Phospholipid/cholesteryl ester microemulsions containing unesterified cholesterol: model systems for low density lipoproteins

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Abstract As models for the effects of unesterified cholesterol (UC) on the lipid organization of low density lipoprotein (LDL), microemulsions containing either egg yolk phosphatidylcholine (EYPC) or dimyristoyl phosphatidylcholine (DMPC) as the surface component, cholesteryl oleate (CO) as the core component, and varying amounts of unesterified cholesterol were prepared by sonication. Gel filtration chromatography showed coelution of each of the lipid components, demonstrating the formation of well-defined microemulsion populations. Unesterified cholesterol incorporation into the microemulsions was proportional to the composition of the original mixture at low unesterified cholesterol compositions, but reached saturation at compositions of approximately 15 and 10 mol% unesterified cholesterol for EYPC/CO and DMPC/CO microemulsions, respectively. The Stokes' radius of the microemulsions was constant and similar to native LDL for initial compositions less than 15 mol% unesterified cholesterol, but increased at compositions above 15 mol%. In both EYPC/CO/UC and DMPC/CO/UC microemulsions, no significant changes were observed for the calorimetric or Van't Hoff enthalpy for the thermal transition of the core cholesteryl ester; however, increases in the transition temperature as a function of increasing unesterified cholesterol composition suggests that unesterfied cholesterol has a stabilizing effect on the core transition. In DMPC/CO/UC microemulsions, the effect of unesterified cholesterol on the surface-located DMPC could be clearly observed as a broadening of the thermal transition of the acyl chains. These results demonstrate that unesterified cholesterol is located primarily in the surface of these protein-free lipid model systems for LDL. —**Reisinger, R. E., and D. Atkinson.** Phospholipid/cholesteryl ester microemulsions containing unesterified cholesterol: model systems for low density lipoproteins. *J. Lipid Res.* 1990. **31:** 849–858.

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Low density lipoprotein is a biological microemulsion of particular importance because of its central role in cholesterol transport and metabolism. The major LDL lipids, phospholipids, and cholesteryl esters, constitute approximately 80% of the lipid weight of the particle. The remaining lipid mass of the particle is primarily unesterified cholesterol (~10%) as well as mono-, di-, and triglycerides (~10%) with triglycerides as the predominant species (1, 2). Trace amounts of free fatty acids and hydrocarbons have also been shown to exist in LDL (3). Approximately 20% of the total weight of the particle is attributable to the glycoprotein apoprotein B-100 (1). In addition to a structural role in LDL, apoB is responsible for the recognition of LDL by a specific receptor and the subsequent control of LDL metabolism via receptor mediated endocytosis (4).

Structurally, LDL consists of a core of neutral lipids, stabilized by a surface monolayer of phospholipids and apoB. In native LDL, a thermal transition for the core components is observed over a range of 20°C to 40°C, which has been attributed to a "radial smectic-like" to isotropic liquid reorganization of the core cholesterol esters (5–7).

Previous studies (8) have demonstrated that well-defined protein-free microemulsion model systems comprised of cholesteryl esters and phospholipid, which mimic the physical behavior of the lipids in native LDL, could be prepared by sonication. It was demonstrated that the core-located cholesteryl esters undergo thermal transitions with an overall calorimetric enthalpy of ~0.7 cal/g of cholesteryl ester. Ir. microemulsions formed with single species of phospholipids, the surface-located phospholipids undergo thermal transitions with an overall calorimetric enthalpy of ~5.0 cal/g of phospholipid. The nature of

Abbreviations: V_o, void volume; V_t, total elution volume; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; EYPC, egg yolk phosphatidylcholine; UC, unesterified cholesterol; CO, cholesteryl oleate; DSC, differential scanning calorimetry; TLC, thin-layer chromatography; LDL, low density lipoprotein; HDL, high density lipoprotein; NMR, nuclear magnetic resonance; apoB, apolipoprotein B-100; kDa, kiloDalton; PL, phospholipid; ΔH°, calorimetric enthalpy; ΔH^*, Van't Hoff enthalpy.

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these transitions was further characterized by proton nuclear magnetic resonance and X-ray scattering and diffraction, and it was shown that the core-located cholesteryl esters undergo the same "radial smectic-like" to liquid transition previously described for native LDL (5–7). Elevated transition temperatures for the cholesteryl esters in the particle core compared to the temperatures for the analogous transition in neat cholesteryl esters suggested that the core cholesteryl esters were stabilized with respect to temperature by the surface phospholipid monolayer. In microemulsions prepared with DPPC as the surface component, concomitant melting of surface phospholipids and core cholesteryl esters suggested coupling between the core and the surface components.

Similar emulsion model systems for cholesteryl ester-rich very low density lipoproteins have been prepared (9). These larger particles (500–1000 Å) prepared by cosonication of DMPC and CO also exhibit two distinct thermal transitions that are attributed to the surface DMPC (31°C in this system) and the core located CO (46.6°C in this system). Increases in the transition temperature for the surface-located DMPC as compared to the transition temperature of DMPC small unilamellar (18°C) vesicles and large multilamellar (23°C) liposomes again suggested that the DMPC transition is stabilized by interactions between the surface and core of the particle, consistent with the coupling phenomena observed for smaller LDL size microemulsions prepared with DPPC as the surface component (8).

However, unesterified cholesterol, the third major lipid component of LDL has not been included in either model system. Unesterified cholesterol has been shown to have significant effects on the structure and physical properties of phospholipid bilayers (for review see 10, 11). Studies on bulk phase lipids extracted from native LDL (6) suggest that the partition coefficient of unesterified cholesterol between native LDL phospholipids and cholesteryl esters is approximately 6 in the absence of apoB. In intact HDL and LDL, ^{13}C-NMR studies have shown that unesterified cholesterol exists in two environments (12, 13) and subsequent ^{13}C-NMR studies (14) have suggested that the partition coefficient for unesterified cholesterol in intact LDL is approximately 3.

