Interaction of Plasma High Density Lipoproteins with Dimyristoyllecithin Multilamellar Liposomes†

Alan R. Tall,*‡ Valerie Hogan, Lora Askinazi, and Donald M. Small

ABSTRACT: Studies of incubated mixtures of lecithin multilamellar liposomes and plasma high density lipoproteins (HDL) have suggested that apoprotein can dissociate from the HDL and solubilize phospholipid by forming discoidal complexes of phospholipid and apoprotein (Tall, A. R., and Small, D. M. (1977), *Nature (London) 265,* 163–164). Mixtures of HDL incubated with dimyristoyllecithin (DML) have been examined by ultracentrifugal techniques in an attempt to separate phospholipid apoprotein complexes from HDL. Equilibrated mixtures of ^{14}C-labeled DML and HDL_2 (density 1.063 to 1.125 g/mL) or HDL_3 (density 1.125 to 1.21 g/mL) were subjected to preparative and equilibrium density gradient ultracentrifugation, and the various fractions were analyzed for lipid and apoprotein content and examined by negative stain electron microscopy. In DML/HDL_3 mixtures, the DML formed a peak at density 1.105 g/mL, in which apoprotein lecithin complexes were identified by electron microscopy as discs on edge or circular particles, both of mean diameter ~180 Å. These complexes were enriched in apoprotein A-1 compared with HDL_3. The HDL_3 lipids were transformed from a single peak at density 1.15 g/mL (in control experiments) into lipoproteins floating between densities 1.105 and 1.08 g/mL. These lipoproteins showed an increase in lipid to protein ratio and a decrease in the apo-A-1 to apo-A-2 ratio. In experiments where HDL_2 was incubated with DML, a fraction was isolated at density ~1.09 g/mL, enriched in DML and apo-A-1, while most of the HDL_2 lipids were associated with lipoproteins floating at density <1.08 g/mL. Thus, both HDL_2 and HDL_3 can solubilize DML multilamellar liposomes, probably via the formation of discoidal complexes enriched in apo-A-1. Very little of the HDL lipid or apo-A-2 is incorporated into these complexes. During the process of complex formation, HDL_2 and HDL_3 are transformed into lipoproteins of decreased density and increased size perhaps by a process involving fusion of apo-A-1 depleted HDL particles.

The plasma high density lipoproteins (HDL)[1] comprise two classes: HDL_2 (*d* 1.063 to 1.12 g/mL) and HDL_3 (*d* 1.125 to 1.21 g/mL). HDL_2 consists of about 60% lipid, 40% protein and HDL_3, 40% lipid, 55% protein (Scanu and Wisdom, 1972). The lipid composition of the HDL_2 and HDL_3 is similar (about 53% phospholipid, 37% cholesterol ester, 5% cholesterol, 3% triglyceride; Tall et al., 1977a). HDL_2 and HDL_3 are both spherical particles of diameters ~110 Å (mol wt = 360 000) (Shipley et al., 1972) and ~95 Å (mol wt = 180 000) (Laggner et al., 1973), respectively, in which the protein and phospholipid surface components enclose a core of apolar lipid. The principal apoproteins of HDL are apo-A-1 (mol wt 28 300) and apo-A-2 (mol wt 17 800) (Lux et al., 1972; Baker et al., 1974).

