Microemulsions of Cholesteryl Oleate and Dimyristoylphosphatidylcholine: A Model for Cholesteryl Ester Rich Very Low Density Lipoproteins†

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ABSTRACT: This study describes the preparation, purification, and characterization of a cholesteryl oleate/dimyristoylphosphatidylcholine microemulsion as a model for the interaction of lipid domains in cholesteryl ester rich very low density lipoproteins. These lipids were chosen specifically because their thermal transitions were distinct from each other, and their differences in chemical structure permitted the motion(s) of each lipid component to be monitored independently by ^{13}C nuclear magnetic resonance (NMR). The model particles were formed by cosonication of cholesteryl oleate and dimyristoylphosphatidylcholine in a 4:1 molar ratio for 45 min at 55–60 °C (above both lipid phase transition temperatures). The crude microemulsion was fractionated by low-speed centrifugation and Sepharose CL-2B chromatography. Microemulsion particles which eluted from the column at a volume similar to that of cholesteryl ester rich very low density lipoproteins had high cholesteryl ester:phospholipid ratios (2.5:1 → 6:1). Electron micrographs of negatively stained particles showed them to be large spheres devoid of multilamellar or unilamellar vesicle structures. Particle size calculated from a simple compositional model correlated well with sizes determined by electron microscopy (500–1000 Å) for various column fractions. Differential scanning calorimetry studies of the microemulsion revealed two thermal transitions for the model particles, at 31.0 and 46.6 °C, which were tentatively assigned to the surface phospholipid and core cholesteryl ester domains, respectively. These assignments were confirmed by ^{13}C NMR which demonstrated that, at temperatures near the lower thermotropic transition, only resonances derived from carbon atoms of dimyristoylphosphatidylcholine (DMPC) were observable. As the temperature was raised to 38.6 °C, resonances from the olefinic carbons in the cholesteryl ester acyl chain appeared in the spectrum. At 46.6 °C, the center of the higher temperature endotherm, resonances from both the steroid ring and remaining acyl chain carbons of cholesteryl oleate became observable in the spectrum. Further increases in temperature did not result in the appearance of new resonances; however, those that were present narrowed and increased in intensity. The elevation in transition temperature for DMPC in these particles (31 °C) as compared to that for DMPC in small unilamellar (18 °C) and large multilamellar (23 °C) vesicles suggested a stabilization of the phospholipid monolayer, possibly by interaction with the nonpolar core lipids. The single thermal transition of cholesteryl esters in the nonpolar core (46.6 °C) suggested that these cholesteryl esters were stabilized relative to their behavior in neat form. Comparison of ^{13}C NMR spectra of the microemulsion with those of neat cholesteryl oleate in both smectic and cholesteric mesophases suggested that core cholesteryl esters form a single liquid mesophase similar to the smectic phase of the neat material.

The plasma level of a cholesteryl ester rich, triglyceride-poor lipoprotein exhibiting β electrophoretic mobility has been implicated in the development of atherosclerosis both in humans with type III hyperlipoproteinemia (Hazzard et al., 1970, 1976; Morganroth et al., 1975; Patsch et al., 1975) and in animals maintained on high cholesterol diets (Gidez et al., 1965; Mahley et al., 1975, 1976; Roth et al., 1983). In type III patients, the very low density lipoprotein (VLDL)[1] fraction consists of spherical particles whose normal core triglycerides have been largely replaced by cholesteryl esters (Patsch et al., 1976; Sata et al., 1972). These lipoproteins, which exhibit a decreased rate of clearance from the plasma and a high apo-E content (Hazzard et al., 1976), are partly of intestinal origin and result from the action of lipoprotein lipase on chylomicrons. Patients with this disorder are at high risk for ischemic heart disease and often exhibit atherosclerosis of coronary and peripheral arteries. Although the reasons for accumulation of these chylomicron remnants and their role in the development of arteriosclerosis have not been conclusively determined (Chait et al., 1977), it has been suggested that their reduced clearance rate may reflect impaired interaction of lipases with the surface components of the lipoprotein (Chait et al., 1978).

The accumulation of cholesteryl ester rich remnant lipoproteins in the plasma and tissue has been observed in a number of species maintained on atherogenic diets (Mahley et al., 1976; Noel et al., 1975). One of the most striking examples of this is the accumulation of cholesteryl ester rich VLDL (CER-VLDL) in the plasma of rabbits fed a 1%

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; CO, cholesteryl oleate; NMR, nuclear magnetic resonance; CER-VLDL, cholesteryl ester rich very low density lipoproteins isolated from hypercholesterolemic rabbit plasma by ultracentrifugation at d = 1.006 g/mL; DSC, differential scanning calorimetry; TLC, thin-layer chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid, Me_4Si [$Si(CH_3)_4$ in figures], tetramethylsilane; LDL, low-density lipoprotein(s); EM, electron microscopy.

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cholesterol diet. Within 4 days on this diet, 60% of the VLDL fraction of these rabbits consists of large chylomicron remnants (Roth et al., 1983). After longer periods, the $d < 1.006$ g/mL material is greatly elevated while the levels of other lipoproteins are drastically decreased. Although the process is not yet completely understood, induction of hypercholesterolemia in rabbits in this manner results in rapid development of atherosclerotic lesions (Zilversmit, 1975). Indeed, Ross & Zilversmit (1977) have demonstrated that these chylomicron remnants are as effective as LDL in producing atherosclerosis when present in equivalent serum cholesterol concentrations. Thus, while cholesteryl ester rich lipoproteins have been directly linked to atherogenesis both in humans and in animals, the mechanism by which this process occurs has yet to be elucidated.

One approach to understanding the role that cholesteryl ester rich lipoproteins play in the development of atherosclerosis has been to examine their physical properties. Toward this end, Morrisett et al. (1984) have recently published a series of experiments aimed at elucidating the physical states of the lipids in the polar surface and nonpolar core of rabbit CER-VLDL. Using a variety of techniques, it was shown that both the neutral lipids in the core and the polar phospholipids on the surface of CER-VLDL exhibit significantly higher acyl chain order parameters than do the corresponding lipid domains in normal triglyceride-rich VLDL. These data were interpreted in terms of interactions between the rigid, highly organized nonpolar core and the polar surface to produce a lipoprotein in which the mobility of surface components is also severely restricted. This restriction might in turn affect critical lipoprotein properties such as receptor binding and lipase interaction. A difficulty encountered in analyzing these data specifically in terms of the core and surface structure and interaction was the compositional and size heterogeneity of the lipoprotein population. This problem is of particular significance to cholesteryl ester rich lipoproteins since even small proportions of triglyceride can cause very large changes in the thermal transitions of constituent cholesteryl esters (Kroon, 1981). A possible solution to this problem is the use of model lipoproteins whose size and composition are well-defined. Such a well-characterized model system would enhance molecular cooperativity and improve the chance of observing subtle structural changes which might go undetected in the native lipoprotein.

Recently, Ginsburg et al. (1982b) have reported a method for preparing an LDL-size microemulsion[2] composed of a cholesteryl ester rich core surrounded by a surface layer of phosphatidylcholine. These particles appear to be stable and have been shown to combine with apo-B to produce an LDL-like population (Ginsburg et al., 1984). Preparation of microemulsion models for larger lipoproteins, however, has been somewhat less successful. In 1975, Lundberg reported that microemulsions of cholesteryl esters and phospholipids could be formed after sonication for 20 min, but the size, composition, and stability of these emulsions was not determined. Using a technique involving injection of a warm ethanol solution of lipids into rapidly stirring buffer, Via et al. (1982) produced emulsions in the LDL to VLDL size range. The injection method, however, produced very small quantities of the microemulsion and did not yield stable particles of cholesteryl ester and phospholipid in the absence of triglyceride. The same technique has been used by Chen et al. (1984) to prepare microemulsions containing nearly equal amounts of egg phosphatidylcholine and cholesteryl oleate measuring about 320 Å in diameter.

