells (oval fat bodies) that were laden with anisotropic (birefringent) droplets when viewed by polarizing microscopy. The mean total cholesterol excretions for the five urine specimens without anisotropic droplets and the six urine specimens with anisotropic droplets were 6.7 mg/L and 35.5 mg/L, respectively. The cellular fractions of the six urine specimens with anisotropic droplets are enriched in cholesterol esters relative to whole urine. Upon heating, the anisotropic droplets underwent phase transitions characteristic of cholesterol esters, as observed by polarizing microscopy. The mean cholesteric to isotropic phase transition temperature of the anisotropic droplets was 41.3°C. These data indicate that the anisotropic droplets of the oval fat bodies were composed of cholesterol esters acetylated largely with monounsaturated fatty acids consistent with cellular origin. The noncellular fractions were subjected to ultracentrifugation (48,000 x g for 2 hours) and then separated into supernate and infranate fractions. The supernate fractions contained minor amounts of lipid, except in two cases of massive lipiduria. In these two cases, the supernate fractions contained many individual anisotropic droplets with lipid composition nearly identical to their cellular fractions. The dispersed lipid of the infranate fractions was invisible by microscope. Nine of the 11 infranate fractions revealed an alpha migrating lipid band by agarose gel electrophoresis. For urine specimens with anisotropic droplets, the infranate fractions contained cholesterol ester fatty acids that were less saturated (as measured by gas-liquid chromatography) than the cellular fractions. Thus, the cholesterol esters of the infranate fractions were derived from a different source (probably high-density lipoproteins) than the cholesterol esters of the cellular fractions. (J LAB CLIN MED 103:798, 1984.)

Abbreviations: acyl cholesterol acyl transferase (ACAT), high-density lipoproteins (HDL), high power field (HPF), lecithin cholesterol acyl transferase (LCAT)

Nephrotic syndrome, which may occur during the course of kidney diseases of varying cause, is characterized by proteinuria, lipiduria, hypoalbuminemia, hyperlipidemia, and edema. In 1913, Munck† noticed that the urine sediment of patients with

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Urine lipids in nephrotic syndrome

Fig. 1. Phase diagram of three-component system, cholesterol (C), phospholipid (PL), and cholesterol ester (CE), in excess water at 37°C and 1 atmosphere pressure. Zone I has a single phase of phospholipid lamellar liquid crystal with up to 33% cholesterol and 2% cholesterol ester. Zone II also has a single phase composed of liquid or liquid crystalline cholesterol ester. Mixtures in zone III have both the cholesterol ester and the phospholipid phase. In zone IV, there is a third phase as well, cholesterol monohydrate crystals. Schematic molecular representation of the phases shown near each apex of the triangle. Phospholipid molecule shown as Δ, cholesterol, O, and cholesterol ester / . Molecules of cholesterol ester form isotropic oil droplets when random, and birefringent droplets when ordered in layers 35 Å thick. (Adapted from Katz SS, Shipley GG, Small DM: Physiological chemistry of the lipids of human arteriosclerotic lesions. Demonstration of a lesion intermediate between fatty streaks and advanced plaques. J Clin Invest 58:200-211, 1976).

nephrotic syndrome contained cells with lipoid droplets. The cells have been termed oval fat bodies, and the droplets are called Maltese crosses because of their anisotropic microscopic appearance when viewed with crossed polarized light. Isolated anisotropic droplets have been reported to be composed of cholesterol esters in two patients. Other researchers have measured urinary lipid in nephrotic patients, normal patients, and patients with other diseases. All previous works were done with whole urine specimens. This study was undertaken to define in greater detail the physical nature of the oval fat bodies and to investigate the presence of other forms of lipid in the urine of patients with nephrotic syndrome.

The possible physical states of the major lipids in urine can be represented by a ternary phase diagram of the model system of cholesterol ester, unesterified cholesterol, and phospholipid at high water concentration (Fig. 1). Cholesterol is virtually insoluble in water and could only account for a minor fraction of the daily excretion of cholesterol.

Cholesterol may exist in the urine in four major physical forms. Each form has a characteristic appearance on polarizing microscopy and a characteristic lipid composition that can be plotted (Fig. 1). Separation of the various forms of urinary lipid by centrifugation can be verified by analyzing the microscopic appearance and the lipid composition of each fraction.