Recombination of the delipidated apoproteins of HDL with phospholipids can result in the formation of phospholipid bilayer discs (Forte et al., 1971; Atkinson et al., 1976). Similar discoidal complexes have been observed as the nascent form of HDL, secreted by the liver (Hamilton et al., 1976). These particles accumulate a core of cholesterol ester when exposed to plasma lecithin:cholesterol acyltransferase and are thus converted to spherical, mature HDL particles (Forte et al., 1971). Studies of recombinants of delipidated HDL apoproteins with dimyristoyllecithin (DML) by differential scanning calorimetry showed that as discs are formed the gel to liquid crystalline transition of the lecithin is diminished in enthalpy, broadened, and shifted from 24 to 26 °C (Tall et al., 1975, 1977b). In separate experiments, it was found that large amounts of apo-A-1 could be removed from HDL by systematic heating and cooling, leaving a stable, apo-A-1 depleted lipoprotein (Tall and Small, 1977). The increased size of the remnant lipoprotein was interpreted as arising from fusion of apoprotein depleted particles. When mixtures of lecithin multilamellar liposomes were incubated with intact HDL, they displayed a lecithin transition typical of discoidal apoprotein lecithin complexes, and ~150 × 55 Å discoidal particles were identified in the mixtures by electron microscopy (Tall and Small, 1977). Thus we postulated that intact HDL was able to give up apoprotein, probably apo-A-1, when incubated with lecithin membranes, leading to the formation of discoidal apo-A-1–lecithin complexes. The residual HDL_3 particles were increased in size by EM, consistent with lipoprotein fusion secondary to loss of apo-A-1.

In this paper we describe our attempts to isolate the apo-A-1–phospholipid complex and the large remnant HDL by centrifugation. We have examined mixtures of HDL with DML by preparative and equilibrium density gradient ultracentrifugation. Each fraction has been fully characterized with respect to chemical composition and has been examined by negative stain electron microscopy.

Experimental Section

Materials

Human HDL_2 and HDL_3 were isolated from the plasma of normal, fasting male donors by preparative ultracentrifugation between densities 1.063 to 1.125 g/mL and 1.125 to 1.21

† From the Biophysics Section, Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118. *Received May 24, 1977.* This work was supported by National Institutes of Health Grants No. GRS605711 (A.R.T., PI) and No. GM00176 (D.M.S., PI).

‡ Present address: Department of Medicine, College of Physicians and Surgeons of Columbia University, The Presbyterian Hospital, New York, N.Y. 10032.

[1] Abbreviations used are: HDL, plasma high density lipoprotein; *d*, density; DML, dimyristoylphosphatidylcholine; apo-A-1, apolipoprotein A-1; apo-A-2, apolipoprotein A-2; EYL, egg yolk lecithins; mol wt, molecular weight.

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g/mL, respectively, as described previously (Tall et al., 1976). Centrifugations were conducted for 36 h at 40 000 rpm in a Beckman 40.3 fixed angle rotor. Each lipoprotein was washed once at its highest density in order to eliminate albumin contamination. For preparation of apo-HDL, HDL was delipidated with chloroform:methanol 2/1 v/v, and washed three times at −15 °C with anhydrous diethyl ether (Scanu and Edelstein, 1971). The apoprotein composition of apo-HDL as determined by Sephadex G-200 chromatography was 60% apo-A-1, 30% apo-A-2, and 10% C peptides. For preparation of recombinants of DML and apo-HDL, apo-HDL was labeled with ^{125}I by the iodine monochloride method of MacFarlane (1958) employing 1 mol of iodine per mol of protein. More than 99% of the ^{125}I counts were precipitable with 10% trichloroacetic acid.

Dimyristoyllecithin was prepared by the method of Cubero Robles and Van den Berg (1969). Glycerolphosphorylcholine was acylated with myristic anhydride in the presence of potassium myristate and purified by silicic acid chromatography to more than 99% purity. For preparation of ^{14}C-labeled DML, DML was digested with phospholipase D, by the method of Kornberg and McConnell (1971) to form dimyristoylphosphatidic acid which was isolated by silicic acid chromatography. The dimyristoylphosphatidic acid was coupled with ^{14}C-labeled choline (New England Nuclear) by the method of Aneja and Chadha (1971), as modified by Sears et al. (1976). The resulting ^{14}C-labeled DML was isolated by silicic acid chromatography. Egg yolk lecithin (EYL) was purchased from Lipid Products. EYL and DML were more than 99% pure by thin layer chromatography (Figure 1).