In this paper, we report a procedure for the production of stable microemulsions of CER-VLDL size containing cholesteryl oleate (CO) and dimyristoylphosphatidylcholine (DMPC). This method represents an improvement over past work in terms of size homogeneity, stability, and amounts of material generated. Analysis of the model system by differential scanning calorimetry and ^{13}C NMR has yielded valuable information about the physical states of the constituent lipids and core–surface interaction which may be extended to interpret data previously obtained on the native lipoprotein.

Materials and Methods

Materials. DMPC (Sigma) was judged >99% pure by thin-layer chromatography in chloroform/methanol/water/acetic acid (65:25:4:1). Cholesteryl oleate (Sigma) was judged >99% pure by TLC in hexane/diethyl ether/acetic acid (90:10:1). Both lipids were used without further purification.

Sonication Conditions. Lipids were weighed out into Corex tubes to give the desired ratio of the two components (4 g:1 g, CO/DMPC in most cases), dissolved in dry 2-propanol, and thoroughly mixed. Most of the solvent was removed by evaporation under a stream of nitrogen; remaining traces were removed by vacuum desiccation for 12–24 h. The buffer used in the sonication experiments was 0.01 M Tris-HCl and 0.1 M NaCl, pH 7.4. Sonication was performed in a Heat Systems sonifier (W-350) equipped with a microtip. A 15-mL Corex tube containing the dried lipids (approximately 40 mg total) was clamped into place and the microtip positioned in the tube. Four milliliters of buffer at 65–70 °C was transferred to the tube and sonication begun immediately so that the temperature of the resuspended lipids was always greater than 52 °C. Sonication was performed for 45 min at 40% power (140 W), and the temperature was maintained by means of a water bath at 55–60 °C throughout the procedure. Following sonication, the lipid mixture was centrifuged for 30 min at 18000*g* and 25 °C. After centrifugation, the tube contained an opaque floating pad of lipid above a large volume of cloudy material. A pipet was inserted down the wall of the tube; care was taken not to disturb either the lipid pad or the small titanium pellet, and the cloudy fraction was carefully withdrawn. The volume of the isolated material was measured, and glycerol was added to 2.5% by weight to stabilize the emulsion.

Column Chromatography. The fraction isolated in the centrifugation step was concentrated via vacuum filtration to approximately 1.5 mL and applied to a 1.6 × 90 cm column of Sepharose CL-2B (Pharmacia Fine Chemicals) equilibrated in 0.05 M malonate, 0.1 M NaCl, 0.025% sodium azide, and 0.01% EDTA, pH 7.4. Chromatography was performed under the following conditions: downward flow rate = 7.2 mL/h; fraction volume = 2.4 mL. Fractions predicted to contain the desired material were eluted into test tubes containing sufficient glycerol so that its final concentration in each fraction was 2.5%.

Lipid Quantitation. The chemical composition of the individual column fractions was analyzed by a combination of methods. The cholesteryl ester content was determined by performing a modified Folch extraction of each fraction ($CaCl_2$ was omitted) followed by flame ionization of the components separated by microscale thin-layer chromatography on silica-coated glass rods using the Iatroscan TH-10 (Ancal, Inc., Los Osos, Ca) (Ackman, 1981; Mills et al., 1979). Phospholipid content was determined via the phosphorus assay of

[2] We have used the word microemulsion to refer to pseudomicellar particles that have a neutral lipid core and phospholipid shell and exhibit a diameter <1000 Å.

Table I: Size of Gel-Filtered Microemulsion Particles (Figure 1) As Determined from the Chemical Composition and Electron Micrographs of Negatively Stained Particles (Figure 2)

	CO:DMPC		radius[a]	
fraction	wt ratio	molar ratio	compn	EM
26	5.94 ± 0.14	6.19 ± 0.15	499[b] 420[c]	546 ± 155
28	3.54 ± 0.11	3.69 ± 0.11	322[b] 272[c]	339 ± 54
31	2.06 ± 0.11	2.15 ± 0.11	213[b] 181[c]	256 ± 74

[a] Radius was calculated from the equation $r = 3[(V_{CO}x + V_{DMPC})/A_{DMPC}]$ where x = moles of CO per mole of DMPC. For these calculations, two sets of molecular dimensions were used. These were taken from Nagle & Wilkinson (1978), Small (1985), and Curatolo et al. (1977). [b] Based on values for DMPC multilayers in the gel phase at 10 °C (A = 50.2 Å^2; V = 1041 Å^3) and cholesteryl stearate in the smectic phase (V = 1180 Å^3). [c] Based on values for DMPC multilayers in the liquid-crystalline phase at 37 °C (A = 60.8 Å^2; V = 1109 Å^3) and cholesteryl stearate in the isotropic liquid phase (V = 1195 Å^3).

Bartlett (1959) using a multiplication factor of 21.87 for DMPC.

Concentration of Microemulsions. When necessary for DSC analysis, fractions in the first peak were pooled and concentrated at ≤6 psi and 4 °C to 2 mL in a 10-mL Amicon ultrafiltration cell equipped with an XM-100 membrane. Fractions were further concentrated to approximately 200 μL (4–5 mg of total lipid) via vacuum filtration in a collodion bag (75 000-dalton cutoff) (Schleicher & Schuell, Kene, NH). Fractions thus concentrated were stored at 4 °C. For NMR studies, fractions were concentrated by placing the microemulsion in well-rinsed dialysis tubing and packing Aquacide (carboxymethylcellulose) around it, which gradually and gently concentrates the material by absorbing the buffer material through the dialysis bag.

Electron Microscopy. Formvar- and carbon-coated copper grids were pretreated with 0.1% bovine serum albumin. Lipoproteins and emulsions were then applied to the grids and negatively stained with 2.0% phosphotungstic acid, pH 7.4. Grids were examined with a Jeol 200CX electron microscope. Magnifications were calibrated by using a Fullam diffraction grating replica. The average particle radius of each sample was determined from measurement of 100 individual particles, and these results are recorded in Table I.

Differential Scanning Calorimetry. DSC was performed on both DSC-4 and DSC-2 instruments (Perkin Elmer) calibrated with an indium standard at full-scale sensitivities of 0.1–0.2 mcal/s. Heating and cooling rates were 5 °C/min. Seventy-five-microliter samples were sealed in large-volume stainless-steel capsules (Perkin Elmer). Reference pans contained 75 μL of buffer obtained as the ultrafiltrate during the concentration steps.

Preparation of CER-VLDL. Lipoproteins were obtained from the plasma of adult female New Zealand white rabbits fed a diet of normal chow pellets coated with 1% cholesterol (Roth et al., 1983). Rabbits were maintained on the diet for 4–6 weeks after which blood was collected in 1% EDTA by cardiac puncture. Cellular components were sedimented by low-speed centrifugation, and Trasylol (Mobay Chemical Co.) was added immediately to inhibit proteolysis. CER-VLDL was obtained by ultracentrifugation for 18 h at plasma density (55 000 rpm, 4 °C). The whitish supernatant was removed and washed with buffered saline. The density of the solution was adjusted to 1.210 g/mL, and the lipoproteins were again ultracentrifuged at 4 °C for 18 h at 55 000 rpm to remove residual albumin. The lipoprotein fraction was removed by decantation, applied to a 2.5 × 180 cm column of Sepharose CL-2B, and eluted at a flow rate of 16 mL/h. The desired fractions were combined, treated with Trasylol, concentrated, and stored under nitrogen at 4 °C until used.

