Incorporation of Phosphatidylcholine into Spherical and Discoidal Lipoproteins during Incubation of Egg Phosphatidylcholine Vesicles with Isolated High Density Lipoproteins or with Plasma*

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Incubation of phosphatidylcholine vesicles with isolated high density lipoproteins (HDL) or with whole plasma results in transfer of phospholipid into HDL. To investigate the mechanisms of this process, small unilamellar vesicles of egg phosphatidylcholine were incubated with human HDL (d 1.125 to 1.210 g/ml) or with whole plasma and were subsequently analyzed by density gradient ultracentrifugation and negative stain electron microscopy. Incubation of vesicles with isolated HDL resulted in formation of both discoidal lipoproteins (1.04 to 1.06 g/ml) and phospholipid-enriched spherical lipoproteins (1.10 to 1.14 g/ml). The lipid bilayer discoidal lipoproteins had dimensions ~22 × 5.4 nm and contained phospholipid, apoA-I, and apoA-II. The phospholipid-enriched spherical HDL were increased in diameter and decreased in density compared to HDL. With maximum phospholipid uptake these lipoproteins had a similar density, size, and lipid composition to HDL3, a phospholipid-rich subfraction isolated from human plasma between 1.100 to 1.125 g/ml. Scanning microcalorimetry of the phospholipid-enriched HDL showed, compared to HDL3, a decrease in the temperature and enthalpy of lipoprotein denaturation. This altered denaturation pattern was similar to that of plasma HDL3. Following incubation of vesicles in plasma, there was an increase in phospholipid-rich lipoproteins within the HDL density range (1.070 to 1.125 g/ml), and disappearance of lipoproteins from the HDL density range (1.125 to 1.210 g/ml). The phospholipid-rich HDL contained a major peak (d 1.095 to 1.125 g/ml), which consisted of only spherical particles and a smaller peak (d 1.070 to 1.095 g/ml), which included a population of lipid bilayer discs.

Thus, during incubation of vesicles with isolated HDL, there is insertion of phospholipid into HDL, producing spherical lipoproteins which have some properties in common with HDL3. Discoidal lipoproteins are also formed, probably as a result of interaction of vesicles with small amounts of apoA-I and apoA-II released from HDL3. The uptake of phosphatidylcholine by HDL in whole plasma occurs by similar mechanisms.

Human plasma high density lipoproteins (HDL) are spherical particles in which a core of apolar lipids (cholesterol esters and triglycerides) is surrounded by a surface layer of more polar molecules (phospholipids, unesterified cholesterol, and apoproteins) (1). The principal apoproteins of human HDL, apoA-I and apoA-II, can be recombined with phospholipids to produce discoidal particles (2-5). Discoidal lipoproteins are also formed during incubation of phospholipid liposomes or vesicles with holo-HDL (6-8). Incubation of multilamellar liposomes of dimyristoyl phosphatidylcholine with holo-HDL resulted in formation of disc of phospholipid and apoA-I, accompanied by decrease in density and increase in size of the spherical HDL. It was suggested that disc formation resulted from release of apoA-I from spherical HDL, while changes in the size and density of spherical HDL were due to lipoprotein fusion or to uptake of phospholipid (6, 7). The interaction of unilamellar vesicles of egg phosphatidylcholine with human (9) or bovine (10) HDL was also found to result in transfer of phospholipid mass into HDL. However, in both of those studies, electron microscopy of the HDL fraction showed only spherical particles, raising the possibility that the interaction of HDL with unsaturated phosphatidylcholine did not lead to formation of discoidal complexes. Incubation of unilamellar vesicles in plasma also causes dissolution of the vesicle structure and incorporation of phospholipid mass into HDL (9). However, the mechanisms of uptake of phosphatidylcholine by HDL in whole plasma have not been well defined.

In the present investigation, we have examined further the interaction of vesicles of egg phosphatidylcholine with human HDL3. Our aims were to re-examine the question of disc formation, to define the properties of the phospholipid-rich spherical HDL, and to compare these lipoproteins with the known subclasses of human HDL (11). Also, we have incubated egg phosphatidylcholine vesicles in whole plasma in order to quantify the uptake of phosphatidylcholine by HDL and to compare the mechanisms of phosphatidylcholine uptake with those found for isolated HDL3. The results indicate that the incorporation of egg phosphatidylcholine into HDL does, in fact, lead to formation of both discoidal lipoproteins and insertion of phosphatidylcholine into pre-existing HDL3. The phospholipid-enriched spherical HDL resemble HDL3, a phospholipid-rich subfraction isolated from normal human plasma.

**MATERIALS AND METHODS**

Blood was collected in tubes containing Na2EDTA from normal human plasma.

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1 The abbreviations used are: HDL, plasma high density lipoproteins; ApoA-I and apoA-II, the major apolipoproteins of human HDL; holo-HDL, intact HDL isolated by preparative ultracentrifugation between 1.063 to 1.21 g/ml; DTNB, dithionitrobenzoic acid; DSC, differential scanning calorimetry.
subjects after a 12- to 16-h overnight fast and the blood plasma was used for preparation of HDL or incubation with vesicles within 48 h. For preparation of HDL, plasma was raised to density 1.125 g/ml by addition of solid KBr, then centrifuged for 36 h in a Beckman 40.3 rotor at 39,000 rpm. The infranatant was raised to density 1.21 g/ml and centrifuged for 36 h and the supernatant HDL was removed and reconstituted with 1.21 g/ml NaBr. For preparation of NaBr density gradient ultracentrifugation, densities were chosen to create a shallow gradient between 1.100 to 1.125 g/ml.