In this study, we have examined the effects of unesterified cholesterol on the lipid composition and lipid physical properties, with respect to the temperature-dependent structural transitions of the surface and core components, of these simplified phospholipid/cholesteryl ester microemulsion model systems for LDL.

MATERIALS AND METHODS

Lipids

Lipid purity was determined by thin-layer chromatography (TLC) on 250 micron silica G. Egg yolk phosphatidylcholine (Lipid Products, Surrey, England) and dimyristoyl phosphatidylcholine (Sigma, St. Louis, MO) were determined to be >99% pure by TLC in chloroform–methanol–water–acetic acid 65:25:4:1. Cholesteryl oleate (Nu-Chek-Prep, Elysian, MN) was determined to be >99% pure by TLC in hexane–diethyl ether–acetic acid 70:30:1. Unesterified cholesterol (Nu-Chek-Prep) was determined to be >99% pure by TLC in chloroform–acetone 96:4. All unlabeled lipids were used without further purification. Each lipid was individually dissolved in chloroform–methanol 2:1 to prepare stock solutions of approximately 0.1 mg/μl. Absolute lipid concentrations were determined by taking dry weights of aliquots of the stock solutions. Cholesteryl[1-^{14}C]oleate, [7(n)-^{3}H]cholesterol, and 1,2-dipalmitoyl-L-3-phosphatidyl[N-methyl-^{3}H]choline (Amersham, Arlington Heights, IL) were used in the preparation of lipid solutions, after determination of activity per unit volume (DPM/μl). Purity of radiolabels was determined by TLC in the same solvent systems as described for the nonlabeled lipid counterparts. TLC plates were scraped and appropriately sized portions of the silica gel were counted by liquid scintillation chromatography in Liquiscint (National Diagnostics, Somerville, NJ), and compared for co-migration with the corresponding unlabeled lipids. When necessary, radiolabels were purified by preparative TLC in the appropriate solvent system; the silica gel was scraped from the appropriate area of the plate and the radiolabel was extracted from the silica three times with chloroform. The chloroform was removed by evaporation and the radiolabel was resuspended in chloroform–methanol 2:1. All lipids were stored at −20°C.

Microemulsion preparation

Phospholipid/cholesteryl oleate microemulsions containing varying amounts of unesterified cholesterol were prepared according to procedures that have been previously described (8). Aliquots of cholesteryl oleate and either egg yolk phosphatidylcholine or dimyristoyl phosphatidylcholine were taken from stock solutions of known concentration to give a 1:1 weight ratio of EYPC or DMPC to CO. Appropriate amounts of [^{14}C]cholesteryl oleate were added to give a specific activity of 100,000 DPM per mg of cholesteryl ester. Unesterified cholesterol was added to the mixture to give the appropriate molar concentration with respect to the combined mass of EYPC or DMPC and CO, which were held constant in each microemulsion preparation. Appropriate amounts of [^{3}H]cholesterol were added to give a specific activity of 1×10^6 DPM per mg of unesterified cholesterol. To insure proper composition prior to sonication, concentration of lipids in the final mixture was quantitated by liquid scintillation counting. The solvent was removed by drying the lipid mixture under a gentle stream of N_2, followed by vacuum desiccation at 4°C for at least 12 h to

insure complete removal of the solvent. The lipids were resuspended in ~10 ml of 0.1 M KCl, 0.01 M Tris-HCl, and 0.025% NaN_3, at pH 8.0, to give an approximate lipid concentration of 1% w/v.

The resulting lipid suspension was sonicated for 5 h using a Branson model W350 sonicator (Branson Sonic Power, Danbury, CT) with a 0.5" horn with a macrotip at a power setting of 4 (approximately 125 W) under a N_2 environment at a temperature at least 5°C higher than the highest melting lipid component (51°C to 55°C; the transition temperature of cholesteryl oleate in these systems is 46°C). Temperature was monitored throughout sonication by a copper/constantan thermocouple (Omega Engineering, Stamford, CT) inserted directly into the vial. Immediately after sonication, the microemulsion containing solution was centrifuged at 195,000 *g* (SW41 rotor, no brake) for 30 min to remove large particles that float at a background density of $d < 1.006$ g/ml as well as titanium fragments from the sonicator tip. After centrifugation, the top 2 ml of solution was removed by slicing the centrifuge tube, and the remaining subnatant was carefully pipetted from the centrifuge tube to avoid disturbing the titanium fragments. The background density of the subnatant was adjusted to d 1.25 g/ml by the addition of solid KBr and recentrifuged for 2 h under identical conditions. The top 2 ml of the tube, which contains the gelatinous microemulsion layer, was resuspended by repeated pipetting to yield the S2 fraction. This fraction was used for further physical studies. Generally, lipid recovery in the S2 fraction was at least 80% with respect to the lipid mixture prior to sonication. A high yield in the S2 fraction reflects the efficiency of the sonication and the final yield of microemulsions as identified by column chromatography. Experience has demonstrated that, for yields in S2 >80% relative to the initial lipid mass prior to sonication, virtually all of the lipid in S2 is in the form of microemulsion particles as indicated by very small void volume peaks on chromatography (8). Analytical TLC of lipids extracted from the S2 fraction by the Bligh and Dyer (15) procedure using the same solvent systems used to assess lipid purity showed that no observable degradation of the lipids (i.e., <1%) occurred during the sonication procedure.