^{13}C NMR. Proton-decoupled Fourier-transform ^{13}C NMR spectra were obtained at 96 kG (100.6 MHz for ^{13}C) on a Bruker AM400 spectrometer equipped with an Aspect 3000 data system. Spectra of emulsions were obtained by using 1.5 mL of sample (15–16 mg/mL total lipid) which had been dialyzed against 0.01 M phosphate buffer and 0.1 M NaCl, pH 7.4. About 0.1 mL of D_2O was added to the sample to obtain a deuterium signal for locking and shimming. The fatty acyl methyl resonance (14.1 ppm) was used as an internal chemical shift reference (Hailstone, 1972; Hamilton et al., 1974). Sample temperature was controlled with the Bruker variable-temperature unit to within ±1 °C. The sample was equilibrated in the probe for 30 min under data collection conditions before each data acquisition sequence was begun. The temperature of the sample was measured with a thermocouple after equilibration under spectral acquisition conditions. After each set of data was collected, the sample was ejected from the probe and visually examined; at no point during the series of experiments was any change in sample stability noted.

Results and Discussion

Production and Stabilization of Microemulsions. Although several methods for production of microemulsions have been reported in the literature (Lundberg, 1975; Via et al., 1983), only sonication appears to produce large stable particles of cholesteryl ester and phospholipid in the absence of triglyceride. By sonicating for long periods of time, Ginsburg et al. (1982b) were able to produce stable cholesteryl ester/phosphatidylcholine microemulsions of LDL size. This technique not only was reproducible but also gave homogeneously sized microemulsions in good yield. Our procedure differs from that of Ginsburg et al. chiefly in the use of much shorter periods of sonication. This shorter time period produces a microemulsion which contains more of the larger sized particles and less of the smaller LDL-sized material. In addition, since the ratio of cholesteryl ester to phospholipid in CER-VLDL is much larger than that in LDL, the initial weight ratio of cholesteryl ester to phosphatidylcholine in the sonication mixture was 4:1. Cholesteryl oleate and dimyristoylphosphatidylcholine were chosen as lipid components of the microemulsion for several reasons. First, both of these lipids exhibit thermal transitions which are distinct from each other, and which are in an experimentally convenient range. Cholesteryl oleate is one of the major cholesteryl ester constituents in CER-VLDL, and although DMPC is not a major phospholipid component of this lipoprotein, it is the only saturated phospholipid with a convenient thermal transition which does not overlap that of cholesteryl oleate. Finally, resolved resonances for the C9′, C10′ olefinic (C=C), and C8′,C11′ aliphatic (*C*–*C*=C–*C*) carbons of the cholesteryl oleate acyl chain allow its motion(s) to be monitored by NMR independently of motions for carbons in the phospholipid acyl chains.

Preparation of the CER-VLDL-sized microemulsion required several steps. Lipids in the appropriate ratio were intimately mixed in solvent, and the solvent was thoroughly removed prior to sonication. Better yields were obtained when buffer at 60 °C was added immediately before sonication was begun so that the hydrated mixture was always above the transition temperature of the cholesteryl ester. The temperature of the mixture was maintained at 55–60 °C throughout the sonication procedure; failure to maintain the temperature above 52 °C resulted in the formation of very large micro-

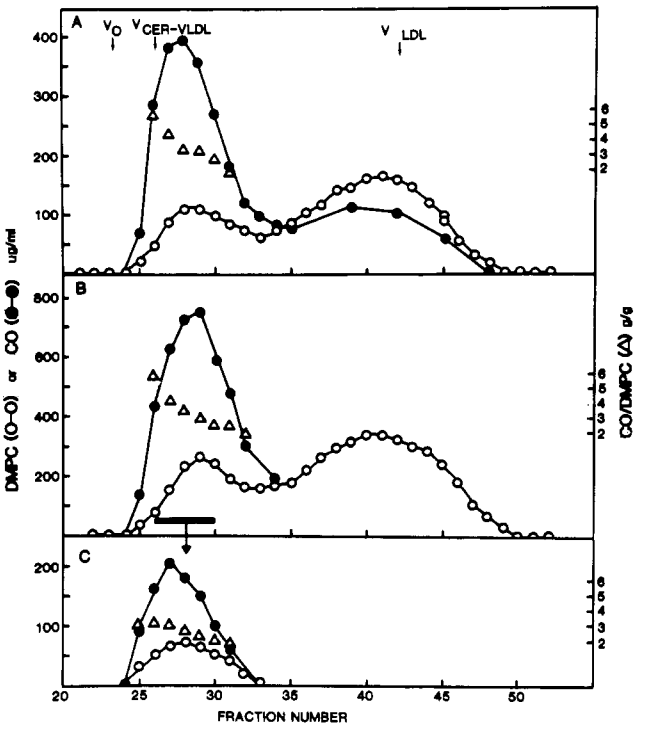

FIGURE 1: Gel filtration chromatography profile of CO/DMPC microemulsions on Sepharose CL-2B. A CO/DMPC mixture (4:1 w/w) was sonicated for 45 min, centrifuged, decanted, stabilized in glycerol (2.5%), and then chromatographed on a Sepharose CL-2B column (1.6 × 90 cm) at about 23 °C. Elution conditions: 7.2 mL/h; 2.4 mL/fraction. (A) Elution profile of a single sonicated mixture containing 32.4 mg of CO and 8.3 mg of DMPC; 8.7 mg of CO and 6.6 mg of DMPC were isolated after centrifugation, then concentrated to 1.5 mL, and applied to the column. About 7.7 mg of CO and 5.5 mg of DMPC were recovered in the eluent. Of this material, approximately 4.4 mg of CO and 1.2 mg of DMPC eluted in the first peak (CER-VLDL-sized material). (B) Elution profile resulting from two sonication mixtures which were centrifuged, fractionated, pooled, concentrated, and applied to the CL-2B column. (C) Fractions 26–30 in (B) were pooled, and a portion of the material was reapplied to the same column. (●) Cholesteryl ester; (○) phospholipid; (Δ) CO/DMPC weight ratio. V_0 represents the void volume of the column; $V_{CER-VLDL}$ and V_{LDL} are the elution volumes of CER-VLDL and LDL, respectively.

emulsion particles and a significant number of phospholipid vesicles. A 45-min sonication period gave optimum yields of the desired material. Shorter sonication times resulted in large amounts of material eluting in the void volumn, while longer sonication produced increased amounts of smaller particles at the expense of the desired material.

The size homogeneity of the microemulsion material was evaluated by gel filtration chromatography. A typical elution profile of the centrifuged material is shown in Figure 1A. Arrows indicate the elution volumes of rabbit CER-VLDL (~950-Å average diameter) and human LDL (200-Å diameter). A large portion of the microemulsion preparation eluted near the elution peak of CER-VLDL. A second peak of smaller material eluted from the column at a larger volume, corresponding approximately to that of LDL. Compositional analysis of material in the first peak indicated a high cholesteryl ester:phospholipid ratio on the leading edge of the peak (~6:1) which decreased toward the end of the profile (2.5:1), suggesting a large size for these particles. Fractions in this peak scattered light and had a whitish, opalescent appearance similar to that of CER-VLDL. Fractions in the second peak which had a fairly constant, but much lower, CO:DMPC ratio (0.7:1) were optically clear. This material was probably similar to the LDL-sized microemulsions of Ginsburg et al. (1982b). Although the microemulsion appeared to be stable overnight, after several days at room temperature particles in the first peak began to float and appeared to have disintegrated. Solid lipid material was visible in the samples.