Small unilamellar vesicles were prepared by the method of Huang (12). Egg yolk phosphatidylcholine, from Lipid Products, and [14C]-choline phosphatidylcholine, from New England Nuclear, were copurified by silicic acid chromatography. Aliquots containing 30 mg of lipid were lyophilized from cyclohexane, suspended in 0.15 M NaCl, 0.01 mM Na2EDTA, raised to pH 8.5 by addition of NaOH, then sonicated in a water-jacketed cell, maintained at 5-10°C, until the solution was clear (about 30 min). The solution was centrifuged at 10,000 × g for 30 min to remove particulate matter, then subjected to chromatography on Sepharose CL-4B column (2.4 × 40 cm). The eluate was monitored by liquid scintillation counting and fractions containing small vesicles were pooled from the top and descending limb of the included volume peak. Typically, about 15 mg of lipid were recovered following preparation of small vesicles.

Incubations and NaBr Density Gradient Ultracentrifugation—Incubations with HDL or vesicles were conducted for 4 h in the presence of 2 mM 5,5'-dithionitrobenzoic acid, at 37°C, under N2, in a shaking water bath. Following incubation the samples were concentrated to a volume of 0.8 ml by ultrafiltration (Amicon) using a PM-10 membrane and the density of the samples was raised by addition of solid NaBr. The samples were then analyzed by NaBr density gradient ultracentrifugation. The gradients were formed by layering successive NaBr density solutions of volumes 1.1, 1.0, 0.8, 1.1, and 1.1 ml in the middle of the fractionation tube. The densities of the solutions were chosen according to the region of interest. Generally, for analysis of material in HDL, the densities were 1.06, 1.08, 1.10, 1.14, and 1.18 g/ml; while for analysis of the region containing discoidal lipoproteins, the densities were 1.01, 1.04, 1.08, 1.11, and 1.14 g/ml. In experiments where HDL was prepared by density gradient ultracentrifugation, densities were chosen to create a shallow gradient in the 1.10 to 1.12 g/ml region, namely 1.08, 1.10, 1.11, 1.12, and 1.15 g/ml (11). After layering of the density solutions, the gradients were centrifuged for 60 h at 49,000 rpm in a Beckman SW 50.1 rotor. Fourteen fractions of equal volume were removed from the tubes by careful pipetting. The densities of individual fractions were determined directly from gradient fractions by refractometry, using an Abbott refractometer at 25°C. The densities obtained in this way were almost identical with those obtained on gradients run without samples. In some instances, the samples were also analyzed by chromatography or a column (1.25 × 120 cm) of cross-linked 6% agarose (Sepharose CL-4B).

Chemical Analyses—The protein concentration was determined directly on individual gradient fractions by the method of Lowry et al. (13), using bovine serum albumin as the standard. The optical densities of blank solutions were obtained from fractions of a control gradient-lacking sample, because the high salt content of these fractions was found to cause a very small increase in the optical density of the blank solution. For lipid analysis, portions of the gradient fractions were extracted by the method of Folch et al. (14). The phospholipid content was determined by the method of Bartlett (15) and total cholesterol by the method of Zlatkis and Zak (16). Cholesterol and phospholipid were separated by thin layer chromatography on hexane/diethyl ether/acetic acid/H2O. The lipid composition of pooled HDL subfractions obtained from density gradients was determined by quantitative thin layer chromatography (17, 18).

For analysis of apoproteins, samples were dialyzed against distilled water, pH 8.5, lyophilized, delipidated by addition of 5 to 10 ml of chloroform/methanol (2:1) and washed twice with saturated NaCl, then treated with 0.2 M Tris buffer, pH 7.2, containing 0.7 M decayl sulfate, quantitated by the method of Lowry et al. (13), and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 15% polyacrylamide. The gels were stained with Coomassie blue. The proteins were identified by comparison of their Rf values with purified human apo A-I or apo A-II. For analysis of the proteins of HDL, discos and phospholipid-enriched HDL, apo A-I was measured by quantitative immuno-electrophoresis as described previously (19).

Electron Microscopy—Prior to electron microscopy samples were dialyzed against 4 changes of distilled water, pH 8.5. Electron microscopy was performed on a Hitachi 11c electron microscope operated at 75 kV. Dilute samples (0.2 to 0.5 mg/ml lipoprotein) were applied to carbon-coated Formvar-Cu grids for approximately 1 min., then negatively stained with 2% NaC1, pH 6.8 for 25 s. Electron micrographs were obtained under observer-blinded condition, selecting areas where particles were not confluent. Electron micrographs were taken at magnification × 67,000. Particles were sized directly from randomly chosen areas of negatives of electron micrographs, employing a magnifying eye piece with a reticle.

Differential Scanning Calorimetry—DSC was performed on a Perkin-Elmer DSC-2, as described previously (4, 20). Prior to analysis, samples were concentrated by vacuum dialysis (final concentration ~100 to 200 mg/ml), then sealed hermetically in 75-μl stainless steel pans. Scanning calorimetry was also performed on lipoprotein samples using an adiabatic scanning calorimeter (Microcal-I, Amherst, MA). In these experiments, lipoproteins obtained by preparative or density gradient ultracentrifugation were used without further concentration and heated at 1°C/min.