Multilamellar liposomes of DML were prepared by adding 0.15 M NaCl, pH adjusted to 8.0 with NH_4OH, to a known amount of DML colyophilized from benzene with a trace amount of ^{14}C-labeled DML. For preparation of recombinants, HDL_2 or HDL_3 or apo-HDL (containing 4 to 15 mg of protein/mL) was added to unsonicated, turbid suspensions of DML and incubated while stirring under N_2 at 26 °C for 12 to 18 h.

Methods

Gradients of NaBr were generated with a peristaltic pump, using the density 1.006 g/mL mixtures of DML/HDL (or apo-HDL) as the limiting buffer. The gradients were centrifuged for approximately $5.4 \times 10^6 g \times h$ in a Beckman SW 56 swinging bucket rotor. The density of each fraction was determined by weighing a 200-μL aliquot. An aliquot of each fraction was counted for [^{14}C]DML and the absorbance at 280 nm measured in an UV spectrophotometer. The remainder of the fraction was dialyzed against 0.15 M saline, pH 8. The dialyzed fractions were examined by negative stain electron microscopy, and extracted with 2/1 v/v chloroform/methanol by the method of Folch et al. (1957) for lipid determination. The apoprotein content was determined using tetramethylurea–polyacrylamide gels by the method of Kane (1973). Apo-A-1 and apo-A-2 were identified by their migration compared with purified apo-A-1 and apo-A-2, and the relative amounts (weight ratio) of each apoprotein quantitated by scanning the gels with a Beckman model gel scanner. The lipid extract of each fraction was quantitated for free cholesterol, fatty acid, triglyceride, cholesterol ester, lysolecithin, sphingomyelin, and lecithin by thin-layer chromatography, following the method of Downing (1968). In preliminary studies (Tall and Small, 1977), it was found that dimyristoyllecithin could be resolved from the lecithins of HDL by quantitative thin-layer chromatography. The lipid extracts were applied to 20 × 20 cm precoated silica gel G plates (Scientific Products),

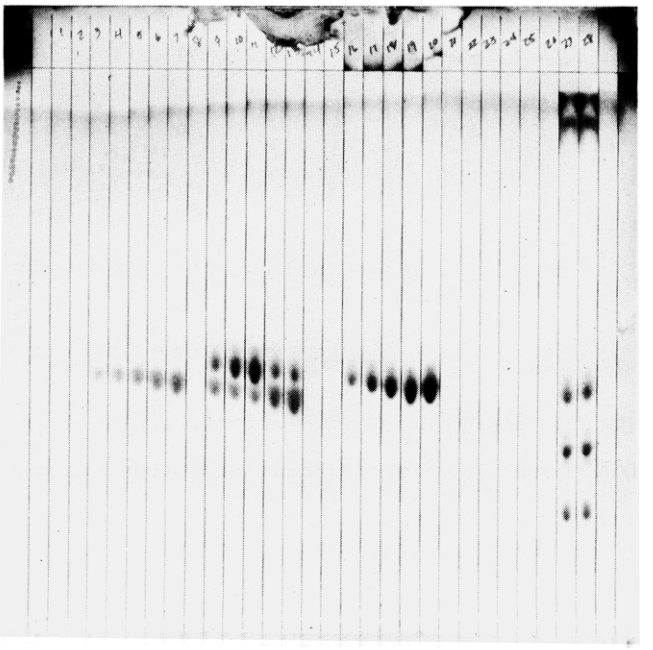

FIGURE 1: Separation of dimyristoyllecithin from egg yolk lecithin by thin-layer chromatography. Increasing amounts of dimyristoyllecithin or egg yolk lecithin were spotted on lanes 3–7 and 16–20, respectively. In lanes 8–12 egg yolk lecithin–dimyristoyllecithin mixtures were applied containing, respectively, weight ratios of 1/1, 2/1, 3/1, 1/2, 1/3. Lanes 27–28 show the mobilities of lipids in the standard mixture containing, from the solvent front, cholesterol ester, egg yolk lecithin, sphingomyelin, lysolecithin. For the experimental procedure, see Methods.