Gel filtration chromatography

Ascending elution column chromatography was performed using Sepharose CL-4B in K 26/40 columns (Pharmacia Fine Chemicals, Uppsala, Sweden) with a flow rate of 20 ml/h at 25°C. Columns were preequilibrated with 0.1 M KCl, 0.01 M Tris-HCl, and 0.025% NaN_3, at pH 8, and pre-saturated with lipid by the fractionation of an egg yolk phosphatidylcholine vesicle preparation, prepared according to the method of Barenholz et al. (16), prior to sample application. Samples were applied in volumes of 2 ml, and 2-ml fractions were collected. Gel filtration columns were calibrated for Stokes' radius with calibration standards (Sigma) consisting of blue dextran (V_o), apoferritin, thyroglobulin, alcohol dehydrogenase, bovine serum albumin, catalase, aldolase, and tryptophan (V_t). Cholesterol and cholesteryl oleate concentration in the column fractions was determined by liquid scintillation counting. Aliquots of 100 μl were counted in 10 ml of Liquiscint (National Diagnostics, Somerville, NJ) using narrow window settings for ^3H and ^{14}C. Efficiencies and ^{14}C spill-over into the ^3H window were determined using ^3H and ^{14}C toluene standards. Phospholipid phosphorus was quantitated by the Bartlett colorimetric phosphorus assay (17). Phospholipid concentration was determined by multiplying the value obtained for the inorganic phosphorus concentration by 25.

Differential scanning calorimetry

Calorimetry experiments were performed on a Microcal MC-2 scanning calorimeter (Microcal, Inc., Northampton, MA) at a scanning rate of 90°/h. Microemulsion samples consisting of the S2 fraction, as used in previous studies by Ginsburg, Small, and Atkinson (8), containing 20 to 24 mg of total lipid in 1.2 ml of Tris-KCl buffer were loaded into the calorimeter sample cell at room temperature and an equal amount of buffer was loaded into the reference cell. Samples were cooled to approximately 5°C and data was collected on heating runs from 5°–80°C. Each sample was heated three times, and samples were held at 5°C for approximately 30 min between each run. The first heating run was generally unstable; the second and subsequent runs were reproducible. The data presented represent the results of the second and third heating runs. Mass normalization and thermodynamic analysis of the calorimetric data was performed on an IBM Personal Computer (International Business Machines, Boca Raton, FL) using the DA2 data analysis software provided by Microcal.

A minimum of three microemulsion preparations was analyzed at each unesterified cholesterol composition. The results obtained reflect the mean of the multiple preparations. Statistical analysis of the data was performed using the RS1 laboratory software package (BBN Software, Cambridge, MA). Selected microemulsion preparations were analyzed for structural integrity after DSC; gel filtration chromatography and transmission electron microscopy demonstrated that the microemulsion preparations remained intact throughout the calorimetry experiments.

The S2 fraction was used in the calorimetry experiments as opposed to fractionated microemulsion particles from column chromatography in order to avoid the significant concentration step that would be involved in using column fractionated material. The column chromatography (see results) demonstrated that for compositions

up to 15 mol% and 10 mol% unesterified cholesterol for the EYPC and DMPC systems, respectively, >80% of the lipid mass eluted in the fractions representing the microemulsion particles. Thus, the thermodynamic analysis will represent the thermal properties of the microemulsion particles reasonably accurately up to these limiting compositions of unesterified cholesterol. When pooled column fractions representing the microemulsion particles were concentrated by pressure filtration and subsequently analyzed calorimetrically, the results were indistinguishable from the results obtained with the S2 fraction (see Results).

Transmission electron microscopy

Microemulsion samples were diluted 1:125 with buffer, and a droplet of the diluted sample was placed on a glow-discharged, carbon and Formvar-coated 300-mesh copper grid. After 10 sec, the droplet excess was removed with filter paper, and a drop of 1% sodium phosphotungstate (pH 7.4) was immediately added. After a few seconds, excess stain was removed, and the grid was air dried. The samples were viewed and photographed in a Hitachi HU-11C electron microscope, calibrated using a carbon grating replica (Ernest F. Fullam, Schenectady, NY).

RESULTS

Microemulsion model systems prepared with egg yolk phosphatidylcholine as the surface lipid component

Gel filtration elution profiles of microemulsions for progressively increasing initial compositions of unesterified cholesterol are shown in **Fig. 1**. In the absence of unesterified cholesterol (Fig. 1A) EYPC and CO co-eluted with a CO/PL molar ratio of approximately 0.8, demonstrating the formation of microemulsion particles as described previously (8). For unesterified cholesterol compositions up to 20 mol% (Fig. 1B–E), the microemulsion particles eluted at a constant volume, consistent with microemulsions prepared without unesterified cholesterol (8) as well as native low density lipoproteins. However, with increasing amounts of unesterified cholesterol in the initial sonication mixture, microemulsion particles showed somewhat broadened chromatographic elution profiles. This suggests that the increase in unesterified cholesterol concentration has a direct effect on the heterogeneity of the microemulsion preparations. The mass contained within the void volume of the chromatographic elution profiles increased from approximately 5% of the total lipid mass for microemulsion preparations without cholesterol to about 15% for microemulsions prepared with 15 mol% unesterified cholesterol.

At an initial starting composition of 30 mol% unesterified cholesterol (Fig. 1F) the particles eluted from the