Spectroscopic Experiments—Intrinsic protein fluorescence spectra of HDL were recorded with an Aminco-Bowman spectrophotofluorometer. Lipoprotein solutions containing 0.7 mg of protein/ml were excited at 280 nm and the emission spectra were recorded from 290 to 400 nm using 1-nm slit widths. Spectrophotometric titrations were performed by addition of 0.2 mM NaOH solution to HDL preparations containing 1 mg of protein/ml. The difference spectra were recorded with a Perkin-Elmer model 554 UV spectrophotometer.

RESULTS

Interaction of Phosphatidylcholine Vesicles with Isolated HDL—Egg Phosphatidylcholine vesicles were incubated with different ratios of HDL and the products were analyzed by NaBr density gradient ultracentrifugation (Fig. 1). Following incubation with vesicles, the HDL was reisolated as a single symmetrical peak. However, there was transfer of phospholipid radioactivity into HDL, an increase in phospholipid mass in the major HDL peak, and a shift of the HDL peak toward lower density. In these and other incubations it was found that with increasing vesicle/HDL ratio there was a progressive decrease in density of the top of the peak, tending toward a minimum value of 1.11 g/ml. The increase in HDL phospholipid mass reached a plateau of 0.25 mg of phosphatidylcholine/mg of HDL protein, between incubation ratios 1.0 to 1.5 mg of phosphatidylcholine/mg of HDL protein. At all ratios of vesicles/HDL, small amounts of protein were recovered along with phospholipid in the top fraction of the density gradient, indicating the presence of additional lipoprotein particles and/or unreacted vesicles.

To define in greater detail the particles found in the top gradient fractions of Fig. 1, lipoproteins were also analyzed by NaBr density gradient ultracentrifugation between 1.03 to 1.13 g/ml (Fig. 2). At all ratios of vesicles to HDL protein there was a major lipoprotein peak of density 1.04 to 1.06 g/ml, containing large amounts of phospholipid and small amounts of protein (Fig. 2). Control vesicles were almost entirely recovered in top fraction of the gradient (Fig. 2B), indicating that the 1.04 to 1.06 peak contained only lipoproteins and no unreacted vesicles.

Negative stain electron microscopy of the major HDL peak of density 1.10 to 1.15 g/ml (middle of gradients in Fig. 1, bottom of gradients in Fig. 2) showed a uniform population of spherical particles. Between incubation ratios 0 to 0.75, there was an increase in the mean diameter of the spherical HDL particles, from 8.0 ± 0.2 nm (n = 313) to 9.2 ± 0.3 nm (n = 250). There was no further increase in the diameter at higher vesicle/protein ratios. Negative stain electron microscopy of the particles isolated at density 1.04 to 1.06 g/ml (Fig. 2) showed a striking population of discoidal particle, forming many rouleaux of lipid bilayer discs stacked on edge (Fig. 3A).
Density gradient ultracentrifugation of egg phosphatidylcholine vesicles incubated with HDLs (1.125 to 1.21 g/ml), using weight ratios of vesicle phospholipid to HDL protein of 2.20 (A), 0.90 (B), 0.63 (C), 0.44 (D), 0.22 (E), and 0.44 (F). Unilamellar vesicles were incubated with HDL, containing 4 mg of protein for 4 h at 37°C, then subjected to NaBr density gradient ultracentrifugation between 1.07 to 1.18 g/ml for 60 h at 49,000 rpm in a Beckman SW 50.1 rotor. The concentrations of protein (A), phospholipid (B), total cholesterol (C), and phospholipid radioactivity (D) were determined in individual fractions of the gradient. In Panels A and B, the phosphate content of the top fraction was off scale. The densities of individual fractions from the gradient were determined by refractive index measurement.

The thickness of these particles, determined from the periodicity of the rouleaux, was that of a lipid bilayer (5.4 nm). The diameters ranged from 16.2 to 40.6 nm (mean ± S.E. = 22.4 ± 0.6 nm). Discoidal particles of similar dimensions were observed in these fractions at all ratios of vesicle phosphatidylcholine to HDL protein from 0.1 to 2.5. The discs were quite different in appearance from control vesicles subjected to density gradient ultracentrifugation (Fig. 3B).

Chemical analysis of the discoidal lipoproteins revealed that they contained, at all incubation ratios, 90% phospholipid and 10% protein, with only trace amounts of cholesterol (less than 2%) and no detectable triglycerides. The phospholipid specific activity of the discs increased with increasing vesicle/HDL ratio, approaching the specific activity of the unreacted vesicles at ratio 2.5 (Fig. 2). The decreased specific activity at lower ratios indicates some phospholipid exchange between discs and HDL. However, the phospholipid specific activity of discs was more than 10 times that of HDL at all ratios, indicating exchange of only a minor fraction of phospholipid molecules.

The apoproteins of the discoidal lipoproteins were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The only protein bands corresponded to apoA-I and apoA-II.

Fig. 1. Density gradient ultracentrifugation of egg phosphatidylcholine vesicles incubated with HDLs (1.125 to 1.21 g/ml), using weight ratios of vesicle phospholipid to HDL protein of 2.20 (A), 0.90 (B), 0.63 (C), 0.44 (D), 0.22 (E), and 0 (F).

