# Interaction of ApoA-1 and ApoE-3 with Triglyceride–Phospholipid Emulsions Containing Increasing Cholesterol Concentrations. Model of Triglyceride-Rich Nascent and Remnant Lipoproteins†

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*Received September 8, 1987; Revised Manuscript Received June 3, 1988*

ABSTRACT: The cholesterol content of triglyceride-rich lipoproteins increases during their catabolism in circulation. We therefore studied the binding of the exchangeable apoprotein apoA-1 and apoE-3 to triolein-rich emulsions with increasing cholesterol content. Five emulsion systems containing 83.1–88.8% (w/w) triolein, 9.3–10.1% egg yolk phosphatidylcholine, and 1.1–7.3% cholesterol were isolated from sonicated lipid mixtures by flotation. Negative stain EM of emulsions containing 1.1 and 7.3% cholesterol showed polydisperse populations of large spherical particles with diameters of 106 ± 39 and 108 ± 57 nm. These values are similar to particle diameters calculated from the lipid composition data. No lamellar structures were observed by EM, even after addition of apoA-1 at a molar ratio to lecithin of $10^{-2}$. Apolipoproteins apoA-1 and apoE-3 bound to the particles in a saturable manner without altering particle morphology. We found a dissociation constant $K_d = 7.4 \times 10^{-7}$ M and a binding capacity $N = 3.9 \times 10^{-3}$ proteins/lecithin for apoA-1 with particles containing 1.1% cholesterol; the $K_d$ and $N$ values for apoE-3 were very similar. When the emulsion particles were saturated with cholesterol at 7.3%, the protein binding capacity $N$ sharply decreased to $0.6 \times 10^{-3}$ (apoA-1) and $0.7 \times 10^{-3}$ proteins/lecithin (apoE-3), but the $K_d$ values were virtually unchanged. No change in $N$ occurred when the particle cholesterol content was increased from 1.1 to 3.7%, which spans the normal physiological range. These results suggest that increases in lipoprotein cholesterol content above 3.7% may be responsible for impaired apoprotein redistribution and altered metabolism of remnants such as β-VLDL.

Chylomicrons (CM) and VLDL are large lipoproteins that carry primarily triglyceride (TG) in the blood to the peripheral tissues (Havel, 1975) where they bind to capillary endothelium and provide substrates for enzymes such as lipoprotein lipase (Korn, 1955). Chylomicrons are formed in the intestine, contain B and A apolipoproteins, are secreted into the lymph, and enter the systemic circulation via the thoracic duct (Ashworth & Johnson, 1963; Cardell et al., 1967). By convention VLDL are formed in the liver, contain B and perhaps other apolipoproteins, and are secreted into the blood (Hamilton et al., 1967; Jones et al., 1967). Nascent chylomicrons and VLDL usually contain a low surface and core cholesterol content (Miller & Small, 1983a, 1987).

Once in the blood, chylomicrons and VLDL undergo similar physical changes and are acted on by the same catalytic and transfer proteins. Lipoprotein lipase, apoproteins C and E, and possibly some apoA bind to the surface, perhaps displacing some phospholipid (Atkinson & Small, 1986; Minari & Zilversmit, 1963) and most of the apoA-4 (Imaisumi et al., 1978). Free cholesterol is transferred from other lipoproteins and cellular elements of blood to these particles (Miller & Small, 1987; Minari & Zilversmit, 1963). Hydrolysis of triglycerides and some phospholipid ensues, resulting in loss of TG with a relative increase in all other components and a concomitant shrinking of the particle. The loss of TG from CM reduces the core size rapidly, resulting in the budding of excess surface monolayer components into bilayers (Miller & Small, 1983; Tall & Small, 1980). When these bilayers are shed, surface lipid and apolipoproteins, primarily apoA and -C, are transferred to the high-density lipoprotein (HDL) fraction where LCAT transacylate phosphatidylcholine and cholesterol into lysophosphatidylcholine (LPC) and cholesteryl esters (CE). The hydrophobic CE migrates into the core, transforming the bilayers into a spherical particle (Tall & Small, 1978). The core remnants derived from chylomicrons still contain $apoB_{48}$ and -E on the surface and are rapidly cleared by the liver. Large VLDL are metabolized and cleared like chylomicrons, but small plasma VLDL remnants remain in circulation and are ultimately converted into LDL [for review, see Havel et al. (1980)]. ApoE-containing chylomicron remnants enter liver cells via membrane receptors that specifically bind with the apoE on the particle surface. A high surface concentration of apoC is thought to inhibit this process (Shelburne et al., 1980), presumably keeping these remnants in circulation. Thus, the exchangeable apoprotein composition of the produced remnants may control their metabolic fate, e.g., which particles are removed and which stay in circulation. The mechanism of redistribution and exchangeable apolipoproteins among plasma lipoproteins during the metabolism of TG-rich lipoprotein is, however, incompletely defined.

Our approach to further define this mechanism is to study the interactions of pure exchangeable apoproteins with lipid emulsions whose particle size and simplified compositions serve as models for TG-rich lipoproteins and their remnants.

During lipolysis of triglyceride-rich lipoproteins, cholesterol may accumulate in the surface of the particles and affect the binding of apoproteins. In this paper we have compared the interaction of exchangeable plasma apoproteins apoA-1 and -E-3 [for review, see Mahley et al. (1984)] with a series of large triolein emulsion particles having surface monolayers consisting of phosphatidylcholine, 6–33% cholesterol, and 1–3% TG (w/w) (Miller & Small, 1982), e.g., apoprotein binding to triolein emulsion as a function of the cholesterol content.