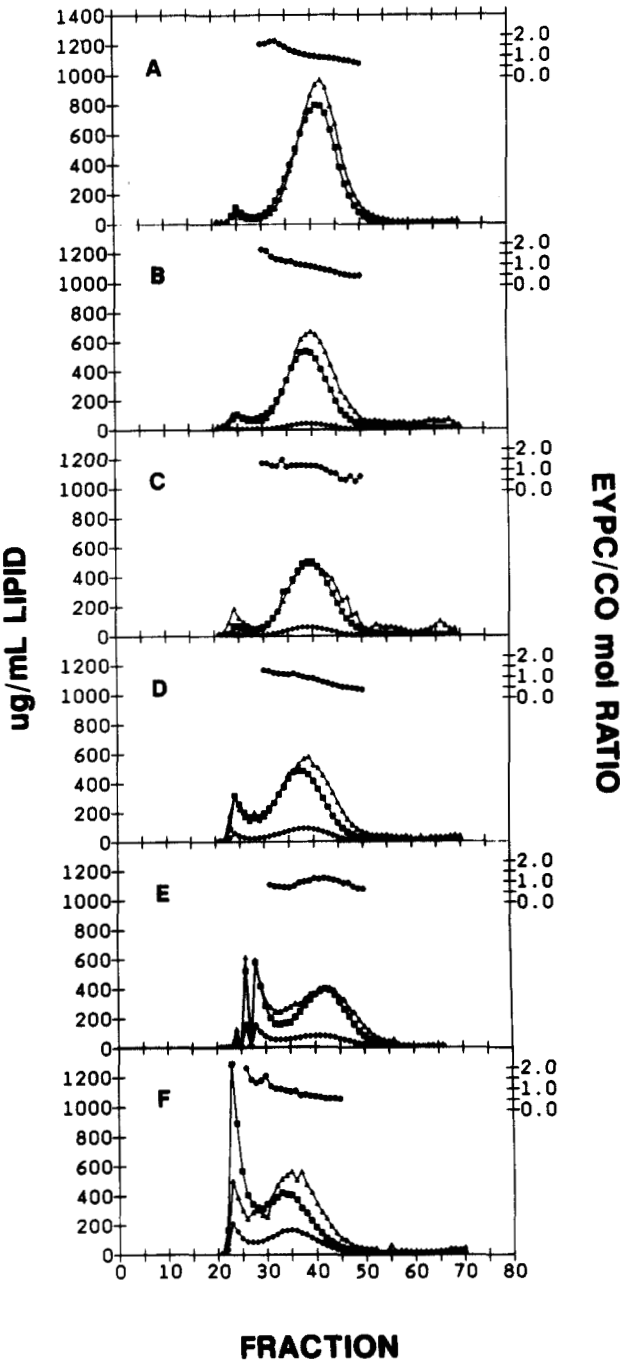

Fig. 1. Elution profile of egg yolk phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions prepared with the following initial concentrations of unesterified cholesterol as described in the text: (A) 0 mol%; (B) 5 mol%; (C) 10 mol%; (D) 15 mol%; (E) 20 mol%; (F) 30 mol%. Crude microemulsion preparation (2.0 ml) was applied to a Sepharose CL-4B column and eluted with 0.1 M KCl, 0.01 M Tris-HCl, 0.025% NaN_3 buffer (pH 8.0). Fractions (2.0 ml) between V_o (~40 ml) and V_t (~140 ml) were collected. The concentration in μg/ml of unesterified cholesterol (♦), egg yolk phosphatidylcholine (▲), and cholesteryl oleate (■) is plotted according to the left hand ordinate. The molar ratio of cholesteryl oleate to egg yolk phosphatidylcholine (●) is plotted against the right hand ordinate.

column at a significantly smaller volume, indicating that the population of particles shifts to a larger mean size. Also, at a composition of 30 mol% unesterified cholesterol, coelution of the radiolabels was no longer observed and the void peak was very pronounced. These chromatographic data, in conjunction with chemical data, suggest that it not possible to prepared microemulsions with a size and composition similar to low density lipoproteins at unesterified cholesterol compositions exceeding approximately 20 mol% with respect to phospholipid and cholesteryl ester. However, for microemulsion preparations containing up to 15 mol% unesterified cholesterol in the starting mixture (Fig. 1A-D), peak elution volume, and hence mean particle size and particle homogeneity, remained relatively constant.

The effects of unesterified cholesterol on the elution volume of microemulsion preparations are further illustrated in **Fig. 2A**, which demonstrates a constant Stokes' radius for microemulsions prepared with initial unesterified cholesterol compositions up to 20 mol%, and a significant increase in Stokes' radius at 30 mol% unesterified cholesterol. Consistent with these results are changes in size observed by transmission electron microscopy of negatively stained microemulsion peak fraction preparations as shown in Fig. 2B. For microemulsion preparations with an initial unesterified cholesterol composition up to 20 mol% with respect to egg yolk phosphatidylcholine and cholesteryl oleate, the mean particle size was approximately 200 ± 12 Å for the peak fraction with a range of 120 to 380 Å for the entire S2 fraction, similar to the mean particle size observed for native LDL and for microemulsions prepared without unesterified cholesterol. For preparations with an initial starting composition of 30 mol% unesterified cholesterol, the observed size was approximately twofold larger. In addition, Fig. 2C illustrates that the composition of unesterified cholesterol in the final microemulsion preparation was similar to that of the initial starting mixture. Also, a small increase in the molar ratio of cholesteryl oleate to egg yolk phosphatidylcholine was observed for the peak fractions as a function of the unesterified cholesterol composition of the peak fractions for microemulsions prepared with unesterified cholesterol compositions less than 20 mol% (i.e., with constant size).

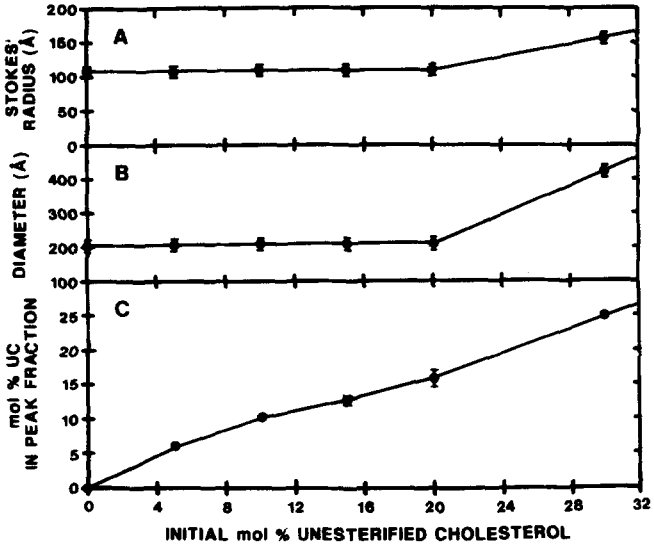

Fig. 2. (A) Stokes' radius for the microemulsion peak fraction as determined by gel filtration chromatography; (B) the mean diameter of the particles in the peak fraction determined by transmission electron microscopy; (C) mol% unesterified cholesterol contained in the peak fraction; as a function of initial mol% unesterified cholesterol in the starting mixture for egg yolk phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions. The points represent the mean values for three independent samples ± SEM. Statistical analysis was performed using the RS1 laboratory software system (BBN Software, Cambridge, MA).