which were developed in a solvent system of chloroform, methanol, water, and acetic acid (65:25:4:1). The plates were then sprayed with 50% sulfuric acid, heated to 220 °C for 45 min, and scanned with a photodensitometer and the individual lipids quantitated from the area under peaks compared with standards. In order to determine the specificity and accuracy of the chromatographic assay, different amounts of DML, egg yolk lecithin (EYL), or DML/EYL mixtures were subjected to quantitative thin-layer chromatography.

Results

Chromatographic Separation of DML and 18-Carbon Lecithins. In mixtures containing between 3/1 and 1/3 DML/EYL, DML had a slower migration than the EYL in the polar solvent employed, and the two lipids were clearly resolved, provided less than about 50 μg of total lipid was applied to the plate (Figure 1). The mobility of EYL was identical with the 18-carbon lecithins in the standard lipid mixture (Figure 1), and the HDL lecithins migrated as a single spot. For DML and EYL, the degree of charring of the lipid spots was proportional to the amount of spotted lipid (Figure 1), giving rise to a linear, accurate quantitation of each lipid.

Turbidity Studies. Turbid suspensions of DML multilamellar liposomes became clear when incubated with both apo-HDL or intact HDL, indicating dissolution of the liposomes. In a previous study (Tall and Small, 1977), it has been shown that this results at least in part from formation of smaller discoidal particles. The rate of clearing of turbidity was studied as a function of lipid/protein ratio as a guide to establishment of equilibrium conditions prior to centrifugation experiments, and so that centrifugation was performed on mixtures which did not contain a large excess of unreacted lecithin or HDL apoprotein. For a given lipid to protein ratio, the rate of clearing was faster for DML/apo-HDL mixtures, than for HDL–DML mixtures. Incubation of mixtures for longer than

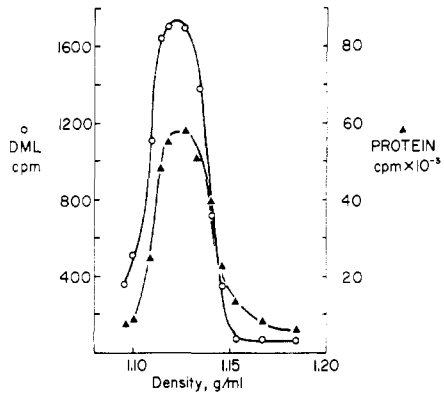

FIGURE 2: Isopycnic density gradient ultracentrifugation of an equilibrated mixture of [^{14}C]DML/[^{125}I]apo-HDL, containing 66/34 w/w DML/apo-HDL. Gradients of NaBr were generated with a peristaltic pump, using the density 1.006 g/mL mixture of DML/apo-HDL as limiting buffer. The gradients were centrifuged at 10 °C for 5.4 × 10$6g$ × h in a Beckman SW 56 swinging bucket rotor. The density of each fraction was determined gravimetrically.

18 h resulted in no further changes in turbidity suggesting that mixtures had attained equilibrium with respect to breakdown of multilamellar liposomes. Mixtures containing more than about 70/30 DML to HDL apoprotein remained partially turbid. However, the clearing point (the maximum ratio of DML/HDL apoprotein which gave rise to a clear solution during an 18-h incubation) varied from about 70/30 to 40/30 between different preparations of HDL. The capacity of HDL_3 to clear mixtures was consistently greater than that of HDL_2 for equivalent DML/total apoprotein ratios.