† This research was supported by Research Grant HL-26335 and Training Grant HL-07291 from the National Institutes of Health.

0006-2960/89/0428-0900$01.50/0 

Particle diameters were calculated (Miller & Small, 1983) and verified by negative stain electron microscopy (EM). Equilibrium binding of apoproteins to emulsions was measured at 22 °C. No major differences were found in the binding parameters of apoA-1 and apoE-3, but an increase in surface cholesterol greater than 34 mol % greatly decreased the surface available for protein binding.

## Experimental Procedures

### *Materials*

Egg yolk phosphatidylcholine was purchased from Lipid Products (Surrey, England), and triolein and cholesterol were obtained from Nu Chek Prep, Inc. (Elysian, MN). These lipids were judged greater than 99% pure by thin-layer chromatography. Radiolabeled [9,10-$^3$H]trioleoylglycerol, [4-$^{14}$C]cholesterol, and 1-palmitoyl-2-[1-$^{14}$C]oleoyl-L-$\alpha$-phosphatidylcholine were purchased from Amersham Corp. (Arlington Heights, IL). The radiochemical purities of these lipids were greater than 98% as verified by analytical thin-layer chromatography. Organic solvents were of HPLC grade, and water was twice distilled and deionized before use. All other chemicals were at least of reagent grade quality.

### *Methods*

*Apoprotein Isolation.* Human delipidated HDL was fractionated by gel chromatography at 8 °C using a 90 × 2.5 cm Sephacryl S-300 column (Pharmacia), equilibrated and run with 3 M Gdn-HCl (Fisher Scientific), 0.1 M Tris-HCl, pH 8.0 (Sigma), 0.01% NaEDTA, and 0.02% sodium azide (Wetterau & Jonas, 1982). The major peak was collected, dialyzed exhaustively against 0.01% EDTA, pH 8.0, and lyophilized. The purity of apoA-1 was verified by SDS–polyacrylamide gel electrophoresis. ApoE-3 was a generous gift of Drs. Weisgraber and Mahley of the Gladstone Laboratories. Both apolipoproteins used in this study were of human origin.

*Radioiodination of Apoproteins.* Apolipoproteins were iodinated with Na$^{125}$I by use of solid-state lactoperoxidase (David & Reisfeld, 1974). Protein (100 $\mu$g) in 67 mM phosphate buffer, pH 7.2, was incubated with 200 $\mu$Ci of Na$^{125}$I in the presence of activated Enzymo beads (Bio-Rad Laboratories, Richmond, CA) for 20 min in a final volume of 100 $\mu$L. The reaction mixture was applied to a 0.7 × 20 cm column containing Sephadex G-50 fine (Pharmacia), equilibrated and eluted with phosphate buffered saline; 70–90% of the protein eluted in the void volume. The efficiency of iodine incorporation in apoA-1 was between 40 and 45% and was between 10 and 15% for apoE-3; 97–99% of the iodine incorporated into both proteins were precipitable with trichloroacetic acid. The specific activity of iodinated apoA-1 ranged from $8 \times 10^5$ to $9.5 \times 10^5$ cpm/$\mu$g of protein and for apoE-3 from $2.5 \times 10^5$ to $3.5 \times 10^5$ cpm/$\mu$g of protein. The specific activities of these labeled apoproteins were reduced about 10-fold on the same day by dilution with buffer and addition of cold apolipoproteins. Iodinated apolipoproteins were used within 7 days.

*Protein Procedures and Analysis.* Unlabeled apoproteins were stored at −80 °C (Revco) until used for experiments. ApoA-1 was stored lyophilized and was dissolved in 0.1 M phosphate buffer (Sorenson), pH 7.4, at a final concentration of 1.6 mg/mL. ApoE-3 was stored in solution in 0.1 M ammonium bicarbonate buffer at a concentration of 1.56 mg/mL. Dissolved apoproteins taken out of the Revco were transferred into the cold room and used within 1 week. Radiolabeled apolipoproteins in phosphate buffer were diluted with sodium chloride, NaEDTA, and sodium azide containing solutions as well as unlabeled apoprotein stock solutions from the cold room. Prior to use, the pH of the radiolabeled apolipoprotein solutions was adjusted to pH 7.4, and final salt concentrations were 10 mM phosphate, 140 mM sodium chloride, 0.01% NaEDTA, and 0.02% sodium azide. Final protein concentrations varied between 170 to 180 $\mu$g/mL with specific activities from 30000 to 60000 cpm/$\mu$g of protein. The protein content of each solution was determined by the method of Lowry, using crystalline bovine serum albumin as a standard (Lowry et al., 1951), and by measuring the absorbance at 280 nm in a Perkin-Elmer Lambda 5 spectrophotometer using molar extinction coefficients of 29.300 and 35.400 for apoA-1 and apoE-3, respectively (Tajima et al., 1983; Wetlaufer, 1962).