Fig. 2. Density gradient ultracentrifugation (1.03 to 1.14 g/ml) of egg phosphatidylcholine vesicles incubated with HDLs, in a weight ratio of vesicle phospholipid to HDL protein of 0.3 (C), 1.4 (D), 1.8 (E), and 2.5 (F). (A) and (B) show, respectively, control HDL and control vesicles. The concentrations of protein (A), phospholipid (B), and phospholipid radioactivity (C) were determined in individual fractions of the gradient. Note that the y axis scales of Panels E and F are twice the relative values of Panels A to D.

Fig. 3. Negative stain electron micrographs of density gradient peaks. A, lipoproteins of density 1.04 to 1.06 g/ml (Fig. 2); B, control vesicles (Fig. 2); C, lipoproteins of density 1.07 to 1.085 g/ml (Fig. 6). Dialyzed, pooled fractions containing 0.2 to 0.5 mg/ml of lipoprotein were negatively stained with Na-phosphotungstate and examined with an Hitachi 11c electron microscope. Magnification of original micrographs was × 69,000. The bar markers indicate 100 nm.

Vesicle-HDL Interactions
ApoC and apoE peptides were absent on sodium dodecyl sulfate gels and could not be detected by immunodiffusion. When quantitated by rocket immunoassay, apoA-I was found to constitute 54% of the total protein of HDL₃ and 63% of the total protein of the discoidal lipoproteins (mean values from 3 samples obtained at incubation ratio 1.0). Since the discs lacked other proteins 37% of their protein was presumably apoA-II.

The density gradient fractions containing discs were also analyzed by chromatography on Sepharose CL-6B. The profile of phospholipid radioactivity showed that the discs eluted at a volume similar to unreacted vesicles (not shown). By contrast, the HDL peak from the density gradients, containing particles of spherical morphology, eluted at the same volume as control HDL. In further experiments, phosphatidylcholine vesicles (5 mg) were incubated with HDL₁ (5 mg of protein), then analyzed by Sepharose CL-6B chromatography. The elution profile of phospholipid radioactivity showed a major peak with an elution volume similar to small vesicles and a smaller peak in the HDL region, as reported previously (9). Electron microscopy showed that these peaks contained particles of discoidal and spherical morphology, respectively.

To compare the phospholipid uptake activity of individual subfractions of HDL, plasma HDL were obtained by preparative ultracentrifugation between densities 1.063 to 1.250 g/ml, then subjected to density gradient ultracentrifugation. The recoveries of total protein in different regions of the density gradient were 67% in HDL₁ (1.125 to 1.21 g/ml), 21% in HDL₂a (1.100 to 1.125 g/ml), and 12% in HDL₂b (1.063 to 1.100 g/ml). Individual gradient fractions were incubated with egg phosphatidylcholine vesicles (0.2 mg of vesicles/0.4 mg of protein). Following incubation, the amount of phospholipid transferred into HDL was determined by centrifugation at density 1.063 (Fig. 4). The greatest uptake of phospholipid mass per mg of HDL protein occurred in HDL₂b, particularly in the more dense fractions of the HDL₂ (fractions 11 and 12). This was also true of transfer of phospholipid radioactivity but in addition there was a secondary peak of activity in fractions 5 and 6 (density 1.12 to 1.13), suggesting greater capacity for phospholipid exchange in these fractions. Since the greatest increment of phospholipid mass per mg of protein occurred in HDL₂b, and since HDL₂b represented the major part of the total HDL protein, these results show that the major capacity for HDL uptake in the whole HDL resided in HDL₂b.

The phospholipid/protein ratios of the HDL fractions before and after incubation with the vesicles are shown in the bottom panel of Fig. 4. For the fractions of HDL₂ representing most of the lipoprotein mass (fractions 6 to 9) the phospholipid/protein ratio following the incubation was similar to that of fraction 5 (density 1.12) prior to incubation, indicating formation of a particle with a phospholipid/protein ratio similar to HDL₂a.

**Interaction of Phosphatidylcholine Vesicles with Plasma**—In further studies we examined the uptake of phosphatidylcholine by HDL in whole plasma. To study the time course of incorporation of phospholipid into HDL, vesicles containing 0.3 mg of phospholipid were incubated with 0.1, 0.2, or 0.4 ml fresh human plasma, in the presence of the lecithin:cholesterol acyltransferase inhibitor, diethionitrobenzoic acid (DTNB). Subsequent analysis by preparative ultracentrifugation showed a time-dependent transfer of phospholipid mass into density > 1.063 g/ml (Fig. 5A). In the presence of 0.1 or 0.2 ml of plasma, a plateau of phospholipid mass transfer was attained between 2 to 4 h of incubation, while in the presence of 0.4 ml of plasma there was continuing transfer between 2 and 4 h. For the 0.1, 0.2, and 0.4 ml incubations, the 4-h time points represent, respectively, transfer of 0.90, 1.10, and 1.21 g/ml. The density of the individual fractions containing discs was 1.063 g/ml, then subjected to NaBr density gradient ultracentrifugation between 1.07 to 1.25 g/ml. The density of the individual HDL fractions is shown in the top panel. Aliquots of each fraction containing 0.4 mg of protein were incubated with 0.2 mg of egg phosphatidylcholine vesicles for 4 h at 37°C, then centrifuged for 20 h at 40,000 rpm in a Beckman 40.3 rotor. The transfer into d > 1.063 g/ml of vesicle phospholipid radioactivity and mass are shown in the middle panels. The phospholipid/protein ratios of the HDL fractions before and after incubation with vesicles are shown in the bottom panel.