Preparative Ultracentrifugation. HDL_2/DML and HDL_3/DML mixtures equilibrated at 26 °C for 12–18 h were centrifuged at densities 1.063, 1.125, and 1.21 g/mL, and the top, middle, and bottom 2 mL of each tube examined for lipid and apoprotein content. Eighty percent of the [^{14}C]DML was distributed in the 1.063 to 1.125 g/mL density range, while 50% of the HDL_2 and 80% of the HDL_3 lipids were found, respectively, at density <1.063 g/mL or between *d* 1.063 to 1.125 g/mL. The density cuts containing the greatest ratio of DML/CE (*d* 1.063 to 1.125 g/mL for HDL_2 and *d* 1.125 g/mL for HDL_3) were also enriched in apo-A-1 compared with the other fractions. These experiments suggested incorporation of DML into DML/apo-A-1 recombinants (density 1.063 to 1.125 g/mL), associated with incorporation of HDL lipids into particles floating at lower density.

Equilibrium Density Gradient Ultracentrifugation. (a) DML/apo-HDL. Density gradient ultracentrifugation of an incubated mixture containing [^{14}C]DML and [^{125}I]apo-HDL in a 66/34 w/w ratio showed that all of the lipid and protein were incorporated into complexes of peak density 1.125 g/mL (Figure 2). Control DML liposomes formed a single peak at density 1.05–1.06 g/mL and control apo-HDL was all found at the bottom of the gradient (*d* >1.21 g/mL). In a series of [^{14}C]DML/[^{125}I]apo-HDL mixtures, the density and stoichiometry of complexes were shown to be determined by the ratio of reactants in the incubation mixture (Tall et al., 1977b).

(b) Control HDL_2 and HDL_3. Equilibrium density gradient ultracentrifugation did not lead to any major alteration in the density distribution of HDL_2 or HDL_3 (Figures 3a and 4a). The small amount of protein at the bottom of the gradient (Figure 3a) probably represents apoprotein liberated from the HDL_3 during centrifugation.