*Preparation of Radioactive Lipid Stock Solutions.* Stock solutions were prepared by codilution of radiolabeled lipid with the same unlabeled lipid using chloroform for triolein and cholesterol and a mixture of chloroform/methanol 1:1 for phospholipid. Specific activities in counts per minute per microgram (cpm/$\mu$g) were determined for each solution by dividing the cpm per unit volume by the dry weight ($\mu$g) per unit volume. Quenching was uniform throughout. In all experiments the lipid specific activities (SA) were 290 cpm/$\mu$g for triolein, 295 cpm/$\mu$g for cholesterol, and 60 cpm/$\mu$g for phospholipid. The stock solutions were stored in the cold room at 4 °C or kept on crushed ice.

*Preparation and Sonication of Lipid Mixtures.* Aliquots of stock solutions containing 40 mg of triolein, 0.75 mg of cholesterol, and 9.25 mg of phosphatidylcholine were dried under nitrogen in a 15 × 45 mm glass vial and vacuum desiccated overnight at 4 °C. In other mixtures cholesterol was increased at the expense of phosphatidylcholine while the triolein content was kept constant. The cholesterol content was varied from 1.5 to 8%. The dried lipids were resuspended in 10 mL of phosphate buffered saline solution, pH 7.4, which was previously degassed and saturated with nitrogen by using a Branson sonifier Model W-350 (Branson Sonic Power Co., Danbury, CT) set at 90-W continuous power. The sample temperature was controlled to 33 ± 5 °C by immersion in an ice–water bath during sonication for 10 min. Only in mixtures containing 8% cholesterol was unemulsified lipid visible after this period of sonication. Fifty microliters of each sonicated emulsion was taken for lipid analysis.

*Isolation of Large Particles from the Sonicated Emulsions.* The emulsions were transferred into polyallomer tubes (14 × 89 mm) and overlayered with 1.5 mL of distilled water. The tubes were placed in an SW-41 rotor and centrifuged in a Beckman L8-70 ultracentrifuge. Spins were for 10 min at 23000 rpm at a temperature of 22 °C. The rotor decelerated without the brake on, and the total accumulated $\omega_2 t$ was recorded at the end of the run. The top 0.6 mL was isolated with a tube slicer and transferred with a 3-mL plastic syringe into a 15-mL plastic conical screw-cap tube. Transfers of the creamy layer were completed with a 200-$\mu$L rinse of buffer. Final volumes were measured and recorded, and 20 $\mu$L was taken for lipid analysis. The volume of the remaining infranatant was also measured and recorded, and 150 $\mu$L was taken for lipid analysis.

*Lipid Analysis.* The lipid composition of the isolated emulsion fractions was determined by liquid scintillation counting after TLC fractionation of lipid classes using the SA of the individual lipid components. Known aliquots of the fractions were applied by Hamilton syringe to glass thin-layer plates (20 × 20 cm) coated with a 0.25-mm layer of silica gel G. Also, 10 $\mu$L of the radioactive lipid stock solutions was

Table I: Lipid Recovery and Yield of the Large Emulsion Particles Floated from Sonicated Triolein–Lecithin Mixtures with Increasing Cholesterol Content[a]

| mix | starting lipid[b] mixtures (%) | | | isolated amounts[c] (emulsion fractions isolated at $\omega^2 t = 475 \times 10^7$ rad$^2$ S$^{-1}$) | | | yields (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CHOL | PC | $N$ | TO | PC | CHOL | TO | PC | CHOL | total |
| A | 1.5 | 18.5 | 11 | 19.4 ± 1.6 | 2.2 ± 0.4 | 0.24 ± 0.03 | 49 | 24 | 32 | 44 |
| B | 3.0 | 17.0 | 3 | 20.4 ± 0.7 | 2.2 ± 0.2 | 0.45 ± 0.02 | 51 | 26 | 30 | 46 |
| C | 5.0 | 15.0 | 2 | 22.1 ± 1.2 | 2.5 ± 0.1 | 0.94 ± 0.02 | 55 | 33 | 37 | 51 |
| D | 7.0 | 13.0 | 2 | 21.9 ± 0.7 | 2.4 ± 0.3 | 1.49 ± 0.07 | 55 | 37 | 42 | 52 |
| E | 8.0 | 12.0 | 3 | 26.7 ± 0.9 | 3.1 ± 0.4 | 2.34 ± 0.14 | 67 | 50 | 59 | 64 |

[a] Sonication, centrifugation, and lipid analysis are performed as described under Methods. Weight values are the mean ± SD. [b] Starting mixtures were composed of 40 mg of triolein (TO), the indicated weight percent cholesterol (CHOL), and egg phosphatidylcholine (PC) to contain 50 mg of total lipid. [c] Milligrams of lipids recovered in the isolated emulsion fraction obtained after flotation.

plated separately. The lipid was separated with hexane/diethyl ether/18 N acetic acid (55:45:1) as developing solvent (Derksen & Cohen, 1973). The individual lipid classes were visualized with iodine vapor and marked. In addition to triolein, cholesterol, and lecithin, traces of free fatty acid and partial glycerides were found in the isolated emulsion and quantitated by GLC (Derksen & Cohen, 1975). No lysophospholipids were detected. The iodine was allowed to evaporate, and the isolated lipids were scraped into individual counting vials. Liquiscint (10 mL) (National Diagnostics, Manville, NJ) was added, and the radioactivity was determined in an LKB 1217 rack $\beta$ liquid scintillation counter. Concentrations of the individual lipid classes were calculated from the SA of the lipid stock solutions. All emulsions used for binding studies were adjusted to contain a final triolein concentration of 18 mg/mL and had small amounts of free fatty acids ranging between 0.2 and 0.3% (w/w) of total lipid.