**Fig. 4.** Transfer of phospholipid mass and radioactivity into d > 1.063 g/ml as a result of incubation of egg phosphatidylcholine (PC) vesicles with fractions of plasma HDL. Human plasma HDL was isolated by preparative ultracentrifugation between 1.063 to 1.25 g/ml, then subjected to NaBr density gradient ultracentrifugation between 1.07 to 1.25 g/ml. The density of the individual HDL fractions is shown in the top panel. Aliquots of each fraction containing 0.4 mg of protein were incubated with 0.2 mg of egg phosphatidylcholine vesicles for 4 h at 37°C, then centrifuged for 20 h at 40,000 rpm in a Beckman 40.3 rotor. The transfer into d > 1.063 g/ml of vesicle phospholipid radioactivity and mass are shown in the middle panels. The phospholipid/protein ratios of the HDL fractions before and after incubation with vesicles are shown in the bottom panel.

**Fig. 5.** Time course of transfer of phospholipid mass (A) and radioactivity (B) into the d > 1.063 g/ml fraction of plasma during incubation of 0.3 mg of unilamellar egg phosphatidylcholine (PC) vesicles with 0.1, 0.2, or 0.4 ml of human plasma. Values shown are mean ± S.E. values for separate incubations performed at each time point on 5 different occasions. Following incubation, samples were chilled on ice, raised to d 1.063 g/ml by addition of solid KBr, then centrifuged for 20 h in a Beckman 40.3 rotor. Material recovered in the bottom 4 ml of the centrifuge tubes was considered to be transferred into the d > 1.063 g/ml fraction. In these experiments, fresh fasting plasma from the same subject was used.
0.89, and 0.68 mg of phospholipid/ml of plasma. The results obtained with 0.1 and 0.2 ml of plasma, where excess vesicles remained at the end of the incubation, indicate a maximum transfer of 0.9 mg of phosphatidylcholine into HDL/ml of plasma. The transfer of phosphatidylcholine mass into HDL (Fig. 5A) was paralleled by transfer of phosphatidylcholine radioactivity (Fig. 5B). At the higher ratios of vesicles to plasma (0.1 and 0.2 ml incubation), there was identical percentage transfer of phosphatidylcholine radioactivity and mass into HDL. Thus, under the conditions of these experiments, transfer of phosphatidylcholine radioactivity into HDL is an accurate indicator of phosphatidylcholine mass transfer. For the 0.4-ml incubation, there was slightly greater transfer of phospholipid mass than radioactivity at the 4-h time point, but the difference was not statistically significant.

To assess variability between different individuals, vesicles containing 0.3 mg of phospholipid were incubated with 0.1 ml of plasma from 4 normal subjects (2 males and 2 females). At all time points, these subjects showed almost identical transfer of phospholipid radioactivity into density > 1.063, indicating little individual variation.

To define the changes in HDL resulting from maximum uptake of phosphatidylcholine, 2.5 ml of fasting plasma from each of 6 normal subjects (4 males, 2 females) were incubated for 4 h with vesicles containing 2.5 mg of phosphatidylcholine and subsequently analyzed by density gradient ultracentrifugation between 1.06 to 1.18 g/ml. Since similar results were obtained in all subjects, the gradients from one subject are shown before (Fig. 6A) and after (Fig. 6B) incubation with the vesicles. In fasting plasma, the top of the major peak of HDL occurred at density 1.13 to 1.14 g/ml, corresponding to HDL{sub}a. Following incubation of plasma with phosphatidylcholine vesicles there was a striking redistribution of lipoprotein components: the top of the major peak of HDL occurred at density ~1.11 g/ml, a shoulder became more prominent at density ~1.07 to 1.09 g/ml, and there was loss of some lipoprotein mass from the HDL{sub}a density range (Fig. 6B). A marked increase in phospholipid concentration (Fig. 6, open circles), and radioactivity occurred in the major HDL peak and in its shoulder. In all subjects there were two distinct peaks of phospholipid specific radioactivity of density ~1.11 g/ml, and ~1.08 g/ml (Fig. 6B, squares). These results suggest the formation of at least two major classes of phospholipid-rich lipoproteins as a result of incorporation of phosphatidylcholine into HDL.

Negative stain electron microscopy was performed on density gradient fractions. Prior to incubation with the vesicles, both regions contained a uniform population of spherical particles, of diameters 8.6 ± 1.5 nm (1.095 to 1.125) and 10.4 ± 0.3 nm (1.070 to 1.095). Following incubation with the vesicles, the 1.095 to 1.125 fraction still contained a uniform population of spherical particles (8.7 ± 0.2 nm). By contrast, the 1.070 to 1.095 g/ml region contained a new population of particles with the morphology of lipid bilayer discs, tending the form rosettes of discs stacked on edge (Fig. 3C). The periodicity of the rosettes was 5.4 nm indicating the particles had the thickness of a lipid bilayer. Their diameters ranged from 14 to 25 nm (mean ± S.E. 18.6 ± 0.5 nm). Although this fraction contained a few larger particles of circular morphology ranging in size up to 39 nm, the median size of the background spherical lipoproteins (10.4 ± 0.2 nm) was unchanged from the control lipoproteins of similar density. When DTNB was omitted from the incubation, no discoidal particles were seen, suggesting that they had been converted into spherical lipoproteins as a result of the action of lecithin:cholesterol acyltransferase.