1. Membranes of cells and organelles have a lipid composition corresponding to zone I, the phospholipid bilayer phase (Fig. 1). Phospholipid bilayers appear as myelin figures or cell
membranes by polarizing microscopy.\textsuperscript{10,12,13} Phospholipid membranes are more dense than water and will sink to the bottom of an aqueous solution with centrifugation.\textsuperscript{15}

2. The lipid composition of microscopically visible lipid droplets, noncellular or cellular, corresponds to zone II in Fig. 1. These large lipid droplets are composed of cholesterol ester and triglycerides, both of which are less dense than water. Consequently, these large lipid droplets will float to the top of an aqueous solution during centrifugation. Microscopically visible droplets of cholesterol esters exhibit anisotropism, or birefringence, when viewed between crossed polarizers.\textsuperscript{16,17} Below the thermal phase transition temperature, droplets of cholesterol esters display the focal conic texture of the smectic mesophase with crossed polarized light. The focal conic texture is commonly referred to as the Maltese cross appearance. With increasing temperature, droplets of cholesterol ester melt to the cholesteric mesophase, which has a more disordered texture. The smectic and cholesteric mesophase are liquid crystal states of matter.\textsuperscript{10} A few degrees above the smectic to cholesteric transition temperature, droplets of cholesterol ester melt to an isotropic, or nonbirefringent, liquid oil.\textsuperscript{11} Droplets of cholesterol esters melt to isotropic liquids at characteristic temperatures, depending on the proportion of various cholesterol ester fatty acids.\textsuperscript{11}

3. The lipid composition of lipoprotein particles\textsuperscript{16} corresponds to zone III of Fig. 1. Lipoproteins are too small to be visualized by polarizing microscopy.

4. Except in the case of supersaturation, crystals of cholesterol monohydrate are present in a sample with a lipid composition in zone IV on Fig. 1.

In this study of urine from patients with the nephrotic syndrome, we separated the urinary lipids by centrifugation into three fractions: a cellular fraction, which includes the urine sediment, a noncellular supernate fraction, and a noncellular infranate fraction. The physical state of each fraction is determined by hot-stage polarizing microscopy. The chemical composition was determined by microanalytic techniques. The electrophoretic mobility was studied by agarose electrophoresis.

**Methods**

**Protocol.** Patients who had had a recent 24-hour urine collection for protein and creatinine clearance evaluation were studied in the Renal Clinic at the Boston University Medical Center. A fresh urine sample was obtained from patients with nephrotic syndrome, and sediment was immedia-

---

**Table I. Clinical and laboratory data of each patient at the time of urine collection**

<table>
<thead>
<tr>
<th>No.</th>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Weight (kg)</th>
<th>Blood pressure (mm Hg)</th>
<th>TP/Alb (gm/dl)</th>
<th>Hematocrit (gm/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A-1</td>
<td>20</td>
<td>M</td>
<td>*</td>
<td>120/80</td>
<td>6.0/3.0</td>
<td>*</td>
</tr>
<tr>
<td>2</td>
<td>A-2</td>
<td>20</td>
<td>M</td>
<td>*</td>
<td>140/80</td>
<td>5.8/2.8</td>
<td>*</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>26</td>
<td>M</td>
<td>110</td>
<td>107/78</td>
<td>6.3/3.5</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>34</td>
<td>F</td>
<td>82</td>
<td>155/90</td>
<td>5.7/3.5</td>
<td>42</td>
</tr>
<tr>
<td>5</td>
<td>D-1</td>
<td>19</td>
<td>F</td>
<td>59</td>
<td>125/85</td>
<td>3.3/2.0</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>D-2</td>
<td>19</td>
<td>F</td>
<td>56</td>
<td>140/90</td>
<td>3.6/2.4</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>E</td>
<td>30</td>
<td>M</td>
<td>75</td>
<td>130/90</td>
<td>6.0/3.7</td>
<td>43</td>
</tr>
<tr>
<td>8</td>
<td>F-1</td>
<td>54</td>
<td>F</td>
<td>102</td>
<td>196/88</td>
<td>6.6/3.5</td>
<td>28</td>
</tr>
<tr>
<td>9</td>
<td>F-2</td>
<td>54</td>
<td>F</td>
<td>102</td>
<td>240/90</td>
<td>6.6/3.5</td>
<td>28</td>
</tr>
<tr>
<td>10</td>
<td>G</td>
<td>34</td>
<td>F</td>
<td>69</td>
<td>150/108</td>
<td>7.7/3.0</td>
<td>29</td>
</tr>
<tr>
<td>11</td>
<td>H</td>
<td>30</td>
<td>M</td>
<td>65</td>
<td>115/84</td>
<td>6.2/4.1</td>
<td>39</td>
</tr>
</tbody>
</table>