*Assay of the Binding of Apoproteins to Emulsion Particles.* Binding assays were performed in duplicate at room temperature. Six polyallomer centrifuge tubes (5 × 20 mm, Beckman) each contained 175 $\mu$L, composed of 40 $\mu$L of isolated emulsion particles and 135 $\mu$L of buffer with the appropriate protein concentration. The mixtures were incubated for 20 min in an A-100/18 rotor followed by centrifugation at 20 psi for 10 min in a Beckman airfuge. After centrifugation, the bottoms of the tubes were punctured with a 27-gauge needle, and 100 $\mu$L was collected with a gastight Hamilton syringe. This aliquot (bottom fraction) containing virtually no lipid and the tube with the leftover (top fraction) containing the emulsion were transferred separately to 10 × 75 mm plastic tubes. $\gamma$ counting was performed in an LKB 1275 Minigamma spectrometer with a sample elevator setting of 0.5 cm. In all experiments, the total amount of protein added to each assay mixture was accounted for by the sum of the bottom and top fractions. In each assay mixture the buffer and emulsion content were kept constant while the protein content was increased to 10, 20, 40, or 60 $\mu$g/mL.

Two rotors were employed during the execution of the binding study protocols comparing either emulsions with different cholesterol compositions and different proteins or both. Twelve tubes could be processed per hour, and protocols were completed within 6 h after the isolation of the large emulsion particles.

*Analysis and Expression of Binding Data.* For each assay mixture the following concentrations were measured or derived: [PC], lecithin concentration; $P_f$, free protein concentration (bottom fraction); $P_T$, total protein concentration (bottom + top fractions); $P_b$, $P_T - P_f$, bound protein concentration. By use of these values, the data were analyzed, assuming a single isothermal equilibrium and a finite number of discrete, equivalent, and noninteracting binding sites on the surface of the lipid particles. This equilibrium has been described mathematically (Yokoyama et al., 1980) according to eq 1,

$$K_d = (N[PC] - P_b)P_f/P_b \quad (1)$$

in which the $K_d$ is the dissociation constant and the term $(N[PC] - P_b)$ represents the concentration of uncoupled binding sites at protein levels below the saturation level.

Equation 1 transforms to $P_f = (N[PC] - P_f/P_b) - K_d$ and yields the parameters $K_d$ and $N$ (the number of binding sites in relation to phospholipid) given in Figure 5 and Table III.

*Electron Microscopy.* The isolated emulsion fractions were diluted with pH 7.4 phosphate buffer and were fixed for 30 min with $OsO_4$. Final lipid and osmium concentrations are 2–4 mg/mL and 0.6–0.8%, respectively. A 5-$\mu$L droplet of fixed emulsion was applied for 10 s to a Formvar carbon-coated 300-mesh copper grid. All film surfaces were made hydrophilic by glow discharge and used within 2 weeks. Excess emulsion was removed by Pasteur pipet and filter paper and replaced by a 5-$\mu$L droplet of 1% sodium phosphotungstate; after a few seconds, excess was removed and the stained residue on the grid was air-dried. Each grid quadrant (field) was photographed with a Hitachi HU-11C electron microscope at an acceleration voltage of 75 kV. The instrument was calibrated with a carbon grating replica (Ernest F. Fullam, Inc.) and magnification was 15513. Each field contained 100–200 particles. Particles were measured systematically by using a Peak 7× magnifier with a graticule 1–20 mm in 0.1-mm divisions. Only discrete spherical particles greater than 260 Å were tabulated, and a minimum of 400 particles was accumulated in a histogram.

*Calculation of Particle Diameter.* The method for the calculation of particle diameter from chemical compositions has been previously described (Miller & Small, 1983b, 1987). This method takes into account that triolein and cholesterol distribute between the emulsion surface and core phases. For larger particles ($D > 100$ nm), as used in this study, the oil phase contains a significant amount of cholesterol. Therefore, the ratio of oil to surface masses for the particle is increased and the diameter becomes about 10% larger than the value estimated by assuming all cholesterol is at the surface (Miller & Small, 1983b).

*Statistical Analysis.* The $\chi^2$ test of homogeneity (Colton, 1974) was used to determine whether the distribution of emulsion particles was from the same population.

## Results

*Characteristics of the Isolated Lipid Emulsion Particles To Be Used for Protein Binding.* Isolated lipid emulsions were characterized by determining their chemical composition and particle size distribution. Table I shows the recovery and yield of the lipid constituents residing in the large emulsion particles

Table II: Average Lipid Composition, Cholesterol Content of Surface and Core, and Diameter of Isolated Emulsion Particles Calculated from Table I

| emulsion | lipid composition[a] TO | PC | CHOL | surface[b] CHOL | core[b] CHOL | diameter[c] (nm) lipid composition | neg stain EM |
|---|---|---|---|---|---|---|---|
| A′ | 88.8 | 10.1 | 1.1 | 7.8 (13.2) | 0.35 (0.72) | 116 | 106 ± 39 |
| B′ | 88.5 | 9.5 | 2.0 | 13.8 (22.8) | 0.63 (1.31) | 116 | |
| C′ | 86.5 | 9.8 | 3.7 | 22.6 (33.5) | 1.03 (2.18) | 100 | |
| D′ | 84.9 | 9.3 | 5.8 | 32.4 (48.2) | 1.47 (3.19) | 90 | |
| E′ | 83.1 | 9.6 | 7.3 | 32.6 (48.5) | 2.96 (6.32) | 86 | 108 ± 57 |