To utilize the large microemulsions for further study, a method was sought to stabilize them for longer periods of time. Although detergents and other surface-active agents are known to stabilize emulsions by lowering interfacial surface tension, they act by forming a penetration complex with the nonpolar lipid region. In the case of our microemulsions, this type of stabilization was undesirable since it raised the possibility of perturbing the surface–core interactions we wished to study. Several authors (Adamson, 1967; Berkman & Egloff, 1941) have reported that carbohydrates and glycerol, which are very water soluble, are frequently able to impart stability to emulsions. This stabilization is presumably due to the ability of these compounds to lower the surface tension at the surface–water interface. For these studies, glycerol was chosen as a stabilizing agent for several reasons. First, since it is not a charged molecule, it does not alter the ionic strength of the buffer solution, although for its size it is one of the best surfactants available as defined by the hydrophile–lipophile balance number (11.25) (Davies & Rideal, 1961). Second, glycerol should contribute relatively few peaks to the NMR spectrum. Third, because of its small size, glycerol should be easily dialyzed from samples when necessary. At low microemulsion concentrations of 1–2 mg of CO/mL, experiments performed with radioactive glycerol at room temperature confirmed that glycerol is easily dialyzed out of the samples with no immediate loss of sample stability. Our results showed that glycerol concentrations of 2.5% by weight were sufficient to stabilize the microemulsions for up to 1 month or more at 4 °C. Thus, glycerol was added routinely to the sample after the centrifugation and isolation step, prior to concentrating and sizing the microemulsions by chromatography. In addition, column fractions were collected in sample tubes containing a glycerol solution and stored at 4 °C.[3]

Gel filtration chromatography of a typical microemulsion preparation is illustrated in Figure 1A. For this column, 32.4 mg of CO and 8.3 mg of DMPC were cosonicated; the cloudy middle zone in the centrifugation step contained 8.76 mg of CO and 6.63 mg of DMPC. Glycerol was added, and this material was concentrated to 1.5 mL by vacuum dialysis and applied to the column. Recovery of material after concentration and chromatography was 88% of CO and 83% of DMPC; approximately 4 mg of CO and 1.3 mg of DMPC were contained in the first peak (CER-VLDL-sized material). In a second experiment, two sonicated preparations were centrifuged; the cloudy lower material was combined, concentrated, and applied to the column. This column profile (Figure 1B) is qualitatively similar to that in Figure 1A, demonstrating reproducibility of the microemulsion preparation. When fractions 26–30, which contain the large microemulsion material of interest, were combined and a portion of this material was rechromatographed (Figure 1C), it eluted as a single peak coincident with the first peak of Figure 1B, demonstrating that the microemulsion was stable and did not disintegrate to smaller microemulsions or vesicles which elute at larger volumes. The overall CO:DMPC ratio was approximately the same for fractions 26–30 of elution profiles B and C of Figure 1, indicating that there was no differential

[3] Control experiments in which differential scanning calorimetry was performed on small unilamellar and large multilamellar DMPC vesicles demonstrated that the presence of 2.5% glycerol in the buffer had no effect on the temperature or enthalpy of the thermal transitions.

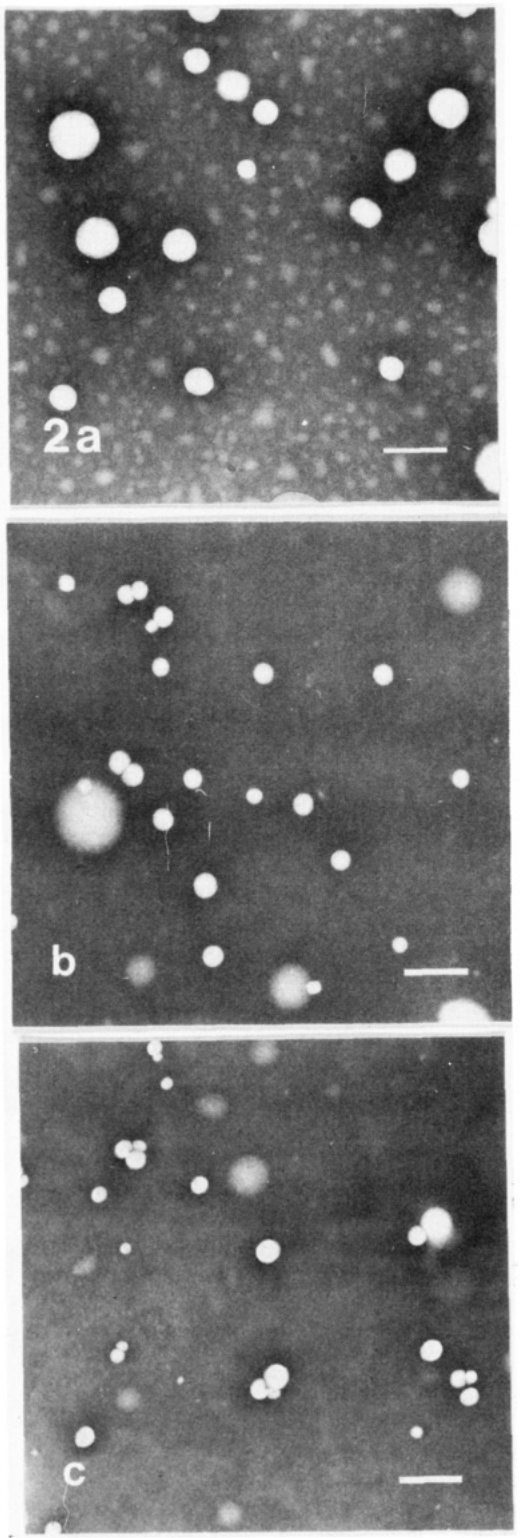

FIGURE 2: Electron micrographs of CO/DMPC microemulsion particles isolated by gel filtration chromatography (Figure 1A). Fractions 24–35 were stabilized in 2.5% glycerol. Aliquots from fractions 26 (a), 28 (b), and 31 (c) were negatively stained with phosphotungstic acid. To prevent excessive aggregation of the particles with resultant distortion of spherical geometry, dilute microemulsion preparations were used. All micrographs are at 42240×. Bars = 0.2 μm.

loss of material in these steps. Thus, this microemulsion preparation appeared to be reproducible and the material obtained to be stable as long as it was maintained in glycerol.

Characterization by Electron Microscopy. Although column chromatography and compositional data predicted a large size for the microemulsion preparation, an electron microscopic

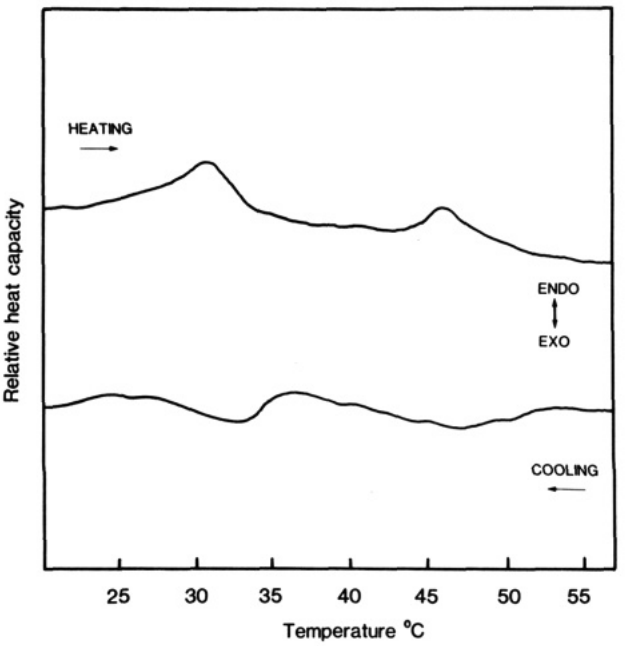

FIGURE 3: Differential scanning calorimetry thermograms for the CO/DMPC microemulsion system. Fractions 26–30 from the CL-2B column (Figure 1) were pooled and concentrated to 200 μL; 75 μL of this material was transferred to a large volume capsule and studied by DSC. Heating and cooling scans were performed at 5 °C/min.

study was undertaken to obtain further information about the size, shape, and homogeneity of the microemulsion particle population. For this study, aliquots from fractions 26, 28, and 31, each in a different region of the large microemulsion peak of column 1A, were taken for study. On the basis of chemical composition, one can calculate an approximate average size for the microemulsion population assuming a spherical shape and exclusive surface and core locations for DMPC and CO, respectively. Results of the calculations for fractions 26, 28, and 31 appear in Table I. Figure 2 is a composite of the micrographs obtained for the different fractions. These micrographs confirm the spherical shape of the particles. The particle sizes based on EM and compositional data correlate to within about 10% for all three fractions, with the EM size exceeding the compositon-determined size in each case. Rabbit CER-VLDL, whose elution peak on the CL-2B column approximately coincides with the large microemulsion peak, has an average particle diameter of 958 Å.