The transfer of phosphatidylcholine into HDL (Fig. 5A) was paralleled by transfer of phosphatidylcholine radioactivity (Fig. 5B). At the higher ratios of vesicles to plasma (0.1 and 0.2 ml incubation), there was identical percentage transfer of phosphatidylcholine radioactivity and mass into HDL. Thus, under the conditions of these experiments, transfer of phosphatidylcholine radioactivity into HDL is an accurate indicator of phosphatidylcholine mass transfer. For the 0.4-ml incubation, there was slightly greater transfer of phospholipid mass than radioactivity at the 4-h time point, but the difference was not statistically significant.

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Negative stain electron microscopy was performed on density gradient fractions. Prior to incubation with the vesicles, both regions contained a uniform population of spherical particles, of diameters 8.6 ± 1.5 nm (1.095 to 1.125) and 10.4 ± 0.3 nm (1.070 to 1.095). Following incubation with the vesicles, the 1.095 to 1.125 fraction still contained a uniform population of spherical particles (8.7 ± 0.2 nm). By contrast, the 1.070 to 1.095 g/ml region contained a new population of particles with the morphology of lipid bilayer discs, tending the form rosettes of discs stacked on edge (Fig. 3C). The periodicity of the rosettes was 5.4 nm indicating the particles had the thickness of a lipid bilayer. Their diameters ranged from 14 to 25 nm (mean ± S.E. 18.6 ± 0.5 nm). Although this fraction contained a few larger particles of circular morphology ranging in size up to 39 nm, the median size of the background spherical lipoproteins (10.4 ± 0.2 nm) was unchanged from the control lipoproteins of similar density. When DTNB was omitted from the incubation, no discoidal particles were seen, suggesting that they had been converted into spherical lipoproteins as a result of the action of lecithin:cholesterol acyltransferase.
Effects of Centrifugation on Phospholipid Uptake by HDL

In experiments performed with isolated HDL3, the maximum phospholipid uptake was 0.25 mg/mg of HDL protein. By contrast, incubation of vesicles in whole plasma resulted in a maximum incorporation of phosphatidylcholine into 

\[ d > 1.063 \text{ g/ml} \]

ranging from 0.7 to 0.9 mg/ml of plasma. In the plasma experiments, the average HDL protein concentration was determined by preparative ultracentrifugation to be 1.4 mg/ml. Plasma was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the average HDL protein concentration in this range. The results obtained from the experiments were as follows:

- At low HDL protein concentrations, the ability to incorporate phospholipid radioactivity into HDL was reduced in proportion to the period of ultracentrifugation (Fig. 7). With 57 h of preparative ultracentrifugation, the HDL top had about 40% of the capacity of whole plasma for phosphatidylcholine transfer activity, in agreement with the results obtained by comparison of activities of ultracentrifugally isolated HDL3 and whole plasma (39% of capacity, see above).

- To examine the possibility that plasma might restore to HDL the phosphatidylcholine transfer activity lost during ultracentrifugation, whole plasma was added to isolated HDL and the ability to incorporate phospholipid radioactivity into 

\[ d > 1.063 \text{ g/ml} \]

was measured. These experiments showed that at low vesicle/HDL ratios, plasma plus HDL had simply additive activities of phospholipid transfer. However, at high ratios, the phospholipid transfer caused by 

\[ d > 1.063 \text{ g/ml} \]

plus HDL was greater than the sum of plasma alone.
distinct from the earlier low density and very low density lipoprotein peaks, but there was considerable overlap with the plasma proteins, shown by measurement of absorbance at 280 nm (Fig. 8, closed circles). The capacity of each column fraction to transfer vesicle phospholipid radioactivity into density > 1.063 was measured. Incubations contained either a constant volume from each fraction (Fig. 8A), or a constant cholesterol content (Fig. 8B). In both instances the capacity for transfer of phosphatidylcholine radioactivity (open circles) and mass (triangles) into d > 1.063 was largely associated with the HDL peak. There was a tendency for phosphatidylcholine transfer to be greater in fractions eluting after the HDL peak with greatest cholesterol content, suggesting greater phospholipid uptake capacity in the smaller HDL particles. The total activity for transfer of phosphatidylcholine radioactivity and mass into d > 1.063 g/ml, obtained by summing activities of individual fractions under the HDL peak, was similar to that of the equivalent volume of whole plasma. For example, in Fig. 8B, total mass of phosphatidylcholine transferred into d > 1.063 was equivalent to 1.3 mg of phosphatidylcholine/ml of plasma, slightly greater than the value of 0.9 mg of phosphatidylcholine/ml of plasma obtained by incubating vesicles in whole plasma. Thus, in contrast to the effects of ultracentrifugation, isolation of HDL by column chromatography did not result in loss of phosphatidylcholine uptake capacity.