GN = Glomerulonephritis; GS = glomerulosclerosis; TP = total protein; Alb = albumin.
*Information unknown.
†Creatinine clearance determined with 24-hour urine specimen.
<table>
<thead>
<tr>
<th>Cholesterol (mg/dl)</th>
<th>C&lt;sub&gt;er&lt;/sub&gt; (ml/min)</th>
<th>Proteinuria (gm/24 hr)</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>*</td>
<td>174</td>
<td>3.7</td>
<td>Henoch-Schoenlein purpura</td>
</tr>
<tr>
<td>*</td>
<td>163</td>
<td>11.0</td>
<td>Henoch-Schoenlein purpura</td>
</tr>
<tr>
<td>329</td>
<td>164</td>
<td>1.0</td>
<td>Chronic GN with hypertension</td>
</tr>
<tr>
<td>234</td>
<td>102</td>
<td>4.1</td>
<td>Focal and segmental GN</td>
</tr>
<tr>
<td>288</td>
<td>50</td>
<td>5.7</td>
<td>Membranoproliferative GN</td>
</tr>
<tr>
<td>*</td>
<td>47</td>
<td>0.8</td>
<td>Membranoproliferative GN</td>
</tr>
<tr>
<td>295</td>
<td>42</td>
<td>5.4</td>
<td>Focal and segmental GS</td>
</tr>
<tr>
<td>252</td>
<td>21</td>
<td>8.2</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>*</td>
<td>20</td>
<td>*</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>231</td>
<td>17</td>
<td>15.0</td>
<td>Rapidly progressing GN</td>
</tr>
<tr>
<td>361</td>
<td>17</td>
<td>4.1</td>
<td>Focal and segmental GS</td>
</tr>
</tbody>
</table>

ately examined by a University Hospital nephrologist. Sodium azide (0.05%) was added to an aliquot to prevent bacterial growth. Portions were taken for polarizing microscopy, agarose gel electrophoresis, and centrifugation. Each urine sample (11 ml) was centrifuged for 20 minutes at 3000 rpm (Model PR-Z, International Equipment Company, Boston, Mass.). The bottom milliliter was the cellular fraction, and the top 10 ml was the noncellular fraction. The noncellular fraction was removed by suction and placed in an ultracentrifuge tube and then overlayed with 1 ml of distilled water. A second centrifugation was performed at 48,000 × g for 2 hours in a Beckman SW41 rotor and a Beckman L5-75 ultracentrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The tubes were sliced near the interface between the urine and the water overlay into an upper supernate fraction and a lower infranate fraction. Because of a small loss of volume that occurs in tube slicing, a complete quantitative recovery was not possible. Aliquots of the cellular fraction, supernate fraction, infranate fraction, and whole urine were analyzed for lipid composition by quantitative thin-layer chromatography.

**Polarizing microscopy.** The anisotropic droplets in the urine sediment were examined on the temperature-controlled stage of a polarizing microscope. The microscope (Model WL, Carl Zeiss, Inc., Oberkochen, West Germany) was equipped with a polarizing filter, an analyzer, a compensator, and a rotating stage, which was heated by a transformer and cooled by compressed carbon dioxide.\(^{11,12}\) Urine sediments were prepared by centrifugation of 10 ml of fresh urine for 20 minutes at 3000 rpm. The supernatant was removed from the sediment by suction. The prevalence of anisotropic droplets in the sediment was classified as many (greater than one droplet per HPF), moderate (one droplet per HPF), few (less than one droplet per HPF), or none (no droplets per many HPF). Individual anisotropic droplets were continuously observed as the temperature was raised 2° C per minute. The melting temperature for the anisotropic to isotropic transition was determined to within 0.5° C.

**Agarose gel electrophoresis.** The infranate fractions were concentrated by evaporation under a gentle stream of nitrogen. Electrophoresis was carried out by Noble's\(^{13}\) method.

**Thin-layer chromatography.** Aliquots of the cellular, supernate, infranate, and whole urine fractions were extracted of their lipid content by the Folch\(^{14}\) procedure. Thin-layer chromatography was used to quantitate unesterified cholesterol, free fatty acid, triglyceride, cholesterols esters, and total phospholipids, according to the method of Downing,\(^{17}\) as modified by Katz et al.\(^{18}\) Control urine specimens from normal volunteer contained undetectable amounts of lipid when subjected to identical procedures as the urine from nephrotic patients.