Characterization by DSC. A DSC study was undertaken to examine the thermal behavior of the particles. For this study, the desired portions of three sonicated preparations were combined, concentrated, and chromatographed on the CL-2B column. Fractions 26–30 from this column were combined and concentrated to approximately 200 μL (~50 mg/mL lipid). Seventy-five microliters of this material (stabilized in 2.5% glycerol) was placed in the sample pan; the reference pan contained 75 μL of 2.5% glycerol-containing buffer. The DSC thermograms indicate two broad, but distinct thermal transitions for the particles (Figure 3); the lower temperature endotherm is centered at 31 °C and the higher one at 46.6 °C. These thermal transitions are different from those observed for the isolated lipid components. Small unilamellar DMPC *vesicles* exhibit a transition at 18–19 °C, while large multilamellar *vesicles* exhibit their transition at 23 °C.[3] Upon heating in the *neat* form, cholesteryl oleate undergoes two thermal transitions corresponding to changes in lipid organization. The lower transition, at 42 °C, corresponds to the smectic → cholesteric mesophase transition, and the higher

Table II[a,b]

assignment	chemical shift (ppm from Me_4Si)	assignment	chemical shift (ppm from Me_4Si)
CO ring C18	12.14	DMPC C2′, CO acyl C2′	34.50
CO C18′	14.18	CO ring C20	36.30
DMPC C14′	14.29	CO ring C22	36.80
CO ring C21	19.08	CO ring C10	37.70
CO ring C19	19.53	CO ring C1	38.70
CO ring C11	21.50	CO ring C24	39.89
CO ring C26 and C27	22.85	CO ring C16	40.50
DMPC C13′, CO C17′	22.98	CO ring C13	42.50
CO ring C15 and C23	24.68	CO ring C9	50.40
DMPC C3′, CO acyl C3′	25.28	DMPC choline methyls	54.30
CO acyl C8′ and C11′	27.50	CO ring C6	122.50
CO ring C25	28.20	CO acyl chain C9′ and C10′	129.70 and 129.90
CO ring C12	28.60	CO ring C5	139.80
DMPC and CO acyl $(CH_2)_n$	29.00–30.75	CO carbonyl	171.20
DMPC C12′, CO ring C7 and C8, and CO acyl C16′	32.30	DMPC carbonyl	173.80

[a] Assignments based on Kroon et al. (1982), Ginsburg et al. (1982a), and Brainard et al. (1984). [b] Large peaks between 61 and 75 ppm result from glycerol and buffer.

transition, at 47.5 °C, corresponds to the cholesteric mesophase → isotropic liquid transition. Because of the differences between the transitions for the isolated lipids and the microemulsion, assignment of the observed DSC transitions in the model system to specific lipid components is tentative in the absence of further data. While it is tempting to assign the lower temperature endotherm to the phospholipids on the surface of the particle, this transition is 10–12 °C higher than that observed for unilamellar vesicles and 7–8 °C higher than that for multilamellar vesicles. The higher temperature microemulsion transition occurring at 46.6 °C correlates well with the cholesteric → isotropic transition of neat cholesteryl oleate, suggesting that this endotherm corresponds to the melting of the cholesteryl ester core. Additional data are needed, however, to confirm this assignment.

Characterization by ^{13}C NMR. ^{13}C NMR is a technique which can be exquisitely sensitive to the physical state of lipids, especially cholesteryl esters. Spectra acquired over a range of temperatures provide useful information about the motional states of lipid components. To improve the detectability of particular critical resonances in the NMR spectrum, DMPC labeled with ^{13}C at either C1′ (carbonyl) or C14′ (terminal methyl) was used in preparation of the microemulsions. As in the DSC experiments, the desired portions of the sonicated mixtures were pooled, concentrated to 1.5 mL, and gel filtered. Fractions in the first peak were then combined, dialyzed against phosphate buffer, and concentrated for study.

A high-resolution ^{13}C spectrum of the microemulsion particles at 55.7 °C, above both DSC transitions, is shown in Figure 4. At this temperature, all of the lipid components appear to be in a fluid state, and a number of resonances from both the phospholipid and cholesteryl ester are well resolved (see Table II for assignments). These resonances include the phospholipid and cholesteryl ester carbonyl resonances at 173.8 and 171.2 ppm, respectively, the steroid ring C5 and C6 resonances at 139.8 and 122.5 ppm, respectively, the olefinic carbons C9′ and C10′ of the cholesteryl ester side chain at 129.7 and 129.9 ppm, respectively, the choline methyl resonance at 54.3 ppm, and steroid C13, C19, C21, and C18 resonances at 42.5, 19.5, 19.0, and 12.1 ppm, respectively. A striking feature of the spectrum (Figure 4) is the excellent resolution obtained at high magnetic field strength. This is well illustrated in the olefinic region, where the resonances for each of the two unsaturated carbons C9′ and C10′ of the cholesteryl ester acyl chain are resolved, a resolution not observed at medium field strength even with the neat material

Table III: Thermal Properties of CO/DMPC Microemulsions and Related Systems

system	T_c (°C)	ΔH (cal/g)
DMPC multilamellar vesicles	23[a]	9.48
DMPC small unilamellar vesicles	18[a]	4.74
CO (neat)	52 (C1 → isotropic)[b]	
	42 (smectic → cholesteric)[b]	0.56
	47.5 (cholesteric → isotropic)[b]	0.25
CER-VLDL	40–42[c]	
	45–48[c]	
CO/DMPC microemulsion	31 (DMPC)	4.8
	46.6 (CO)	0.7

[a] Melchior & Stein (1976). [b] Small (1985). [c] Morrisett et al. (1984).

(Ginsburg et al., 1982a). Furthermore, partially resolved resonances for the terminal methyl carbons on DMPC (14.3 ppm) and CO (14.1 ppm) were observed. Thus, at this temperature, above the second DSC thermal transition for the microemulsion particles, resonances from carbons in both the steroid ring and the acyl chain of the cholesteryl ester are detectable and reasonably narrow, as are those of the phospholipid. To assign the DSC transition to specific lipid components and assess the physical state of the cholesteryl ester core, NMR spectra were acquired at six different temperatures over the range 29.5–55.7 °C, spanning the thermal transitions of the model particle.