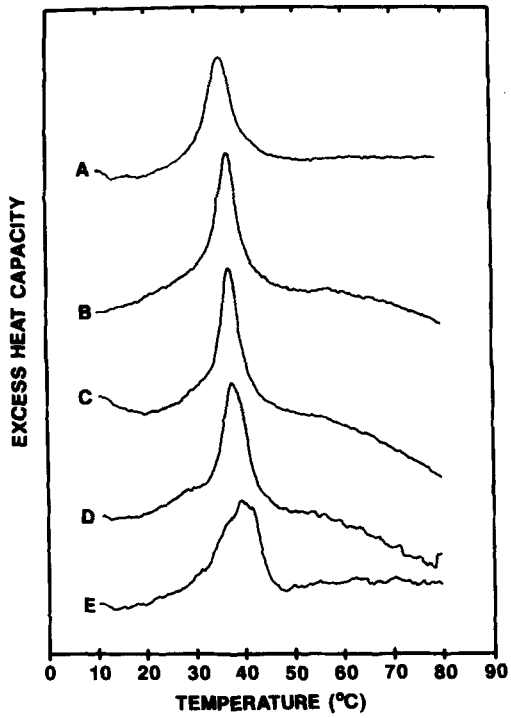

Fig. 3. Representative differential scanning calorimetry thermograms for egg yolk phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions showing the cholesteryl oleate core transition at ~40°C. The microemulsions were prepared with the following initial concentrations of unesterified cholesterol as described in the text: (A) 0 mol%; (B) 5 mol%; (C) 10 mol%; (D) 15 mol%; (E) 20 mol%. The change in heat capacity (Cp) in cal/g is plotted versus temperature. Each sample was scanned three times; the plots are the results of either the second or third heating run.

Representative differential scanning calorimetry traces for the S2 fraction of egg yolk phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions at increasing unesterified cholesterol compositions are shown in **Fig. 3**. At all unesterified cholesterol compositions over

the range from 0 to 20 mol%, a single endothermic transition was observed which corresponds to the order to disorder transition of the core cholesteryl esters as observed in LDL (5, 6) and microemulsions prepared without unesterified cholesterol (8). This transition exhibited a peak temperature (Tm) at 40°C (± 0.5°) and a calorimetric enthalpy (ΔH°) of 0.6 cal/g (± 0.1 cal/g). No gross differences in peak shape or area were observed for this cholesteryl oleate transition for unesterified cholesterol compositions from 0 to 20 mol%. However, an increase in noise (superimposed on the cholesteryl oleate transition) was apparent in the 20 mol% unesterified cholesterol DSC trace (Fig. 3E). This increase in noise probably occurred as a result of the increased heterogeneity observed in microemulsion preparations containing more than 15 mol% unesterified cholesterol. **Fig. 4** presents the calorimetric data for the cholesteryl ester transition as a function of unesterified cholesterol composition. No significant changes were seen in either the calorimetric enthalpy (Fig. 4A) or the Van't Hoff enthalpy (Fig. 4B) for the core cholesteryl oleate transition. However, a small increase in the transition temperature as a function of increasing unesterified cholesterol composition was apparent (Fig. 4C).

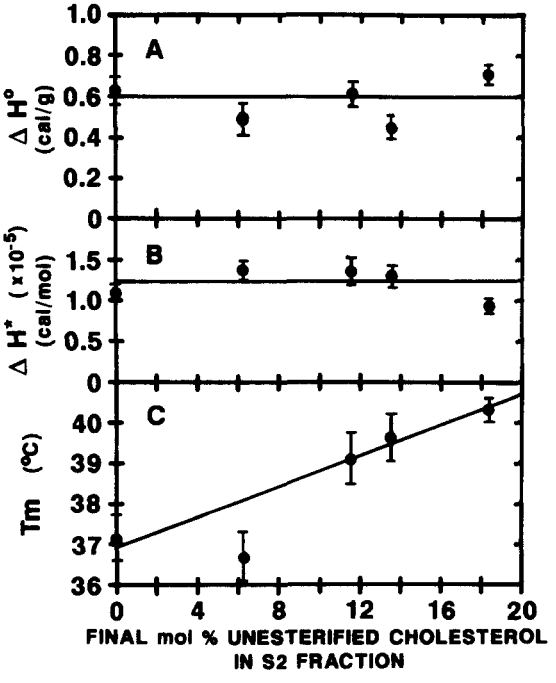

Fig. 4. Calorimetric data for the cholesteryl ester core transition of egg yolk phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions as a function of the mean final unesterified cholesterol composition in the S2 fraction (the second supernatant fraction obtained by centrifugation); (A) calorimetric enthalpy; (B) Van't Hoff enthalpy; and (C) transition temperature. Data were acquired from the second and third heating runs from three independent samples for each data point and are presented as the mean ± SEM. Linear regression was performed using the RS1 laboratory software system. The analysis for ΔH° and ΔH^* gave fitted lines with slopes not significantly different from 0. The T_m data gave a correlation coefficient of 0.91.