[a] The lipid compositions are averages, and values represent weight percent. [b] The surface and core content of cholesterol was calculated by the phase analysis method (Miller & Small, 1983b, 1987). Values represent weight percent and (mole percent). [c] The particle diameter was calculated from the lipid composition data with the aid of a computer program (Miller & Small, 1987), assuming spherical emulsion structure and the appropriate partition of triolein and cholesterol into core and surface. The surface to core distribution ratio for cholesterol was 22.0 for unsaturated conditions such as emulsions A′–D′ and 11.0 for emulsion E′, which was saturated with cholesterol. Also tabulated are the mean diameters ± SD of the particles in emulsions A′ and E′ as visualized by negative stain electron microscopy (see also Figures 1 and 2).

that were floated up by centrifugation of the sonicated lipid mixtures. Five mixtures (A–E) increasing stepwise in cholesterol content from 1.5 to 8% (w/w) are compared. In the range studied more lipid floated up when more cholesterol was present in the starting mixture. The amount was greatest when the lipid mixture was saturated with cholesterol at 8% (w/w) (bottom, Table I). Since all lipid mixtures (A–E) contained 50 mg of total lipid, the yields of total floated lipid increased with increasing cholesterol content of the starting mixture. When the cholesterol content of the lipid mixture was increased from 1.5 to 8% (w/w) and the phosphatidylcholine content correspondingly was decreased from 18.5 to 12%, the yields of cholesterol as well as phosphatidylcholine in the isolated emulsion fraction doubled. The average lipid composition, surface cholesterol content, and calculated diameter of the isolated emulsion particles are listed in Table II. The isolated emulsions designated A′–E′ have a weight percent cholesterol content that is always less than the sonicated lipid mixtures from which they were isolated. This reduction in cholesterol content is greatest (about 30%) in the low-cholesterol emulsions (A′) but becomes smaller (A′–E′) and is 9% in the highest cholesterol emulsion (E′). Phosphatidylcholine behaves similar to cholesterol in this context. Conversely, the triolein contents of the isolated emulsions were always higher than the parent mixtures. The increments in triolein content were largest in the low-cholesterol emulsions (A′ and B′), gradually became smaller, and was smallest in the saturated cholesterol emulsion (E′). With increasing cholesterol content of particles, surface, and core, the calculated average particle diameter of the isolated emulsions (A′–E′) decreased.

Since the protein binding study involves separation of emulsion from unbound protein in an airfuge (see Methods), we refloated the isolated emulsion A′ or E′ in an airfuge without protein at 20 psi for 10 min. All the emulsions floated and gave a constant triolein/phospholipid ratio of the recovered particles in the cream layer, indicating that the lipid composition of the particles was not changed by centrifugation.

An electron micrograph of emulsion A′ in Figure 1A shows that the isolated fraction contains large circular (assumed to be spherical) particles without lamellar structures. The size frequency distribution of the particle diameters in Figure 1A are graphed as a histogram in Figure 1B. The large particles had diameters ranging between 32 and 277 nm (106 ± 39 nm was the mean ● SD of 407 particles measured in the electron micrograph), which are comparable with chylomicrons (Sata et al., 1972). The particle size frequency distribution of emulsion E′ was similar (108 ± 57 nm), but the range was expanded to 368 nm, with 0.5–1% of the particle diameters ranging between 277 and 368 nm (Figure 2). Comparison of the particle size distribution of emulsions A′ with E′ by $\chi^2$ test of homogeneity revealed that the high-cholesterol emulsions were most likely to have a greater number of particles with diameters greater than 187 nm ($p = 0.0001$) (results not shown graphically). Addition of apoproteins apoA-1 to emulsion A′, to contain a protein to phospholipid molar ratio in excess of those used in the binding studies, did not alter the structure of the spherical particles nor induce the formation

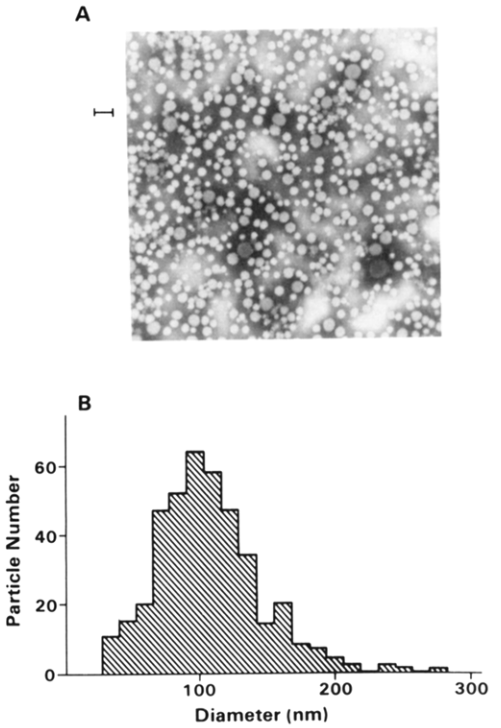


FIGURE 1: Negative staining electron micrographs of large particles from emulsion A′. (A) Emulsions were diluted and fixed with osmium, plated on formvar grids, and stained with 1% sodium phosphatungstate, pH 7.2. Grids were observed and photographed with a Hitachi HU-11C electron microscope. The bar is equivalent to 258 nm, and magnification is 23 270. (B) Histogram of the particle size frequency distribution from emulsion A′. See Methods for the measurements of particle sizes.