**Gas-liquid chromatography.** Cholesterol esters were isolated by preparative thin-layer chromatography in the solvent system hexane-ether–acetic acid (80:20:1, vol/vol/vol). Cholesterol esters were transmethylated by the method of Morrison and Smith\(^{19}\) using methanolic BF<sub>3</sub> (Supelco Inc., Bellefonte, Pa.). Relative amounts of methyl esters were determined by multiplying peak height by retention time.
Results

The clinical diagnosis and the laboratory values in each patient at the time of urine collection are ranked in Table I by decreasing creatinine clearance. The 11 patients are between 19 and 54 years of age (mean, 31 years). All patients had episodes of proteinuria >4 gm/day. All patients with nephrotic syndrome had low to low-normal serum albumin (mean, 3.2 gm/dl), and high to high-normal serum cholesterol values (mean, 284 mg/dl). Most of the patients had low hematocrit levels (mean, 35.5 gm/dl). Patient F had diabetes and hypertension.

The descriptions of the sediments of the urine samples, which are used for additional analysis, are presented in Table II. Leukocytes in the urine are abundant only in Patient G, who had rapidly progressing glomerulonephritis. Fatty casts are present in the urine of two patients. Granular casts are present in six of the 12 specimens. Small amounts of glucose (1+) were present in the urine of the diabetic patient (F), and trace amounts of glucose were found in the urine of patient C.

Anisotropic lipid droplets were observed in the sediment in six of the 12 urine samples. In two (samples D-1 and D-2), the sediment contained many anisotropic droplets; in two it (A-Z and G) contained a moderate number of anisotropic droplets; and in two (E and F-Z), only a few anisotropic droplets. After a span of 3 weeks, fresh urine samples from three patients were obtained and analyzed. One urine sample (patient D) had a decrease in the number of anisotropic droplets, whereas two (patients A and F) contained anisotropic droplets that were not present in the earlier examination.

Fig. 2 shows a series of photomicrographs of the urine sediments. Photographs A and B are of the same field of an oval fat body and a squamous epithelial cell at room temperature with noncrossed and crossed polarized light, respectively. With crossed polarized light, only the droplets with a liquid crystalline nature appear light on a dark field. The small, slightly out of focus particles outside the oval fat body and squamous cell are erythrocytes. The oval fat body is much larger than the erythrocytes, approximately the size of squamous epithelial cells.

Photograph C is of a fatty cast under partial crossed polarized light at room temperature.
**Fig. 2.** Photographs of urine sediments. A, An oval fat body and a squamous epithelial cell from specimen D-2, viewed with non-crossed polarized light. (Original magnification ×400, 26° C). B, Same field as A, viewed with crossed polarized light. C, Fatty cast, partially crossed polarized light (specimen D-2). (Original magnification ×400, 26° C). D, Two individual lipid droplets viewed with crossed polarized light at 30° C (specimen D-1). (Original magnification ×100). E, Same field as D at 41° C.

Fig. 2, D and E, are photographs of two free lipid droplets by crossed polarized light. In D, the two droplets display the focal conic texture, which is referred to as the Maltese cross appearance. The optical sign of birefringence for the urine droplets in this study in the focal conic texture is invariably positive, which is indicative of the smectic liquid crystalline mesophase. The photograph E is of the same field at a higher temperature. The droplets at this temperature display a more disordered birefringence, which is characteristic of the cholesteric liquid crystalline mesophase. The liquid crystalline nature of these droplets can be verified by depressing the coverslip and observing the flow of the droplets. Most droplets undergo a smectic to cholesteric transition approximately 3° C below the final melting to an isotropic liquid. These changes are reversible within 1° C. Starch granules can be introduced into biologic samples from surgical gloves and can be confused with true lipid droplets. Starch granules are anisotropic and are often the same size as free lipid droplets. They can be identified and distinguished from lipid droplets by their jagged edges, crystalline nature, swelling at the melting point of 55° to 57° C, and absence of recrystallization upon cooling. No crystals of cholesterol monohydrate were observed in any of the urine sediments.

The hot-stage polarizing microscopic analysis of the anisotropic lipid droplets, which
were found in six urine samples, is summarized in Table III. The melting points reported in this work are the transition temperatures between cholesteric liquid crystalline mesophase and the isotropic liquid oil. The mean melting point for all droplets was $41.3^\circ\pm 0.4^\circ$ C. The melting point of individual droplets encompasses a wide range of temperatures, even within the same oval fat body.