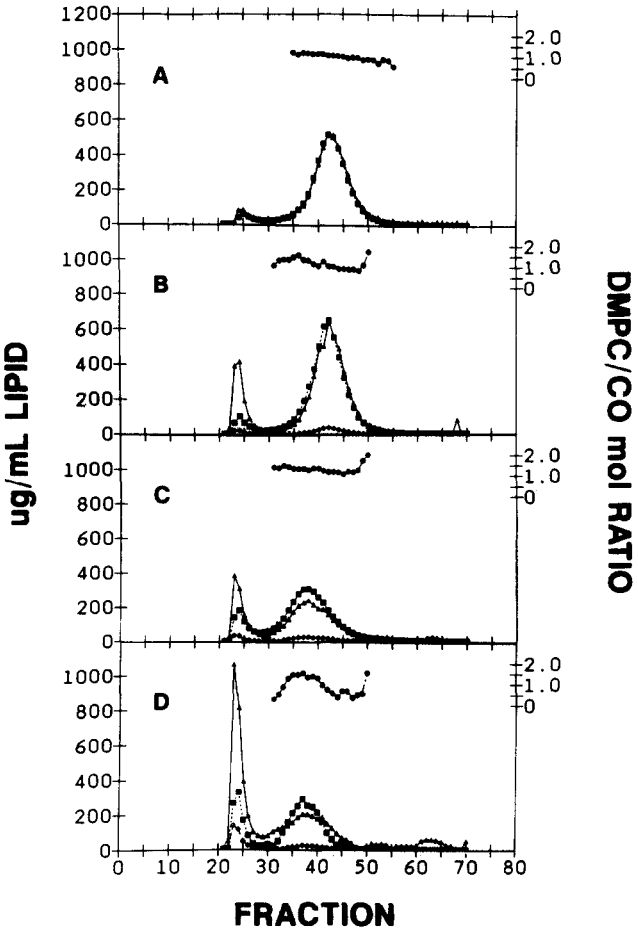

Fig. 5. Elution profile of dimyristoyl phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions prepared with the following initial concentrations of unesterified cholesterol as described in the text: (A) 0 mol%; (B) 5 mol%; (C) 10 mol%; (D) 15 mol%. Crude microemulsion (2.0 ml) was applied to a Sepharose CL-4B column and eluted with 0.1 M KCl, 0.01 M Tris-HCl, 0.025% NaN_3 buffer (pH 8.0). Fractions (2.0 ml) between V_o (~40 ml) and V_t (~140 ml) were collected. The concentration in μg/ml of unesterified cholesterol (♦), dimyristoyl phosphatidylcholine (▲), and cholesteryl oleate (■) is plotted according to the left hand ordinate. The molar ratio of cholesteryl oleate to dimyristoyl phosphatidylcholine (●) is plotted against the right hand ordinate.

Microemulsion model systems prepared with dimyristoyl phosphatidylcholine as the surface lipid component

The same trends in composition with respect to increasing unesterified cholesterol composition were observed for microemulsions prepared with dimyristoyl phosphatidylcholine as the surface component (**Fig. 5**). However, in contrast to the egg yolk phosphatidylcholine system, at 15 mol% unesterified cholesterol in the starting mixture, only approximately 8 mol% unesterified cholesterol was incorporated into the final microemulsion preparation. In addition, it proved difficult to prepare dimyristoyl phos-

phatidylcholine/cholesteryl oleate microemulsions at unesterified cholesterol compositions of 20 mol% and higher. Similar increases in the molar ratio of cholesteryl oleate to phospholipid were seen, as observed for microemulsions prepared with EYPC as the surface lipid component.

Representative differential scanning calorimetry traces for dimyristoyl phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions at increasing unesterified cholesterol compositions are shown in **Fig. 6**. For initial unesterified cholesterol compositions up to 15 mol% unesterified cholesterol, two distinct endothermic transitions were observed. The first corresponds to the gel to liquid crystalline transition of the surface-located dimyristoyl phosphatidylcholine which occurred at a peak temperature (Tm) of ~25°C (± 0.2°) in these microemulsion systems (8). The phospholipid transition was progressively broadened as a function of increasing unesterified cholesterol composition, and an increase in the peak transition temperature was also observed. The second transition was the core cholesteryl ester order to disorder transition which occurred at a peak temperature

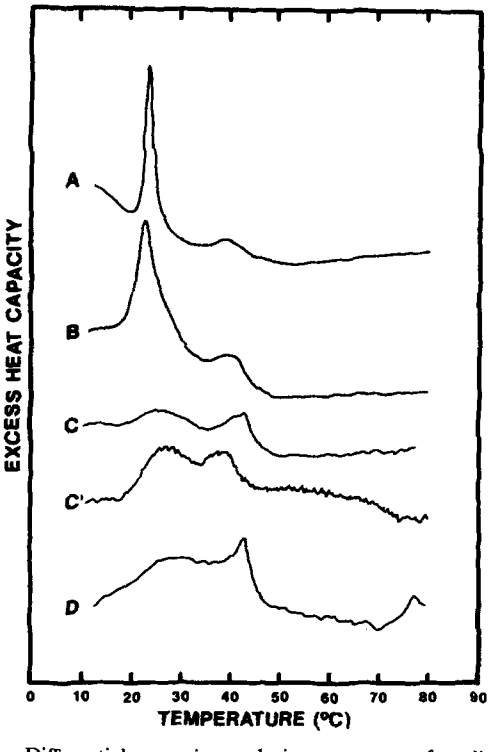

Fig. 6. Differential scanning calorimetry traces for dimyristoyl phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions showing the dimyristoyl phosphatidylcholine surface transition at ~25°C and the cholesteryl oleate core transition at ~40°C. The microemulsions were prepared with the following initial concentrations of unesterified cholesterol as described in the text: (A) 0 mol%; (B) 5 mol%; (C) 10 mol%; (C') 10 mol% column fractionated preparation; (D) 15 mol%. The change in heat capacity (Cp) in cal/g is plotted versus temperature. Each sample was scanned three times; the plots are the results of either the second or third heating run.