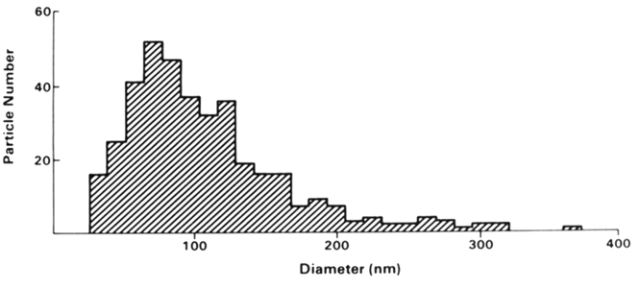


FIGURE 2: Histogram of the particle size frequency distribution from emulsion E′. See Methods for the measurements of particle sizes.

Table III: Parameters for the Binding of ApoA-1 and ApoE-3 to Large Triolein–Lecithin Emulsion Particles with Low and High Cholesterol Content[a]

| apoprotein | binding surface, wt % CHOL in surface | $K_d$ | | $N$ | | |
|---|---|---|---|---|---|---|
| | | M | g/L | mol of protein/mol of PC | g/g | protein molecules/particle $(x)^b$ |
| apoA-1 | 7.8 | $7.4 \times 10^{-7}$ | $2.0 \times 10^{-2}$ | $3.9 \times 10^{-3}$ | $14.8 \times 10^{-2}$ | 227 (116) |
| | 32.6 | $8.1 \times 10^{-7}$ | $2.3 \times 10^{-2}$ | $0.6 \times 10^{-3}$ | $2.3 \times 10^{-2}$ | 14 (86) |
| apoE-3 | 7.8 | $11.7 \times 10^{-7}$ | $4.0 \times 10^{-2}$ | $3.8 \times 10^{-3}$ | $17.5 \times 10^{-2}$ | 221 (116) |
| | 32.6 | $10.5 \times 10^{-7}$ | $3.6 \times 10^{-2}$ | $0.7 \times 10^{-3}$ | $3.5 \times 10^{-2}$ | 16 (86) |

[a] The conditions of the binding assay are described under Methods. Substrate concentrations are as listed in the legend for Figure 4. $K_d$ is a dissociation constant, and $N$ is the upper limit of binding. The values correspond to the intercept on the oridinate ($K_d$) and to the slope ($N$) of the least-squares regressed line in Figure 5. The surface content of cholesterol was calculated by the phase analysis method (Miller & Small, 1983b). Values represent the average of two experiments (see Figures 4 and 5). Surface cholesterol is expressed in weight percent. [b] $(x)$ = average particle diameter in nanometers.

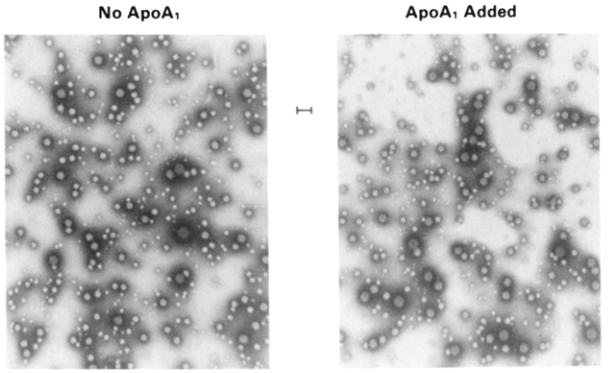


FIGURE 3: Negative staining electron micrographs of large particles from emulsion A′ in the absence (A) or presence of apoA-1 (B). Electron micrographs were taken as described in Figure 1. The molar ratio of apoA-1 to phospholipids was $10^{-2}$. The bar equals 440 nm, and magnification is 11 390.

of lamellar structures when examined by negative stain EM (compare parts A and B of Figure 3). Statistical comparison of the particle size distribution of emulsion A′ with or without protein revealed that the protein-containing preparation was more likely to include particles with smaller diameters in the population visualized with negative stain EM ($p = 0.09$). Emulsion E′ with or without apoA-1 gave the same result but with a higher statistical significance ($p = 0.001$).

*Effect of Cholesterol on Binding of ApoA-1 and ApoE-3 to Large Emulsion Particles.* Apoproteins apoA-1 and apoE-3 reproducibly bound to the surface of large triglyceride-rich particles in phosphate buffer, pH 7.4 at room temperature. As the protein concentration in the incubations was increased, larger amounts of apoproteins bound to the surface of the particles (Figure 4). More proteins bound to the low-cholesterol particles (A′) than to the high-cholesterol particles (E′) at each of the protein concentrations. The shape of the binding curves indicates that in the protein range studied saturation was approached only at the higher protein levels for particles saturated with cholesterol. Plotting the free protein, $P_f$, against the phosphatidylcholine concentration multiplied by free protein/bound protein ($[PC]P_f/P_b$) yields a linear plot (Figure 5) from which $K_d$ ($y$ intercept) and $N$ (slope) could be estimated (Table III).