The lipid concentrations and the lipid compositions in the whole urine specimens and the cellular fractions are given in Table IV. In every case, lipid is concentrated in the cellular fraction by centrifugation. In relation to whole urine, the cellular fractions contained a higher concentration of total lipid, unesterified cholesterol, esterified cholesterol, and phospholipid. The urine can be divided into two groups, depending on the presence or absence of anisotropic droplets. The specimens that contained anisotropic droplets had a higher mean total cholesterol excretion (35.5 mg/L) than the specimens without anisotropic droplets (8.7 mg/L). The specimens that contained anisotropic droplets had a higher mean relative amount of cholesterol esters in the sediment fractions (52.1%) than in the whole urine (41.5%) ($p < 0.01$); whereas urine samples that did not contain anisotropic droplets had a decreased mean relative amount of cholesterol esters in the sediment fractions (34.3%) than in the whole urine (40.5%) ($p < 0.01$).

The amount of lipid in the supernate fractions, the concentration of the total lipid in the infranate fractions, and the lipid composition of both fractions are tabulated in Table V. The supernate fractions are separated from the infranate fractions by tube slicing to prevent contamination of the infranate fractions by the supernate fractions. Consequently, each supernate fraction may be contaminated by a small amount of infranate fraction.

In samples D-1 and D-2, the supernate fractions contained a grossly visible yellow layer on top of the liquid. By polarizing microscopy, these yellow layers were composed of many free anisotropic droplets, with similar thermal phase behavior and melting points as the anisotropic droplets in their respective sediments. The relative lipid compositions of the supernate fractions of samples D-1 and D-2 were similar to their cellular fractions in both cases; specifically, they were composed of predominantly cholesterol esters.

The infranate fractions were void of microscopically visible lipid droplets. This dispersed lipid had a mean percent composition of 7.2% unesterified cholesterol, 5.4% triglyceride, 35.9% cholesterol ester, and 51.6% phospholipid. The infranate fractions contained a smaller concentration of total lipid than whole urine or the cellular fractions.

The lipid compositions of the infranate fractions were determined in five of the urine samples that contained anisotropic droplets. In these cases, the average relative amount of cholesterol esters was lower ($p < 0.05$) in the infranate fractions (31.6%) than in cellular
Table IV. Mean concentrations of lipid and lipid composition weight percentages for the whole urine specimens and the cellular fractions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Whole urine</th>
<th>Cellular fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of lipid (mg/L)</td>
<td>Percentage composition</td>
<td>Concentration of lipid (mg/L)</td>
</tr>
<tr>
<td></td>
<td>UC</td>
<td>TG</td>
</tr>
<tr>
<td>Sediment contained anisotropic droplets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-2</td>
<td>18.2</td>
<td>12.0</td>
</tr>
<tr>
<td>D-1</td>
<td>182.9</td>
<td>12.3</td>
</tr>
<tr>
<td>D-2</td>
<td>67.5</td>
<td>7.7</td>
</tr>
<tr>
<td>E</td>
<td>8.9</td>
<td>7.9</td>
</tr>
<tr>
<td>F-2</td>
<td>52.4</td>
<td>6.9</td>
</tr>
<tr>
<td>G</td>
<td>14.1</td>
<td>16.7</td>
</tr>
<tr>
<td>Mean</td>
<td>57.2</td>
<td>10.6</td>
</tr>
<tr>
<td>SEM</td>
<td>24.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Sediment did not contain anisotropic droplets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-1</td>
<td>19.3</td>
<td>10.1</td>
</tr>
<tr>
<td>B</td>
<td>25.5</td>
<td>4.2</td>
</tr>
<tr>
<td>C</td>
<td>7.2</td>
<td>6.4</td>
</tr>
<tr>
<td>F-1</td>
<td>29.0</td>
<td>8.2</td>
</tr>
<tr>
<td>H</td>
<td>14.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Mean</td>
<td>19.0</td>
<td>6.5</td>
</tr>
<tr>
<td>SEM</td>
<td>3.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

UC = Unesterified cholesterol; TG = triglyceride; CE = cholesterol esters; PL = total phospholipid.

fractions (52.1%, Table IV). When anisotropic droplets were present, centrifugation concentrated the cholesterol esters in the cellular fractions. Compositional differences of the different urine fractions are clearly shown by a plot of the fractions of sample D-2 on triangular coordinates (Fig. 3). The whole urine of sample D-2 has a composition between the cholesterol ester--rich fractions (cellular and supernate fractions) and the infranate fraction, which did not contain visible cholesterol ester. Thus, the cellular and supernate fractions approach the cholesterol ester--rich phase in composition (zone II, Fig. 1), and the infranate falls closer to the phospholipid-rich phase (zone I, Fig. 1). The lipid composition of the infranate was very similar to HDL<sup>4</sup> and could have been caused by small lipoproteins like HDL.