Specific regions of the spectra at four selected temperatures are presented in Figure 5. At 29.5 °C, slightly below the lower DSC endotherm, the only discernible features of the spectrum are the carbonyl (173.8 ppm) and terminal methyl (14.3 ppm) resonances of the DMPC acyl chains (both of which are isotopically enriched), the choline methyl resonance at 54.3 ppm, and a low-intensity, broad envelope of acyl chain resonances between 20 and 32 ppm. No new resonances are apparent in the spectrum at 35.2 °C (data not shown), although the previously observable DMPC resonances begin to narrow at this temperature. As the temperature is raised further, a change in the spectrum is detected at 38.6 °C, between the two DSC thermal transitions (Table III). At this temperature, a cholesteryl ester resonance, due to the olefinic acyl chain carbons, first appears as a broad peak at 129.8 ppm. In addition, a peak appears at 27.5 ppm which we have assigned to carbons C8′ and C11′ of the cholesteryl ester acyl chain (α to the olefinic carbons) (Gunstone, 1977). No resonances

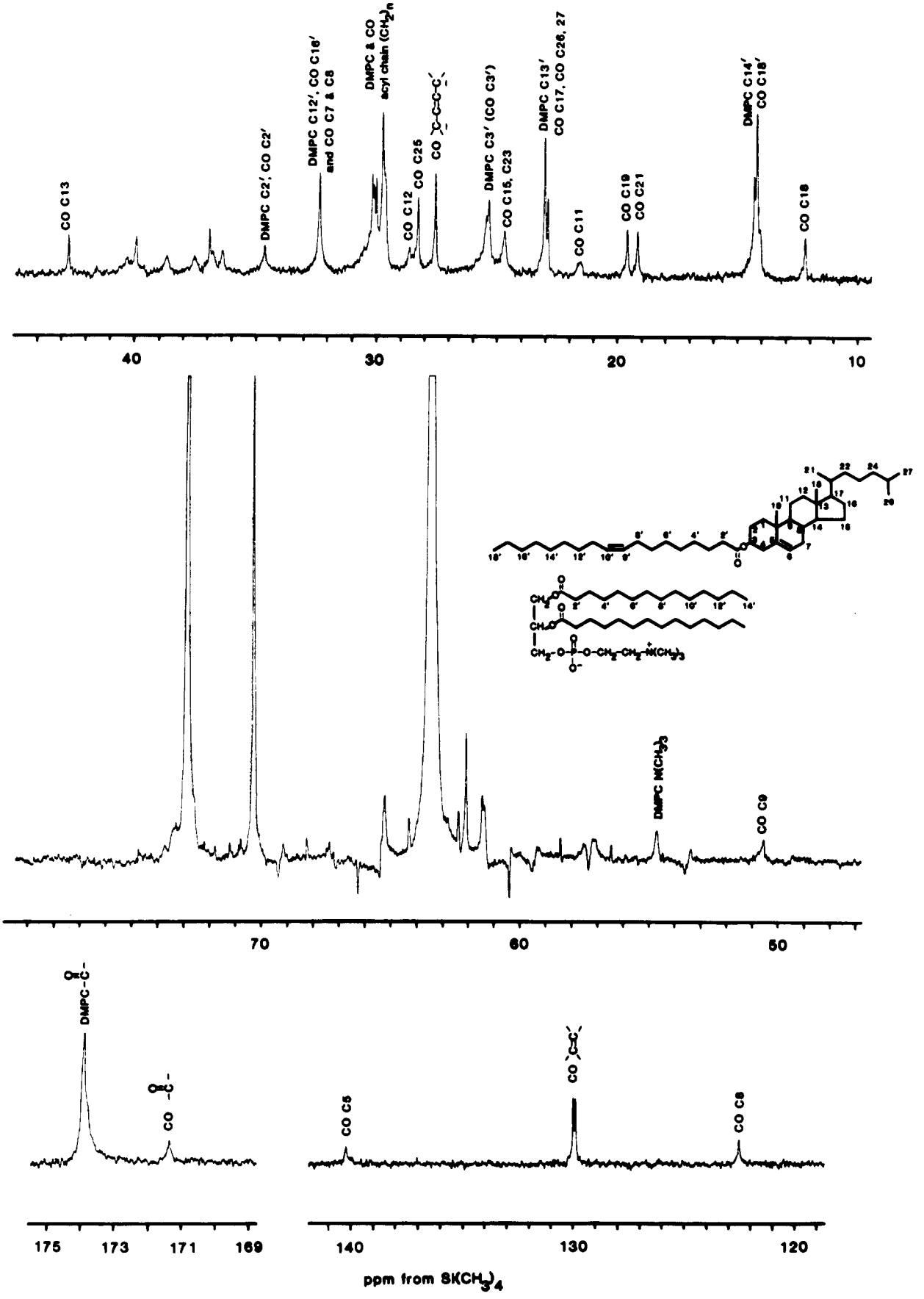

FIGURE 4: Proton-decoupled Fourier-transform ^{13}C (100 MHz) NMR spectrum of the CO/DMPC microemulsion at 55.7 °C. DMPC enriched at C1′ (carbonyl) and DMPC enriched at C14′ (fatty acyl methyl) for both *sn*-1 and *sn*-2 chains have been mixed before preparation of the microemulsion. Conditions: 16 384 accumulations; 2.4-s pulse interval; 20 833-Hz spectral width; 16 384 time domain points. Line broadening of 2 Hz was applied to improve the signal to noise ratio. Large peaks between 60 and 75 Hz are derived from glycerol and buffer material.