(c) [^{14}C]DML/HDL_3 and [^{14}C]DML/HDL_2. Density

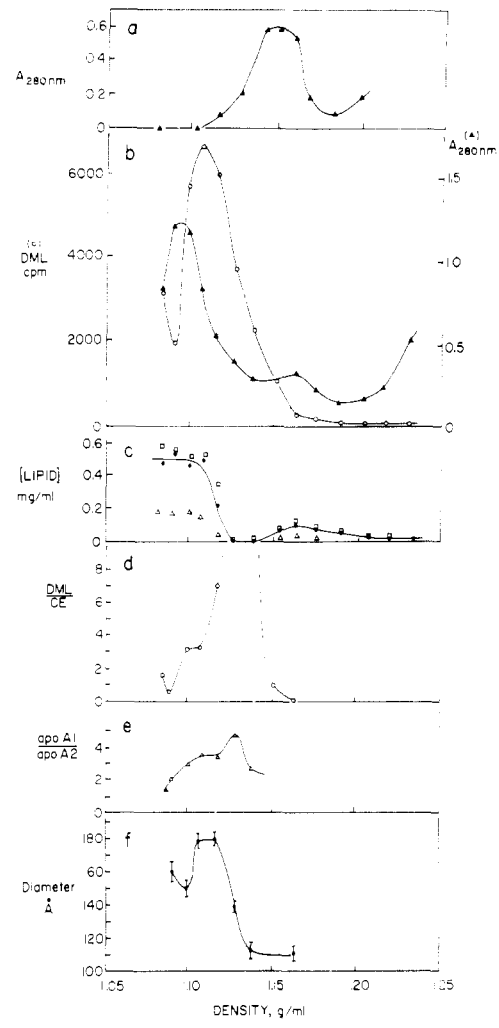

FIGURE 3: Isopycnic density gradient ultracentrifugation of (a) HDL_3 control and (b–f) DML/HDL_3 mixtures containing 60/40 DML/HDL_3. Experimental procedure was as described in the legend to Figure 2. (b) Shows the distribution of apoprotein and [^{14}C]DML. (c) Distribution of HDL lipids; (squares) 18-carbon phospholipids; (circles) cholesterol ester; (triangles) unesterified cholesterol. (d) Ratio of DML to CE as determined by quantitative thin-layer chromatography. (e) Ratios of apo-A-1 to apo-A-2. (f) The mean ± SEM diameter of circular particles (n = 100) as determined by negative stain electron microscopy. The DML peak represents DML/apoprotein complexes. HDL_3 polar and apolar lipids (c) are found primarily between densities 1.07 to 1.125 g/mL, indicating transformation of HDL_3 into less dense particles. The small peaks of apoprotein and HDL lipids at density 1.16 g/mL represent primarily unreacted HDL_3. The fractions containing the highest ratio of DML/CE also had the highest ratio of apo-A-1/apo-A-2. For each fraction, random areas from three to four electron micrographs from each of two grids were examined. The density gradients and lipid protein analyses were performed in duplicate. Each point is the mean of the duplicate analyses.

gradient ultracentrifugation was performed in duplicate on mixtures containing 60/40 DML/HDL_3 and 40/60 DML/HDL_2. These ratios were chosen because they were close to the DML/HDL apoprotein ratio of the clearing point of phospholipid turbidity. For both HDL_3 and HDL_2 a major portion of the [^{14}C]DML was incorporated into a complex of peak density 1.105 g/mL and 1.09 g/mL, respectively (Figures 3b and 4b). Part of the DML was also distributed into fractions of lower density, especially in the HDL_2 experiment, where the top two fractions of the gradient contained free DML, as indicated by turbidity. In the DML/HDL_3 experiments, all fractions were optically clear.

The distribution of the HDL lipids was determined following dialysis, chloroform/methanol extraction, and quantitative

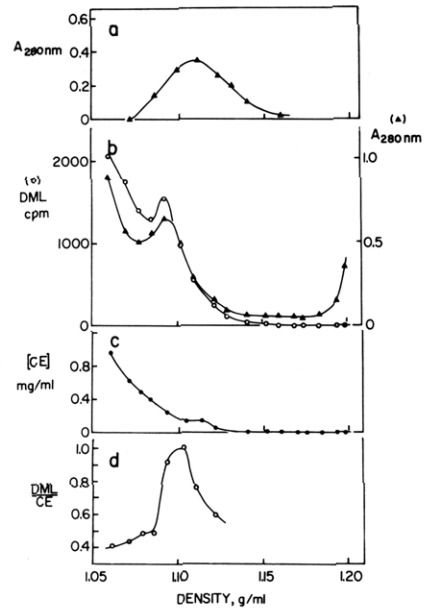

FIGURE 4: Isopycnic gradient ultracentrifugation of (a) HDL_2 control and (b–d) DML/HDL_2 mixture containing 40/60 w/w DML to HDL_2 apoprotein. (b) Shows the distribution of apoprotein and [^{14}C]DML. (c) Distribution of cholesterol ester. (d) Ratio of DML to cholesterol ester.