Increasing the cholesterol content in the surface of the emulsion particles from 7.8 to 32.6% did not significantly change the dissociation constant $K_d$ for apoA-1 or apoE-3. However, the number of protein molecules bound per 1000 molecules phospholipid at saturated cholesterol content was greatly reduced for both apoproteins (Table III). Because of this marked decrease in protein binding at the surface of particles saturated with cholesterol, binding studies were carried out at several intermediate concentrations of cholesterol, using the five emulsions described in Table II. Figure

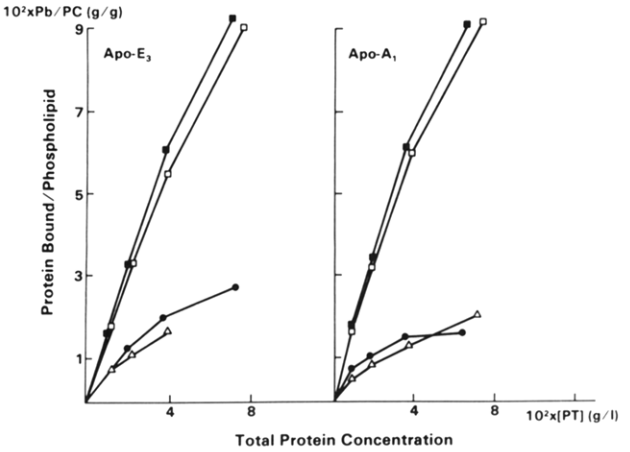


FIGURE 4: Binding profiles of apoA-1 and apoE-3 to large particles of emulsion A′ and E′ with low-cholesterol (square symbols) and high-cholesterol (other symbols) content. Assays with emulsion A′ contained 420 μM PC and with emulsion E′ had 700 μM PC. ApoA-1 and apoE-3 varied from 0.25 to 2.0 μM. Free apoproteins were separated from bound apoproteins with an airfuge as described under Methods. Two separate experiments are graphed (open versus solid symbols).

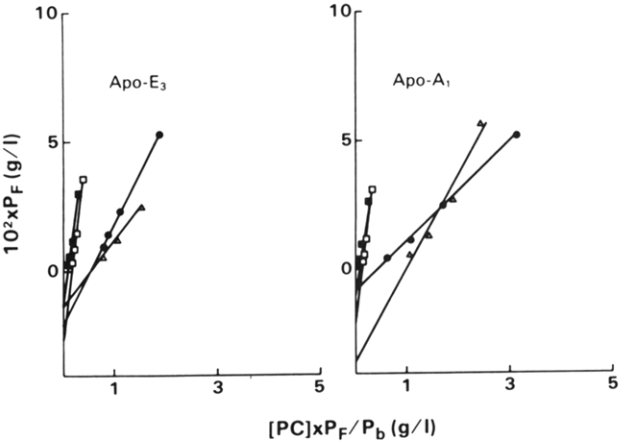


FIGURE 5: Linearized plots of the data from Figure 4 according to eq 1. The solid lines represent the least-squares fit to the data. $K_d$ and $N$ calculated from two lines are given in Table III. For an explanation of the symbols see Figure 4.

6 shows that the maximal capacity of emulsion particles to bind apoA-1 or apoE-3 at high protein concentration changes as a function of the cholesterol content in a similar way for both apolipoproteins. The maximal protein binding capacity is unaltered by increases in the particle cholesterol content from 1.1 to 3.7 wt % but declines continuously with subsequent increases in cholesterol content until the particles are saturated with cholesterol at 7.3 wt %. In the high-cholesterol range (3.7–7.3 wt %) where protein binding becomes more and more

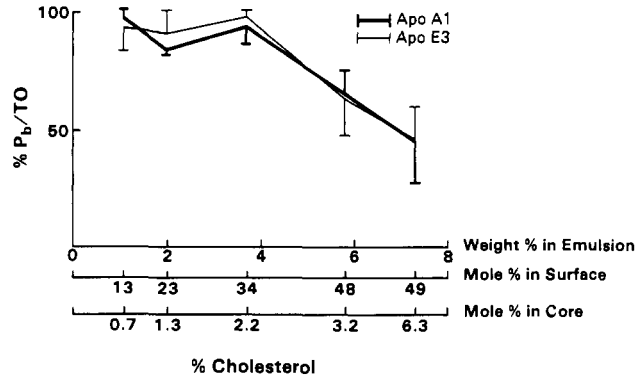


FIGURE 6: Percent maximal binding of apoA-1 and apoE-3 to emulsions A′–E′ as described in Table II. The triolein and protein concentrations in the reaction mixture were respectively 4.1 and 0.05 mg/mL. Values are the mean ± SD of four experiments.

impaired, an almost maximal particle surface cholesterol concentration of 48.2 mol % is attained at an emulsion cholesterol content of 5.8 wt %. A further increase in emulsion cholesterol content from 5.8 to 7.3 wt % primarily doubles the mole percent cholesterol concentration of the core from 3.19 to 6.32 mol % with little calculated change in the surface cholesterol content (bottom, Table II).

## Discussion

In the present paper we have used defined emulsion systems to compare the binding interactions of apoA-1 and apoE-3 with the phospholipid–cholesterol surface of large triolein-containing particles that serve as models for triglyceride-rich lipoproteins and their remnants. The major experimental findings of the present study are the following: (a) The isolated emulsion fractions contain only populations of large spherical particles with compositions and size ranges that imitate native chylomicrons and their remnants. (b) A large increase of cholesterol in the lipid mixture increases the yield of the isolated emulsion fraction, increases the C/PL ratio in the surface, and greatly decreases the available surface of the isolated particles for apoprotein binding when the surface cholesterol content exceeds 34 mol %. (c) The apoproteins apoA-1 and apoE-3 form complexes with these emulsions without changing particle morphology or inducing the formation of lamellar structures. (d) The binding parameters of apoA-1 and apoE-3 to large emulsion particles are affected in the same way by increases in the cholesterol content.