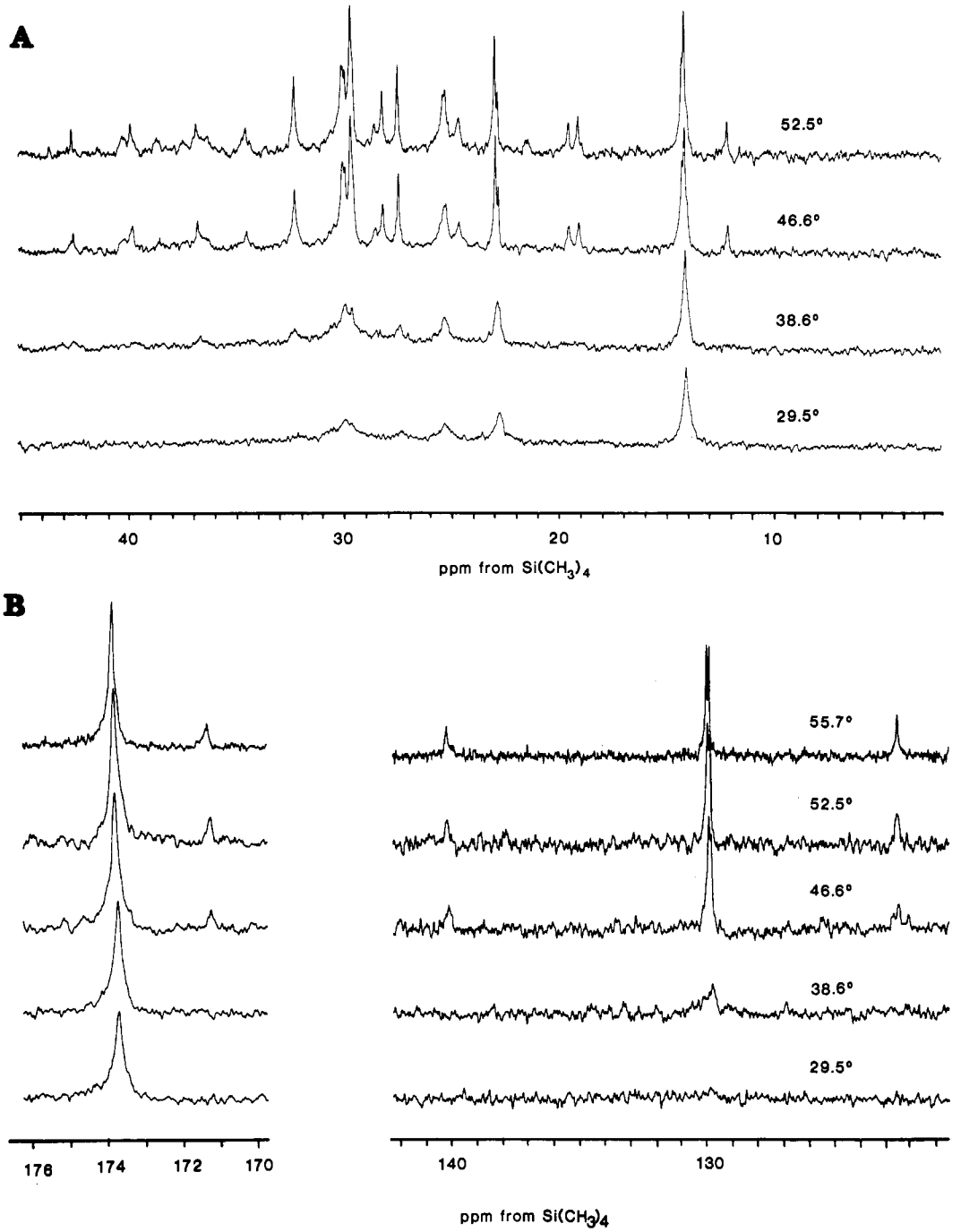

FIGURE 5: Temperature dependence of aliphatic and olefinic/carbonyl regions of ^{13}C NMR spectra of the CO/DMPC microemulsion. Conditions: 4096 accumulations; 2.4-s pulse interval; 16 384 time domain points; 20 833-Hz spectral width; 4-Hz line broadening.

attributable to steroid ring carbons are observable at this temperature. At 46.6 °C, the center of the higher DSC transition, resonances from carbons in the steroid ring, as well as the cholesteryl ester carbonyl, first become evident. Steroid ring carbons C5 and C6 appear as low-intensity, broad peaks in the spectrum as does the carbonyl resonance. Resonances from cholesteryl ester methyl groups C18, C19, and C21, which protrude from the steroid ring system or are part of the isooctyl side chain, appear as sharp, well-resolved peaks in the upfield region of the spectrum. In addition, a second peak due to the acyl chain terminal methyl carbon (C18′) of cholesteryl oleate appears at 14.1 ppm as an intense shoulder on the upfield side of the large DMPC peak. At 129.8 ppm, the olefinic resonances of the ester acyl chain have narrowed and begun to resolve into two peaks. Increasing the temperature still further to 52.5 and 55.7 °C does not result in the appearance of additional resonances; however, those which are present become sharper and increase in intensity.

Qualitatively, the spectra confirm that the first thermal transition does not involve the cholesteryl ester core but is the result of the gel → liquid-crystalline transition of the DMPC on the surface of the particle. Although the higher thermal transition is clearly related to a change in the physical state of the cholesteryl esters in the core of the particle, it is difficult to evaluate this change in terms of the changes in state previously documented for neat cholesteryl esters. Clearly, at 46.6 °C, the cholesteryl ester components have begun to undergo some sort of thermal transition. The cholesteryl ester resonances present at 38.6 °C most closely resemble (in line width) those of neat cholesteryl oleate in the smectic mesophase, with only those carbons in the distal portion of the acyl chain producing resonances narrow enough to detect (Ginsburg et

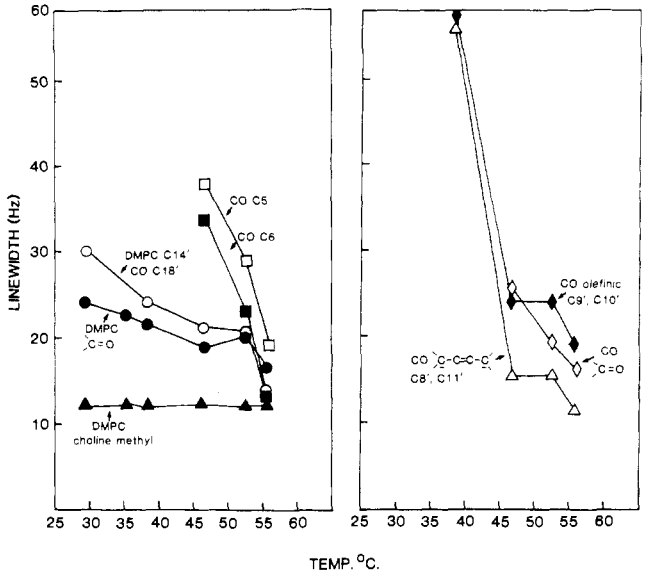

FIGURE 6: Plot of ^{13}C line width as a function of increasing temperature for selected resonances: the choline methyl groups, carbonyls, and C14′ of DMPC; ring carbons C5 and C6 of CO; C9′,C10′ olefinic (C=C) and C8′,C11′ aliphatic (*C*–C=C–*C*) carbons of CO.

al., 1982a). At 46.6 °C, however, one begins to see not only the carbonyl resonance at 171.2 ppm but also resonances from steroid ring carbons, indicating that the cholesteryl esters have begun to enter the isotropic phase with the steroid ring undergoing rapid reorientation. Thus, in the model particles, the single high-temperature transition seems to be due to a melting of the cholesteryl esters from a smecticlike mesophase to a liquid (isotropic) phase without passing through the cholesteric mesophase. A similar conclusion was reached in natural-abundance ^{13}C NMR studies of CER-VLDL (Morrisett et al., 1984).

The line widths of several key resonances are plotted vs. temperature in Figure 6. Notably, the choline methyl resonance is detectable at all temperatures and maintains a constant line width and intensity. The line width of the DMPC carbonyl resonance decreases from about 24.2 Hz at 29.5 °C to about 16.5 Hz at 55.7 °C. Similarly, the line width of the fatty acyl methyl envelope decreases from about 30.5 Hz at 29.5 °C to 14.0 Hz at 55.7 °C. This value is somewhat overestimated due to the presence of the partially overlapping CO C18′ resonance at 46.6 °C and above. The steroid ring carbon resonances C5 and C6 are observable only above 46.6 °C; with increasing temperature, these resonances narrow rapidly and at the same rate. Thus, line-width data also confirm the DSC data and indicate that the surface phospholipids melt at a temperature lower than the core cholesteryl esters and are probably the source of the lower temperature endotherm. At 46.6, 52.5, and 55.7 °C, temperatures which span the second DSC transition, the cholesteryl esters melt to an isotropic state, and the line widths narrow considerably. Interestingly, the phospholipid line widths continue to narrow in the temperature range of the higher DSC transition, an observation which suggests that the surface monolayer may be affected by melting of the core cholesteryl esters.

Interpretation and Conclusions. Electron microscopy demonstrated that the isolated microemulsion particles are indeed spherical. No structures suggestive of single-bilayer vesicles of multilayer liposomes appear in micrographs of fractions used in the DSC and NMR studies. Correlation of particle size as determined by EM with a simple compositional model is good, a result which confirms that a structure consisting of a core of neutral cholesteryl ester surrounded by a monolayer of polar phospholipid is reasonable. Hamilton & Small (1982) and others have shown that small amounts of cholesteryl ester are able to partition into phospholipid bilayers. The structure we propose here does not preclude the presence of a small amount of CO on the surface monolayer. However, partitioning of 2–3% CO into the DMPC monolayer would not significantly alter the particle size determined from compositional data. Thus, these particles appear to be similar in size and structure to CER-VLDL and should serve as a good model for understanding core–surface lipid interaction.