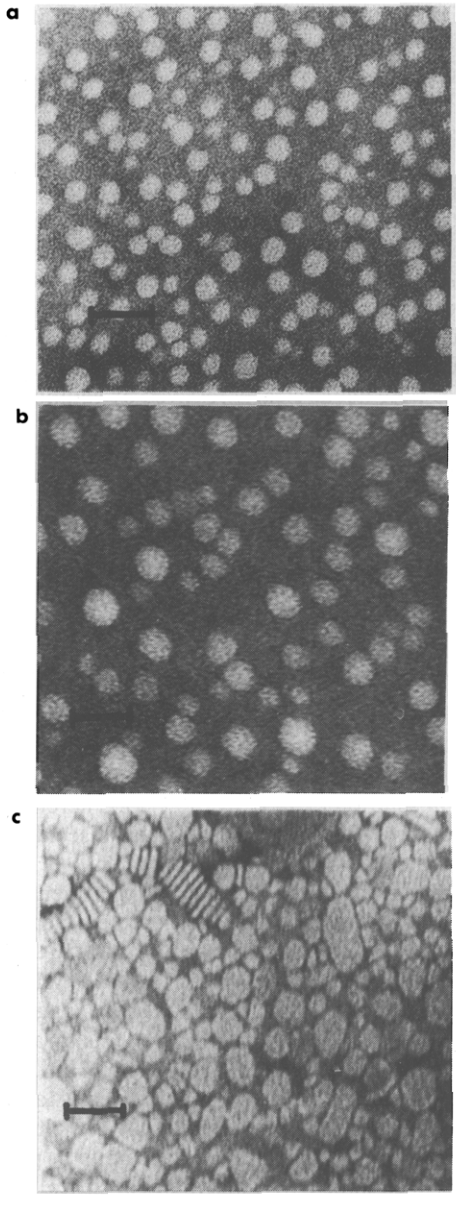

FIGURE 5: Electron micrographs, negatively stained with sodium phosphobungstate, of HDL_3/DML mixture. (a) Control HDL_3 preparation; (b) gradient fraction, d = 1.08 g/mL; (c) gradient fraction, d = 1.105 g/mL. The bars represent 350 Å.

thin-layer chromatography. The HDL_2 and the HDL_3 lipids were found in fractions of lower density than the control HDL preparations (Figures 3c and 4c). Since DML gave rise to a spot on the TLC plates that could be quantitated separately from that of the phospholipids of HDL)see Methods), the relative ratio of DML to HDL lecithins, cholesterol ester, cholesterol, sphingomyelin, lysolecithin, and triglyceride could be determined accurately for each fraction. Although there was considerable overlap in the distribution of DML and HDL lipids, especially in the HDL_2 experiments, there was a clear-cut maximum in the ratio of DML to cholesterol ester (Figures 3d and 4d), and in the HDL_3/DML mixtures some fractions contained pure lecithin/apoprotein complexes. The lipid composition of HDL remained constant in all fractions, indicating that there was no preferential distribution of any HDL lipid into fractions containing DML/apoprotein complexes (Figure 3c).

The HDL apoproteins were found associated with both the transformed HDL lipids and the DML peaks. The distribution of protein suggested from the ultraviolet absorbance at 280 nm was confirmed by Lowry protein analysis of dialyzed fractions. In the DML/HDL_3 experiment, there was a sharp rise in the ratio of apo-A-1 to apo-A-2 to about 5/1, which paralleled the ratio of DML/CE (Figure 4). Control HDL_3 showed an apo-A-1/apo-A-2 ratio of about 2.5/1. Thus, fractions containing pure apoprotein/DML complexes were enriched in apo-A-1, while those containing most of the HDL lipids were relatively enriched in apo-A-2. The distribution of C peptides was similar to apo-A-2.

Negative stain electron microscopy showed rouleaux of discs (180 × 55 Å) on edge in the top fractions of the DML peak (Figure 5c). The discs were of similar diameter to the circular particles observed in these micrographs and were identical in size to DML/apo-HDL recombinants containing a 2/1 lipid/protein ratio. Thus, the circular particles probably represent discs laying flat on the grid. Similar discoidal structures were not observed in fractions containing the bulk of the HDL lipids. These fractions contained spherical particles of mean diameters 150–160 Å (Figure 5b), which were increased in size compared with control HDL_3 (Figure 5a, mean ± SEM diameter = 91 ± 2.8 Å). Although the peaks of apoprotein and HDL lipid at d 1.16 g/mL probably represent primarily unreacted HDL_3, the slightly increased mean particle diameter (110 Å) may reflect the presence of small amounts of DML/apoprotein complex.