The weight percentages of the cholesterol ester fatty acids were determined for the cellular fractions and the infranate fractions in five urine samples that contained anisotropic droplets (A-1, D-1, D-2, F-2, and G). The mean composition and the SEM for the cellular fractions were: 15.5% ± 6.4%, cholesteryl palmitate; 8.2% ± 3.7%, cholesteryl stearate; 50.6% ± 13.5%, cholesteryl oleate; 19.9% ± 8.9%, cholesteryl linoleate; and 5.7% ± 2.5%, cholesteryl arachidonate. The infranate fraction values were: 20.9% ± 5.1%, cholesteryl palmitate; 8.6% ± 2.5%, cholesteryl stearate; 27.4% ± 2.3%, cholesteryl oleate; 37.6% ± 6.0%, cholesteryl linoleate; and 5.5% ± 1.2%, cholesteryl arachidonate. The infranate fractions contained more cholesteryl linoleate (<i>p</i> < 0.02) and less cholesterol oleate than the cellular fraction. Overall, the infranate fractions contained cholesterol esters that were less saturated and more like HDL<sup>4</sup> than the cellular fractions.

With the exception of samples G and A-2, all urine samples contained alpha migrating
Table V. Mean total lipid weights of the supernate fractions, mean lipid compositions of infranate fractions, and the mean lipid composition weight percentages for the supernate and infranate fractions

<table>
<thead>
<tr>
<th>Patient</th>
<th>Total lipid (mg/L)</th>
<th>Percentage composition</th>
<th>Concentration of lipid (mg/L)</th>
<th>Percentage composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UC</td>
<td>TG</td>
<td>CE</td>
<td>PL</td>
</tr>
<tr>
<td>Sediment contained anisotropic droplets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-2†</td>
<td>12.4</td>
<td>3.5</td>
<td>36.1</td>
<td>30.2</td>
</tr>
<tr>
<td>D-1†</td>
<td>289.1</td>
<td>5.3</td>
<td>0.0</td>
<td>58.7</td>
</tr>
<tr>
<td>D-2†</td>
<td>113.7</td>
<td>5.7</td>
<td>0.0</td>
<td>82.8</td>
</tr>
<tr>
<td>E†</td>
<td>5.9</td>
<td>0.0</td>
<td>0.0</td>
<td>21.2</td>
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<tr>
<td>F-2†</td>
<td>66.2</td>
<td>1.4</td>
<td>22.1</td>
<td>12.0</td>
</tr>
<tr>
<td>Mean</td>
<td>97.5</td>
<td>3.2</td>
<td>11.6</td>
<td>41.0</td>
</tr>
<tr>
<td>SEM</td>
<td>46.3</td>
<td>1.0</td>
<td>6.7</td>
<td>11.7</td>
</tr>
<tr>
<td>Sediment did not contain anisotropic droplets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-1</td>
<td>23.0</td>
<td>2.3</td>
<td>21.3</td>
<td>65.4</td>
</tr>
<tr>
<td>B</td>
<td>8.1</td>
<td>1.7</td>
<td>19.2</td>
<td>60.3</td>
</tr>
<tr>
<td>F-1</td>
<td>13.6</td>
<td>4.3</td>
<td>0.0</td>
<td>67.3</td>
</tr>
<tr>
<td>H</td>
<td>8.1</td>
<td>1.6</td>
<td>2.6</td>
<td>37.2</td>
</tr>
<tr>
<td>Mean</td>
<td>13.2</td>
<td>2.5</td>
<td>10.8</td>
<td>57.6</td>
</tr>
<tr>
<td>SEM</td>
<td>3.0</td>
<td>0.5</td>
<td>4.8</td>
<td>6.0</td>
</tr>
</tbody>
</table>

UC = Unesterified cholesterol; TG = triglyceride; CE = cholesterol esters; PL = total phospholipid.

l lipid material on agarose gel electrophoresis. The urine sample from patient G (the only patient in our study with rapidly progressive glomerulonephritis) revealed a pre-beta migrating lipid band. Sample A-2 contained neutral lipids only at the origin. Sample A-1 displayed both alpha and beta migrating lipid material.