Toward this end, ^{13}C NMR and DSC studies were undertaken to obtain information about the properties of the two lipid domains. DSC suggested and NMR confirmed that the phospholipids undergo a transition which is distinct from that of the core cholesteryl esters. This surface phospholipid transition occurs at a temperature significantly higher than that of the DMPC vesicle transition. A similar, though smaller, temperature shift was observed by Ginsburg et al. (1982b) in the LDL-sized model system. This increase in temperature for phospholipid chain melting may be due in part to a curvature effect, with smaller particles exhibiting less stable phospholipid structures which melt at temperatures lower than those at which the larger particles melt. Alternatively, this phenomenon might result from interaction of the core and surface lipids. This could occur directly through interdigitation of the acyl chains of DMPC and CO, thereby rigidifying the phospholipid surface monolayer with respect to the vesicle. It is also possible that the presence of a large nonpolar core forces an increased proportion of cholesteryl ester to partition into the monolayer, thereby altering its physical properties. At present, the data available do not allow us to distinguish between these possibilities.

The second DSC transition has been assigned to the particle core on the basis of ^{13}C NMR spectra which indicate melting of the cholesteryl ester in this temperature range. The spectrum obtained at 38.6 °C is similar to that of neat cholesteryl oleate in the smectic mesophase. At the next higher temperature point, 46.6 °C, NMR spectra indicate that the cholesteryl ester has begun to melt directly to the isotropic, not the cholesteric, phase. Gray (1962) has noted that a pitch of at least 4000 Å is necessary to accommodate a true cholesteric phase; thus, it seems likely that the structure of the core between thermal transitions is a smecticlike phase, possibly radial as suggested by Ginsburg et al. (1982b). If this phase is indeed smectic, then like the phospholipid surface, the core is stabilized relative to its transition in the neat state.

Clearly, the CO/DMPC system has distinct advantages over vesicles as a model for interpreting lipid–lipid interactions in large lipoproteins. First, the microemulsion particles are much larger than vesicles and thus have radii of curvature similar to those of native lipoproteins. Second, the surface of the microemulsion is a phospholipid monolayer, not a bilayer as in vesicles. This distinction may prove very important in future studies where core–surface interactions are more closely probed and apoproteins are recombined with the microemulsion to provide true lipoprotein models. By manipulating the composition of the model system, we were able to independently monitor by NMR the phospholipid acyl chain, steroid ring, and cholesteryl ester acyl chain motions. Thus, this system also provides an advantage over the native lipoprotein in terms of ease of spectral interpretation.

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Registry No. DMPC, 18194-24-6; CO, 303-43-5; glycerol, 56-81-5.

REFERENCES

Ackman, R. G. (1981) *Methods Enzymol. 72*, 205–251.

Adamson, A. W. (1967) *Physical Chemistry of Surfaces*, p 511, Interscience, New York.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Berkman, S., & Egloff, G. (1941) *Emulsions and Foams*, p 175, Reinhold, New York.

Brainard, J. R., Knapp, R. D., Morrisett, J. D., & Pownall, H. J. (1984) *J. Biol. Chem. 259*, 10340–10347.

Chait, A., Brunzell, J. D., Albers, J. J., & Hazzard, W. R. (1977) *Lancet 1*, 1176–1181.

Chait, A., Hazzard, W. R., Albers, J. J., Kushwaha, R. P., & Brunzell, J. D. (1978) *Metab., Clin. Exp. 27*, 1055–1059.

Chen, G. C., Guo, L. S. S., Hamilton, R. L., Gordon, V., Richards, E. G., & Kane, J. P. (1984) *Biochemistry 23*, 6530–6538.

Curatolo, W., Sakura, J. D., Small, D. M., & Shipley, G. G. (1977) *Biochemistry 16*, 2313–2319.

Davies, J. T., & Rideal, E. K. (1961) *Interfacial Phenomena*, Academic Press, New York.

Gidez, L. I., Roheim, P. S., & Eder, H. A. (1965) *J. Lipid Res. 6*, 377–384.

Ginsburg, G. S., Small, D. M., & Hamilton, J. A. (1982a) *Biochemistry 21*, 6857–6867.

Ginsburg, G. S., Small, D. M., & Atkinson, D. (1982b) *J. Biol. Chem. 257*, 8216–8227.

Ginsburg, G. S., Walsh, M. T., Small, D. M., & Atkinson, D. (1984) *J. Biol. Chem. 259*, 6667–6673.

Gray, G. W. (1962) *Molecular Structure and Properties of Liquid Crystals*, pp 42–54, Academic Press, New York.

Gunstone, F. D., Pollard, M. R., Scrimgeour, C. M., & Vedanayagam, H. S. (1977) *Chem. Phys. Lipids 18*, 115–129.

Hailstone, R. K. (1972) M.S. Thesis, Indiana University.

Hamilton, J. A., & Small, D. M. (1982) *J. Biol. Chem. 257*, 7318–7319.

Hamilton, J. A., Talkowski, C., Childers, R. F., Williams, E., Allerhand, A., & Cordes, E. H. (1974) *J. Biol. Chem. 249*, 4872–4878.

Hazzard, W. R., & Bierman, E. L. (1976) *Metab., Clin. Exp. 25*, 777–801.

Hazzard, W. R., Lindgren, G. T., & Bierman, E. L. (1970) *Biochim. Biophys. Acta 202*, 517–525.

Kroon, P. A. (1981) *J. Biol. Chem. 256*, 5332–5339.

Lundberg, B. (1975) *Chem. Phys. Lipids 14*, 260–262.

Mahley, R. W., Weisgraber, K. H., Innerarity, T., Brewer, H. B., & Assmann, G. (1975) *Biochemistry 14*, 2817–2823.

Mahley, R. W., Weisgraber, K. H., & Innerarity, T. (1976) *Biochemistry 15*, 2979–2985.

Melchior, D. L., & Steim, J. M. (1976) *Annu. Rev. Biophys. Bioeng. 5*, 205–238.

Mills, G. L., Taylaur, C. E., & Miller, A. L. (1979) *Clin. Chim. Acta 93*, 173–180.

Morganroth, J., Levy, R. I., & Fredrickson, D. S. (1975) *Ann. Intern. Med. 82*, 158–174.

Morrisett, J. D., Gaubatz, J. W., Tarver, A. P., Allen, J. K., Pownall, H. P., Laggner, P., & Hamilton, J. A. (1984) *Biochemistry 23*, 5343–5352.

Nagle, J. F., & Wilkinson, D. A. (1978) *Biophys. J. 23*, 159–175.

Noel, S.-P., Dolphin, P. J., & Rubenstein, D. (1975) *Biochem. Biophys. Res. Commun. 63*, 764–771.

Patsch, J. R., Sailer, S., Braunsteiner, H., & Forte, T. (1975) *Eur. J. Clin. Invest. 5*, 44–55.

Patsch, J. R., Sailer, S., Braunsteiner, H., & Forte, T. (1976) *Eur. J. Clin. Invest. 6*, 307–310.

Ross, A. C., & Zilversmit, D. B. (1977) *J. Lipid Res. 18*, 169–175.

Roth, R. I., Gaubatz, J. W., Gotto, A. M., Jr., & Patsch, J. R. (1983) *J. Lipid Res. 24*, 1–11.

Sata, T., Havel, R. J., & Jones, A. L. (1972) *J. Lipid Res. 13*, 757–768.

Small, D. M. (1985) *The Physical Chemistry of Lipids from Alkanes to Phospholipids*, Chapter 11, Figures 11–13, Plenum Press, New York.

Via, D. P., Craig, I. F., Jacobs, G. W., Van Winkle, W. B., Charlton, S. C., Gotto, A. M., & Smith, L. C. (1982) *J. Lipid Res. 23*, 570–575.

Zilversmit, D. B. (1975) *Am. J. Cardiol. 35*, 559–566.