Discussion

We have previously inferred the presence of discoidal DML/apoprotein complexes in mixtures of DML liposomes with HDL_3 from altered calorimetric behavior of the DML and from the appearance of rouleaux of discs on edge by negative stain electron microscopy (Tall and Small, 1977). In the current investigation we have attempted to isolate apoprotein/DML complexes from these mixtures by equilibrium density ultracentrifugation. The evidence for the occurrence of complexes was the presence of a peak in the distribution of DML (at density 1.105 g/mL) that was associated with HDL apoprotein. There was some overlap in the distribution of DML and the HDL lipids, but there was a sharp maximum in the

ratio of DML to HDL lipids and some fractions contained pure DML/apoprotein complexes (Figure 3d). Examination of these fractions by negative stain electron microscopy demonstrated the presence of discoidal particles. Although the stacking of complexes in rouleaux is an artefact of EM preparation (Morrisett et al., 1974), this phenomenon readily permits recognition of the discoidal morphology, substantiated from x-ray scattering techniques (Atkinson et al., 1976). Although the circular particles (Figure 5c) probably represented discs laying flat on the grid, the presence of other types of particles in these fractions cannot be excluded. HDL_2 also formed complexes when incubated with DML liposomes but this process did not occur as readily as for HDL_3, and the complexes were not as clearly resolved by equilibrium density gradient ultracentrifugation.

The ability of HDL to give up part of its apo-A-1 to DML liposomes reflects the relatively loose association between apo-A-1 and the HDL particle. Thus, apo-A-1 can be readily removed from the other components of HDL by heating (Tall and Small, 1977), exposure to low concentrations of guanidinium hydrochloride (Nichols et al., 1976), or by flash evaporation (Nichols et al., 1972). Apo-HDL in HDL exchanges readily with ^{131}I-labeled apo-HDL in solution (Sodhi and Gould, 1967). Calorimetric studies indicate that apo-A-1 in HDL is only slightly more thermodynamically stable than free apo-A-1 in solution, while apo-A-1 in discoidal apo-A-1/DML complexes is considerably more stable than free apo-A-1 in solution (Tall et al., 1977a). Therefore, an equilibrium of apo-A-1 between HDL and discoidal apo-A-1/DML complexes would favor the latter.

Heat or guanidinium hydrochloride treatment of HDL resulted in fusion of the residual apoprotein-depleted HDL, producing spherical lipoproteins, 100 to 200 Å in diameter (Nichols et al., 1976). EM of mixtures of DML and HDL_3 showed a paucity of particles of HDL_3 size, suggesting transformation of HDL_3 into larger lipoproteins by an analogous fusion process. In the current investigation HDL_2 and HDL_3 were changed into particles of decreased density and increased size during the process of DML/apo-A-1 complex formation. It is likely that lipoprotein fusion accounts in part for this. However, since some DML was found in optically clear fractions containing the HDL lipids, there may have also been an exchange of DML for apoprotein at the lipoprotein surface resulting in an increased lipid to protein ratio and a decreased density of the particle.

Mechanisms of HDL transformation involving release of apoprotein, with subsequent uptake of phospholipid or fusion of remnant HDL particles, may operate in vivo. Infusion of egg yolk lecithin unilamellar vesicles into rats resulted in an HDL-mediated transformation of the vesicles into particles floating in the HDL density range (Krupp et al., 1976). intravenous administration of Intralipid (phospholipid–triglyceride emulsions) in rats resulted in accumulation of vesicular complexes containing lecithin, unesterified cholesterol, and protein in the LDL density range (Franklin et al., 1976). This process was accompanied by a depletion of HDL and a marked increase in the HDL lipid to protein ratio. It is possible that in these studies HDL gave up part of its apoprotein to the egg yolk lecithin vesicles, resulting in apoprotein phospholipid complexes. Finally, the transformation of HDL_3 into particles of density <1.063 g/mL suggests a possible mechanism for the metabolism of HDL, where small HDL particles are transformed into progressively larger particles as apoprotein is removed from HDL by interaction with other lipoproteins or cell membranes.

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