Discussion

Previous studies of urinary lipid in patients with nephrotic syndrome have not considered the physical state of the lipids. In our study of 11 patients with nephrotic syndrome, the urinary lipid is separated by centrifugation into three fractions: a cellular fraction, which includes the sediment, a noncellular supernate fraction, which is enriched in free droplets of cholesterol esters and triglycerides, and a noncellular infranate fraction, which contains submicroscopic dispersed lipid droplets. The urine samples were divided into a group of six samples that contained anisotropic droplets and a group of five samples, that did not contain anisotropic droplets.

Studies of urinary proteins in humans with proteinuria20,21 and studies of the clearance of dextran polymers in experimental nephrotic syndrome in rats22 have shown that serum proteins are allowed into the glomerular filtrate by abnormally leaky glomeruli present in nephrotic syndrome. In 13 patients with nephrotic syndrome, Klahr et al.4 demonstrated that the amount of glomerular permeability correlated positively with the urinary total cholesterol excretion. The glomerular permeability is calculated as the minimum value of the albumin clearance divided by the creatinine clearance.23 Klahr et al.4 concluded that the lipiduria resulted from the filtration of serum lipoproteins at the glomerulus. In our study, the glomerular permeability and the proteinuria were not related
Fig. 3. Phase diagram similar to Fig. 1. The lipid compositions of each fraction of the urine from D-2 are displayed. The whole urine fraction (A) is separated into a phospholipid-rich fraction, the infranate (B), and cholesterol ester-rich fractions, the cellular (C), and the supernate (D) fractions. C and D compositions approach the CE phase (zone II), and B has the relative lipid composition of HDL. to the amount of lipiduria in the fresh urine sample or in any fraction of the urine (compare Tables I, IV, and V). However, because we were worried that the sediment and the lipids might deteriorate during storage, lipiduria was measured in the same fresh urine samples that were used for microscopy and not in the 24-hour urine samples used for protein and creatinine. Thus, specific correlation of lipiduria with creatinine clearance could not be done.

Several studies have measured urinary cholesterol excretion in normal humans and in humans with disease. The mean daily excretion of lipid was 1.14 mg of unesterified cholesterol in one study of 16 normal male patients; it was 0.76 mg of unesterified cholesterol and 0.92 mg cholesterol ester in another study of 62 normal men. In another study, the mean daily excretion of total cholesterol was 2.5 mg for five normal patients, 6.8 mg for four patients with hyperlipidemia of nonnephrotic cause, and 51.8 mg in 17 urine samples from patients with nephrotic syndrome. The mean total cholesterol excretion in our study was 35.5 mg/L for six urine samples from patients with nephrotic syndrome with anisotropic droplets and 8.7 mg/L for the five patients without anisotropic droplets. Our study has confirmed earlier work that large amounts of cholesterol are eliminated by the urine in patients with the nephrotic syndrome. Also in our study, the excretion of cholesterol is higher when urine contains anisotropic droplets than when anisotropic droplets are absent.

Zimmer et al. isolated an anisotropic droplet from the urine of two patients with nephrotic syndrome by centrifugation in hypertonic sucrose. Qualitative paper chromatography revealed that the neutral lipids of the isolated droplets contained cholesterol esters and perhaps minor amounts of unesterified cholesterol. In our study, microscopic anisotropic droplets, both individually and in oval fat bodies, were concentrated by centrifugation and analyzed by quantitative lipid analysis. Individual anisotropic droplets were concentrated in the supernate fractions of samples D-1 and D-2. In these two cases, the lipids of the supernate fractions were predominantly cholesterol esters. Intact oval fat bodies were concentrated in the sediment fractions in the six urine samples that contained oval fat bodies. In all six cases, the lipid compositions of the cellular fractions were enriched in cholesterol esters relative to the whole urine. These data suggest that the lipids of aniso-
tropic droplets are composed largely of cholesterol esters, which supports the qualitative impression of Zimmer et al.\(^3\) When oval fat bodies lyse, the individual cholesterol ester droplets float to the top of an aqueous solution. Intact oval fat bodies sink with centrifugation because of the dense protein and nuclear components of the cell.

The anisotropic droplets of the oval fat bodies behave physically like droplets of cholesterol esters in model systems.\(^{10,11}\) The liquid crystalline anisotropic droplets of cholesterol esters display the focal-conic texture of the smectic mesophase at room temperature under polarizing microscopy. Upon heating, the smectic liquid crystalline droplets convert to the cholesteric liquid crystalline mesophase, which has a more disordered texture; at higher temperatures, the droplets melt to a liquid, which appears isotropic with polarizing microscopy. Thus, thermal mesomorphic phase behavior, which is characteristic of cholesterol esters, was observed in the anisotropic droplets of the urine sediments.