The isolated emulsion fractions used in this study adequately model triglyceride-rich lipoproteins and their remnants because of the analogous lipid compositions (Table II), particle size frequency distributions (Figures 1B and 2), and polydispersity (Sata et al., 1972; Fraser, 1973). Microemulsion-sized particles (diameter < 45 nm) contained less than 2% of the lipid, and no lamellar structures were detected by EM. The average particle diameter in the histogram (Figures 1B and 2) is in close agreement with the calculated diameter obtained from analysis of the lipid composition (Table II). Apparently, several small inconsistencies occurred in particle sizes as viewed by EM. First, high-cholesterol emulsion fractions were more likely to have larger particles, while the composition data would predict slightly smaller ones. Second, addition of protein to emulsions shifted the distributions to particles with slightly smaller sizes. These discrepancies suggest that the cholesterol or protein may potentially induce either larger or smaller particles in the population to remain associated with the grid after washing and staining. Also, large particles may flatten out on the grid. Therefore, sizes and distributions obtained by EM must be interpreted with caution. Importantly, however, addition of apoA-1 to emulsions in a concentration 5-fold higher than used in the binding studies was shown by EM not to disrupt the morphology of the particles (Figure 3), indicating apoA-1 is binding to the surface and not pulling off lipids to form bilayers.

The mode of binding of apoA-1 to emulsion surfaces appears to be similar to that of apoE-3: (a) Both bind to the particles with a similar isotherm (Figure 4) without disruption of the spherical structures (Figure 3). (b) The affinities and capacities of the particles are comparable (Table III). (c) Cholesterol affects the binding capacity for both by about the same extent (Figure 6) without changing the affinity. For both proteins binding probably occurs primarily through insertion of amphiphilic helical segments of peptides between the head groups of phospholipids, as has been suggested for apoA-1 (Segrest et al., 1974). The apoA-1 and apoE-3 binding data are consistent with published binding constants measured with triolein–phospholipid emulsion (Tajima et al., 1983), even though their particles were either smaller or larger than the particles used in this study and did not contain cholesterol. Collectively, these data indicate that a common binding site for exchangeable apoproteins exists on large emulsion particles, analogous to findings obtained with various apolipoproteins and dipalmitoylphosphatidylcholine vesicles (Cardin et al., 1982).

The effect of cholesterol on the binding of apolipoproteins to the surface of large emulsion particles can be studied exclusively when the presence of other surface-active compounds such as fatty acids, diglycerides, or lysolecithin is avoided or controlled to a minimal amount. The emulsions used in this study contain between 0.2 and 0.3% free fatty acid. Small changes in fatty content may bring about some fluctuation in the binding capacity of exchangeable apoproteins, particularly at saturating cholesterol concentration (Derksen et al., 1986).

A cholesterol content of 34 mol % or larger in the surface of the particles (Figure 6) begins to decrease the binding capacity of exchangeable apoproteins. This result corroborates the cholesterol effect observed on the association of apoA-1 with L-$\alpha$-dimyristoylphosphatidylcholine vesicles (Pownall et al., 1979). At these higher concentrations cholesterol probably alters the steric properties of the phospholipid surface monolayer, decreasing the free volume in the surface (Smaby et al., 1984) and making it more difficult for the helical peptide segments to penetrate into the surface. Cholesterol at this concentration or higher has been reported to sqeeze CE (Spooner et al., 1986) and TG (Spooner & Small, 1987) out of phospholipid bilayers. Within the physiological range of particle surface cholesterol (between 13 and 34 mol %) (Miller & Small, 1987) the binding capacities of apoA-1 or apoE-3 remained maximal; however, at these lower concentrations, cholesterol probably competes less effectively with apoprotein amphiphilic helical segments for the apolar region of the surface phase since it can also partition into the core phase to a limited extent (Figure 6 and Table II). Thus, apoA-1 or apoE-3 might alter the surface to core ratio of cholesterol. The argument is as follows: The free energy of association of cholesterol with the surface is sufficiently large to be nearly quantitative. If apoA-1 binds to phospholipid with a free energy of association that is greater than the free energy of transfer of cholesterol from the surface to the core, then apoA-1 would alter the surface to core distribution. This possibility suggests that during the metabolism of normal TG-rich lipoproteins in normal subjects surface cholesterol does not markedly alter the binding and distribution of exchange-

able apoproteins. β-VLDL has increased surface cholesterol (Miller & Small, 1983a, 1987), and its apoproteins and metabolism are different from those of normal VLDL (Havel & Kane, 1973). Increases in surface cholesterol above 34 mol % may be responsible for impaired apoprotein redistribution and altered metabolism of such remnants.

ACKNOWLEDGMENTS

We are grateful to Drs. Weisgraber and Mahley of the Gladstone Laboratories for Cardiovascular Disease, University of California, San Francisco, for the kind gift of apoE-3. We thank D. Gantz for electron microscopy and Drs. H. L. Kayne and S. Levenson of the School of Public Health, BUMC, for statistical analysis. We also thank Anna M. Tercyak and Cheryl Oliva for technical assistance and Irene L. Miller for help in preparing the manuscript.

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