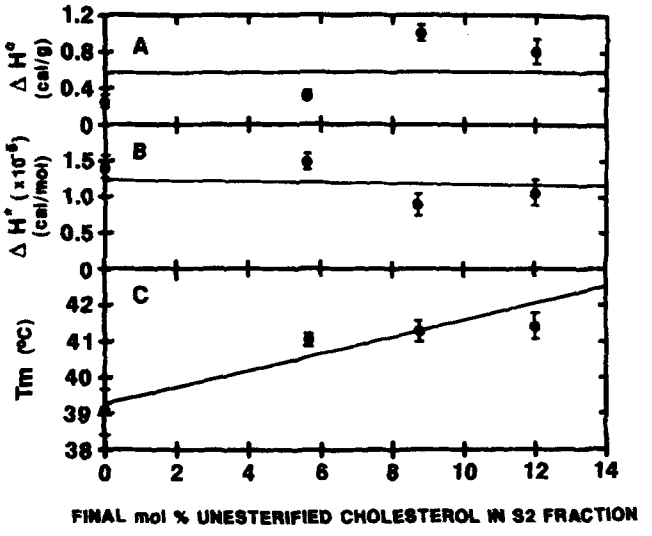

Fig. 7. Calorimetric data for the cholesteryl ester core transition of dimyristoyl phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions as a function of the mean final concentration of cholesterol in the S2 fraction (the second supernatant fraction obtained by centrifugation). (A) calorimetric enthalpy; (B) Van't Hoff enthalpy; and (C) transition temperature. Each point represents the mean of three samples ± SEM. Linear regression was performed using the RS1 laboratory software system. The analysis for $\Delta H°$ and ΔH^* gave fitted lines with slopes not significantly different from 0. The T_m data gave a correlation coefficient of 0.92.

similar to that for the egg yolk phosphatidylcholine system (~40 ± 0.7°C). At increasing unesterified cholesterol compositions, the broadened surface transition began to overlap the core transition.

Fig. 6C and 6C' compare the calorimetric profiles for unfractionated (S2) and fractionated and concentrated microemulsions. The higher noise apparent in the thermogram for the fractionated material (Fig. 6C') results from the significantly lower concentration of material placed in the calorimeter. Qualitatively, the data for the fractionated material was very similar to that for the S2 fraction. The values obtained for the calorimetric enthalpy and Tm for both the DMPC and CO transitions ($\Delta H°$ = 1.14 cal/g DMPC, Tm = 25.8°C, and $\Delta H°$ = 0.74 cal/g CO, Tm = 38.5°C) were in reasonable agreement with the data obtained from the S2 fraction. Similar agreement was obtained for fractionated DMPC/CO microemulsions prepared with 5 mol% unesterified cholesterol ($\Delta H°$ = 0.91 cal/g DMPC, Tm = 21.7°C, and $\Delta H°$ = 0.60 cal/g CO, Tm = 37.5°C).

For the core cholesteryl ester transition, no significant changes were observed in the calorimetric enthalpy (**Fig. 7A**), or the Van't Hoff enthalpy (Fig. 7B). As with egg yolk phosphatidylcholine/cholesteryl oleate microemulsion systems, an increase in the transition temperature as a function of increasing unesterified cholesterol composition was observed (Fig. 7C). For the phospholipid transition, a slight progressive decrease was observed in the

calorimetric enthalpy as a function of unesterified cholesterol composition (**Fig. 8A**). Also, a significant decrease was observed in the Van't Hoff enthalpy (Fig. 8B). An increase in the transition temperature for the surface as a function of increasing unesterified cholesterol composition was also observed (Fig. 8C).

DISCUSSION

The studies presented here extend previous studies on model lipid systems for the lipids of LDL (8) to include the effects of unesterified cholesterol. Gel filtration chromatography demonstrated that critical size considerations are relevant with respect to increasing unesterified cholesterol compositions for these microemulsion model systems. For EYPC/CO/UC model systems, it is impossible to prepare well-defined microemulsion particles with the size characteristics of LDL at initial unesterified cholesterol compositions above 15 mol% UC with respect to total lipids. For microemulsions with DMPC as the surface lipid component, the "limiting" initial unesterified cholesterol composition occurs at 10 mol% UC. Above saturating unesterified compositions for both EYPC/CO /UC and DMPC/CO/UC microemulsions, these systems become extremely polydisperse, as illustrated by the increase in the material which elutes with the void volume of the column, and the overall yield of microemulsion particles decreases.

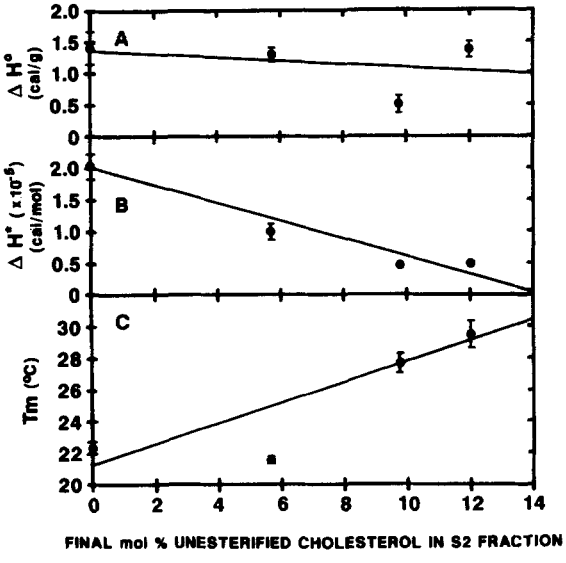

Fig. 8. Calorimetric data for the dimyristoyl phosphatidylcholine surface transition of dimyristoyl phosphatidylcholine/cholesteryl oleate/unesterified cholesterol microemulsions as a function of the mean final concentration of cholesterol in the S2 fraction (the second supernatant fraction obtained by centrifugation); (A) calorimetric enthalpy; (B) Van't Hoff enthalpy; and (C) transition temperature. Each point represents the mean of three independent samples ± SEM. Linear regression was performed using the RS1 laboratory software system. The analysis for $\Delta H°$ gave a fitted line with a slope not significantly different from 0. The ΔH^* gave a correlation coefficient of −0.95 and the T_m data gave a correlation coefficient of 0.85.