Comparison of Phospholipid-enriched HDLα with HDLβ—Anderson and co-workers (11) have shown that HDLα contains a subfraction, designated HDLα2, which is enriched in phospholipid and which has a density (1.100 to 1.255 g/ml) and size (9.1 nm) similar to the HDL produced by incubation of HDLα with phosphatidylcholine vesicles. In order to compare HDLα with the phospholipid-enriched HDL produced in vitro, human HDL was fractionated by density gradient ultracentrifugation using a shallow gradient (1.08 to 1.15 g/ml) to maximize resolution of HDLα subclasses (11). In fasting plasma, HDLα2 was isolated as a shoulder on the HDLα peak. The compositions of HDL present in the different density regions of fasting plasma are shown in Table II. HDLα2 were considerably enriched in phospholipids compared to HDLα, resulting in a high phospholipid/cholesterol ester ratio (Table II). The phospholipid/cholesterol ester ratio of HDLα2 was significantly greater than that of HDLα (p < 0.001). The composition of HDLβ was, in fact, similar to that of phosphatidylcholine-enriched spherical HDL obtained at maximum phospholipid uptake (phosphatidylcholine/HDL incubation ratio 1.0). The latter lipoproteins had 51% protein, 1% cholesterol, 1% triglyceride, 15% cholesterol ester, and 32% phospholipid.

In order to define changes in lipoprotein stability associated with uptake of phosphatidylcholine, the phosphatidylcholine-enriched spherical HDL were examined by scanning calorimetry and compared to HDLα and HDLβ. In the initial studies, lipoproteins were examined at concentration of 50–100 mg of protein/ml and heating rates of 5°C/min using a DSC-2 (Perkin-Elmer). The later studies employed an adiabatic scanning microcalorimeter (Microcal-I), using lipoprotein concentrations of 3 to 10 mg of protein/ml and heating rates of only 1°C/min. Although the scanning microcalorimeter gave much greater base-line stability (Fig. 9), the thermodynamic parameters of lipoprotein denaturation were similar with both instruments and have been grouped in Table III.

On heating HDLα (1.125 to 1.160 g/ml) there was an irreversible, double-peaked denaturation (Fig. 9A), similar to that observed previously (21). However, using the Microcal-I the lipoprotein denaturation was associated with an upward step in the base-line, showing an increased heat capacity of the heat-denatured sample (change in heat capacity ~0.049 cal/g of protein/°C). When the heat-denatured HDLα was re-heated from 10–106°C, two reversible transitions were present (Fig. 9B). The transition between 15–30°C, absent from the intact HDL, is due to liquid crystalline transitions of released cholesterol esters (21) and the transition present between 41–68°C is due to a reversible conformational change of apoA-I (20).

Table II

<table>
<thead>
<tr>
<th>Percentage composition of subfractions of human HDL</th>
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<tr>
<td>Isolated from fasting plasma by preparative ultracentrifugation followed by density gradient ultracentrifugation (11). Protein was determined by the method of Lowry et al. (13) and lipids by quantitative thin layer chromatography (17). Values shown are mean ± S.E. for 4 different donors (two males, two females).</td>
<td></td>
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<tr>
<td>Pro*</td>
<td>Chol.</td>
</tr>
<tr>
<td>HDLα (1.063-1.100 g/ml)</td>
<td>37 ± 3.6</td>
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<tr>
<td>HDLα (1.100-1.125 g/ml)</td>
<td>48 ± 4.3</td>
</tr>
<tr>
<td>HDLα (1.125-1.160)</td>
<td>61 ± 2.3</td>
</tr>
<tr>
<td>HDLα (1.160-1.21 g/ml)</td>
<td>78 ± 3.4</td>
</tr>
</tbody>
</table>

* The abbreviations used are: Pro, protein; Chol, cholesterol; TG, triglyceride; chol. ester, cholesterol esters; lyso, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine.
* ND, not detected.
HDL₃ was also denatured with a double-peaked endotherm (Fig. 7C) and showed new transitions of cholesterol esters and apoA-I following denaturation (not shown). In samples from 5 different donors, the second component of the denaturation endotherm of HDL₃ was at lower temperature than in HDL₂. On the average, the peak temperature of the second component (T₂) was 10°C lower than in HDL₂ (p < 0.001), while the first component (T₁) was at the same temperature (76°C) (Table III). In addition, the total enthalpy of lipoprotein denaturation, expressed in calories per g of lipoprotein, was significantly lower (p < 0.001) in HDL₃ compared to HDL₂ (Table III). The enthalpy of denaturation of HDL₃ was lower than in HDL₂ when expressed as calories per g of protein (Table II), but this did not quite reach statistical significance (p = 0.10). The denaturation of HDL₂ was associated with an increase in heat capacity of the sample of about 0.046 cal/g of protein/°C. The more dense subfractions of HDL₃ (1.16 g/ml, respectively, dialyzed against 0.15 NaCl, degassed and examined without further concentration. Phospholipid-enriched HDL was prepared by incubation with vesicles (incubation ratio = 1.67 to 1.21 g/ml) were even more heat-stable than HDL₃ of 1.12 to 1.16 g/ml. Analysis of 2 samples with the DSC-2 showed that the second denaturation peak had a temperature of 111°C.

Following phospholipid-enrichment, the thermal denaturation of HDL₂ resembled that of HDL₃ (Fig. 5D). In 7 experiments, the mean temperature of the second component of denaturation was decreased from 98 to 85°C (p < 0.001). Table III and the enthalpy of denaturation was decreased from 1.22 cal/g of lipoprotein to 0.81 cal/g of lipoprotein (p < 0.001) or from 2.08 to 1.83 cal/g of protein (not significant, p = 0.20). Although the mean temperature of the second component of lipoprotein denaturation was slightly lower for phospholipid-enriched HDL₂ compared to HDL₃, these differences were not statistically significant (Table II). Denaturation of phospholipid-enriched HDL was associated with an increase in heat capacity of about 0.066 cal/g of protein/°C.