Anisotropic droplets of cholesterol esters undergo phase transitions at characteristic temperatures, depending on the fatty acid composition of the cholesterol esters.\(^{11}\) The major effect is caused by the introduction of cis–double bonds in the acyl chain. Thus, cholesteryl stearate has a liquid crystal to isotropic transition at 79° C, cholesteryl oleate (1 cis–double bond) at 47° C, and cholesteryl linoleate (2 cis–double bonds) at 37° C.\(^{11}\) Knowing the cholesterol ester fatty acid composition and comparing it with model mixtures of cholesterol esters\(^{10,11}\) allows us to predict that the mean anisotropic to isotropic transition temperature for the cholesterol ester droplets of the cellular fractions (Table IV) should be 43.4° C. The actual mean anisotropic to isotropic transition temperature of the droplets in the cellular fraction was quite similar (41.3° C), which indicates that the anisotropic droplets are composed of nearly pure cholesterol esters. Furthermore, the presence of a smectic to cholesteric phase transition suggests that the cholesterol ester core of the droplets was not contaminated by large amounts (>4% by weight) of other lipid substances such as triglycerides because such contaminating lipid molecules abolish the cholesteric phase.\(^{24}\)

In this study, the lipid of the infranate fractions were different from the lipid of the cellular fraction in physical and chemical character. The infranate fractions contained small lipid particles, which were not visible by microscope. The dispersed lipid particles of the infranate fractions were not concentrated in the supernate fractions or the cellular fractions upon centrifugation. The lipid particles of the infranate fractions had alpha mobility on agarose gel electrophoresis, in nine of the 11 patients with nephrotic syndrome. Fractionation of the whole urine specimens that contained anisotropic droplets (n = 5) into the cellular and infranate fractions resulted in greatly different relative lipid compositions in the two fractions (see Fig. 3), whereas urine samples without anisotropic droplets (n = 4) possessed approximately the same relative lipid composition in the cellular and infranate fractions. For urine samples that contained anisotropic droplets, the cholesterol ester fatty acids of the infranate fractions were more unsaturated than the cholesterol ester fatty acids of the cellular fractions. In summary, using the parameters of size, density, agarose gel electrophoretic pattern, relative lipid composition, and the proportions of cholesterol ester fatty acids, the lipid particles of the infranate fraction can be differentiated from the lipid of the cellular fractions.

The dispersed lipid of the infranate fractions in this study may be derived from serum HDL. The average lipid composition of the infranate fractions is similar to published values for HDL\(_p\).\(^{14}\) HDL is an alpha migrating lipoprotein on agarose gel electrophoresis. The cholesterol esters of HDL are produced by LCAT. LCAT utilizes more unsaturated fatty acids for cholesterol esterification than does ACAT, which produces intracellular choles-
The density of HDL is between 1.063 and 1.200\(^2\); therefore, with our protocol, HDL would not concentrate in the cellular or supernate fractions by centrifugation.

Patients with nephrotic syndrome possess decreased amounts of serum HDL\(^2\) and have increased incidence of atherosclerosis\(^5\)\(^-\)\(^8\) The low level of serum HDL in patients with nephrotic syndrome may result from the urinary excretion of HDL in the same manner as hypoalbuminuria in patients with nephrotic syndrome is thought to result from albuminuria.\(^9\) Furthermore, because HDL possesses only a slightly larger molecular volume than albumin, as seen with agarose chromatography,\(^10\) HDL and albumin may be filtered at the renal glomerulus simultaneously. ApoC-II, which is an apoprotein loosely attached to HDL and functions as a cofactor of the serum enzyme lipoprotein lipase\(^12\)\(^-\)\(^14\) has been found in low levels in urine.\(^15\) Lower levels of lipoprotein lipase activity have been found in patients with nephrotic syndrome.\(^16\)\(^,\)\(^17\)

In summary, all 11 urine samples from patients with nephrotic syndrome in this study displayed high levels of cholesteroluria, but only six urine samples contained oval fat bodies. The anisotropic droplets of the oval fat bodies were composed of largely monosaturated cellular cholesterol esters, as shown by the lipid composition of the separate fractions and by the microscopic thermal behavior of the droplets. A pool of cholesterol esters separate from the anisotropic droplets of the cellular fraction exist as dispersed lipid droplets in the infranate fractions. This fraction may come from HDL that has leaked into urine.

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REFERENCES