At saturating unesterified cholesterol compositions, the ratio of unesterified cholesterol to phospholipid is approximately 1:2 mol/mol. Models for the packing of unesterified cholesterol in bilayer systems have suggested that approximately two phospholipid molecules are required for the acyl chain moieties to solubilize each unesterified cholesterol molecule without the occurrence of cholesterol-cholesterol interactions (18, 19). Although it is possible to prepare bilayer systems with cholesterol compositions up to 1:1 mol/mol (10) a similar size increase is observed in unilamellar phospholipid vesicles as a function of increasing cholesterol composition (20). NMR studies (21) as well as X-ray (22) and neutron diffraction studies (23) have shown that the steroid nucleus of cholesterol restricts the flexibility of the first 10 methylene groups of the acyl chains of a phospholipid molecule. This restriction of flexibility may have a direct effect on the packing of unesterified cholesterol into the highly curved surface of these microemulsion systems as well as in the surface of a small unilamellar vesicle. In spite of a common trend, the DMPC/CO/UC system reaches a plateau for UC incorporation at approximately 8 mol% unesterified cholesterol rather than approximately 15 mol% unesterified cholesterol as observed with EYPC/CO/UC microemulsions, indicating that acyl chain length and homogeneity are also important considerations in the amount of unesterified cholesterol that can be accommodated in these LDL-like microemulsion model systems.

A small increase in the CO/PL molar ratio for both EYPC/CO/UC and DMPC/CO/UC microemulsion model systems as a function of increasing unesterified cholesterol composition indicates that, in these model systems, surface-located phospholipid is replaced by unesterified cholesterol. These data further suggest that the majority of the unesterified cholesterol is located primarily in the surface of the particle, suggesting a relatively high value for the partition coefficient of unesterified cholesterol between the surface and the core. This conclusion is consistent with the partition coefficient of unesterified cholesterol between phospholipids and cholesteryl esters extracted from native LDL, in which the partition coefficient for unesterified cholesterol is approximately 6 (6) as opposed to the partition coefficient for intact LDL which has been shown to be approximately 3 (14).

Because of the heterogeneity present in the acyl chain moiety of EYPC, only the "radial smectic-like" to isotropic liquid transition of the core-located cholesteryl oleate is observed in the EYPC/CO/UC systems. The calorimetric enthalpy ($\Delta H°$) for these systems is similar to that observed in EYPC/CO microemulsions prepared without unesterified cholesterol (8) as well as native LDL (6). No major effects of unesterified cholesterol are observed in

the calorimetric traces for these systems relative to the transition observed for EYPC/CO systems prepared without unesterified cholesterol. No significant effects are seen for the calorimetric parameters ($\Delta H°$, ΔH^*) as a function of increasing unesterified cholesterol composition, further suggesting that only small amounts of unesterified cholesterol partition into the core of the particle consistent with previous studies of lipids extracted from native LDL (6). However, increases are noted in the transition temperature for the core cholesteryl oleate transition for EYPC/CO/UC microemulsion systems as a function of increasing unesterified cholesterol composition, unlike bulk mixtures of cholesteryl esters and unesterified cholesterol which exhibit relatively ideal solubility. In such systems slight decreases in transition temperature are observed with the presence of a eutectic between 5 and 15 mol % unesterified cholesterol relative to cholesteryl ester (24). This increase in transition temperature suggests that the presence of unesterified cholesterol may either have a stabilizing effect on the core transition or, alternatively, may enhance interactions between the surface and core of the particle in these microemulsion model systems.

Differential scanning calorimetry thermograms for DMPC/CO/UC microemulsion systems show that, as with the EYPC/CO/UC systems, no major changes are seen for the core-located cholesteryl oleate "radial smectic-like" to isotropic liquid phase transition. As with microemulsion systems prepared with EYPC, an increase in both the calorimetric enthalpy and the transition temperature is observed, again suggesting that unesterified cholesterol influences direct interactions between the surface and core of the microemulsion particle or, alternatively, that the core may be stabilized by the small amount of unesterified cholesterol that partitions into the core of the particle.

However, for systems with dimyristoyl phosphatidylcholine as the surface lipid component, the order-disorder transition of the surface-located DMPC broadens progressively as a function of increasing unesterified cholesterol composition. In these systems, the surface transition is never completely abolished; the thermograms for microemulsions prepared with initial unesterified cholesterol compositions of 10 and 15 mol % unesterified cholesterol appear quite similar, consistent with the saturating level of unesterified cholesterol in these systems (approximately 8 mol %) and not the unesterified cholesterol composition of the initial starting mixture. Unlike planar bilayer systems, in which unesterified cholesterol can be accommodated up to a concentration of 1:1 mol/mol, the DMPC/CO/UC system can accommodate unesterified cholesterol up to a concentration of 1:2 mol/mol as observed in the EYPC/CO/UC systems, again suggesting the potential importance of interactions between adjacent unesterified cholesterol molecules on the stability of these systems.

A decrease in the calorimetric enthalpy for the surface-located dimyristoyl phosphatidylcholine as a function of increasing unesterified cholesterol composition is observed in the DMPC/CO/UC systems, in conjunction with a dramatic decrease in the Van't Hoff enthalpy for these systems, demonstrating that the presence of unesterified cholesterol has a direct effect on the size of phospholipid domains on the surface of microemulsion particles. Of note is the fact that the enthalpy of the surface phospholipid transition is never completely abolished in these systems; when unesterified cholesterol composition exceeds 1:2 mol/mol, these systems become polydisperse and no longer behave as well-defined microemulsions.

CONCLUSIONS

These studies clearly demonstrate that, for unesterified cholesterol compositions up to 1:2 mol/mol with respect to phospholipid, it is possible to prepare well-defined, protein-free microemulsion model systems that mimic the physical properties of the lipids of native LDL. The results of chemical and thermodynamic analysis show that in these model systems the majority of the unesterified cholesterol is associated with the surface-located phospholipid monolayer. Increases in the transition temperature as a function of increasing unesterified cholesterol composition for the "radial-smectic like" to isotropic liquid transition of the core-located cholesteryl esters demonstrate that unesterified cholesterol has a stabilizing effect on the core transition. ^{13}C-NMR studies to further quantitate the partition coefficient and examine the molecular dynamics of unesterified cholesterol in these model systems are currently in progress.

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