To assess potential conformational changes of HDL apoproteins associated with uptake of phosphatidylcholine, the phospholipid-enriched HDL were examined by spectrophotometric titration between pH 8 and 12. Ionization of tyrosine residues at higher pH values resulted in development of a marked increase in absorption in the ultraviolet region, with a maximum difference spectrum developing at 295 nm. Both HDL₂ and phospholipid-enriched HDL displayed reversible titration. Between pH 8 to 12, the change in absorbance at 295 nm was identical for HDL₂ and phospholipid-enriched HDL₂. In further experiments, HDL₃, HDL₄, and phospholipid-enriched spherical HDL were found to give rise to similar intrinsic protein fluorescence emission spectra, all with maxima at 332 nm. Based on total protein content (13), the intensity of fluorescence of phosphatidylcholine-enriched HDL (71%) was decreased relative to HDL₃ (100%) and HDL₄ (110%).

**DISCUSSION**

In earlier studies, incubation of egg phosphatidylcholine vesicles with human HDL was shown to result in partial transfer of phosphatidylcholine into particles of density and size similar to HDL (9). Some phospholipid was also found to be associated with apoprotein, eluting in the same region as small vesicles on agarose columns. This suggested that HDL protein had been adsorbed to vesicles (9). In the present study, we have shown that discoidal particles of constant phospholipid/protein ratio and density are formed at all ratios of vesicles to HDL. Because of their large diameter, these lipoproteins elute at a similar volume to small vesicles when examined by agarose chromatography. Thus, the interaction of egg phosphatidylcholine vesicles with human HDL is similar to that of dimyristoyl phosphatidylcholine in that discoidal lipoproteins are a major product (6-8). However, in contrast to the results obtained with saturated phospholipids (8), lipid-free apoA-I was not recovered from incubations conducted at low phosphatidylcholine/HDL ratio (Fig. 1).

The discoidal lipoproteins were probably formed as a result of increasing the density of the particles by the addition of phosphatidylcholine vesicles.

**TABLE III**

<table>
<thead>
<tr>
<th>Thermodynamic parameters of lipoprotein denaturation</th>
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<td>Mean ± S.E. values determined by scanning calorimetry using DSC-2 (n = 3 or 4) or Microcal-I instruments (n = 3). Using the DSC-2 or Microcal-I, the mean values were, respectively, HDL₂ ΔH = 1.3 or 1.2 cal/g, T₁ = 75 or 77°C, T₂ = 99 or 95°C; HDL₃, ΔH = 0.73 or 0.86 cal/g, T₁ = 76 or 76°C, T₂ = 88 or 88°C; HDL₄ + PC, ΔH = 0.84 or 0.89 cal/g, T₁ = 77 or 74°C, T₂ = 85 or 83°C.</td>
</tr>
<tr>
<td>Enthalpy*</td>
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<tr>
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<tr>
<td>cal/g lipoprotein</td>
</tr>
<tr>
<td>HDL₂₃</td>
</tr>
<tr>
<td>HDL₃₄</td>
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<tr>
<td>HDL₄ + phosphatidylcholine</td>
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* Determined from area enclosed by thermogram and extrapolated base-line (dashed line). The base-line extrapolation was made on the assumption that the increased heat capacity of the heat-denatured sample was largely due to release of apoA-I, associated with the first component of the endotherm (21). Similar enthalpy values were obtained when the base-line was drawn without a step from the beginning to the end of the denaturation endotherm.
of interaction of phosphatidylcholine vesicles with small amounts of apoA-I and apoA-II released from HDL. In view of the high phospholipid specific activity of discs relative to HDL (Fig. 2), it seems unlikely that vesicles first combine with HDL and then give rise to discs. The interaction of discs was accompanied by generation of phospholipid-enriched spherical HDL particles. The increase in size and decrease in density of the latter, relative to HDL, suggest they were formed as a result of insertion of phosphatidylcholine into pre-existing HDL. The simultaneous formation of discs and phospholipid-enriched spherical HDL particles suggests there may have been substitution of phosphatidylcholine molecules for apoA-I or apoA-II in the HDL surface. Alternatively, uptake of phosphatidylcholine by spherical HDL may involve flexibility in the HDL surface that allows insertion of phosphatidylcholine without displacement of protein (10). Such flexibility could result from the conformational adaptability of apoA-I and apoA-II (20, 23, 24). However, in the present study, the spectroscopic results suggest similar solvent accessibility of tryptophan and tyrosine residues in HDL and phosphatidylcholine-enriched HDL. Although not conclusive, this seems inconsistent with a major apoprotein conformational change resulting from phosphatidylcholine uptake.

We have quantitated the uptake of phosphatidylcholine by HDL in whole plasma and examined the mechanisms of this process. In experiments where the vesicle/plasma ratio was varied, with either the amount of vesicles constant, or the amount of plasma constant, there was maximum transfer into HDL of, respectively, 0.9 or 0.7 mg of phosphatidylcholine/ml of plasma. The identical transfer of phosphatidylcholine radioactivity into HDL noted in different individuals, probably reflects the fact that phosphatidylcholine uptake is largely due to an interaction of vesicles with HDL, and that different individuals have similar amounts of HDL (11).

The phosphatidylcholine mass and radioactivity transferred into HDL was recovered largely in the HDL density range (1.070 to 1.125 g/ml), causing increments in two partially resolved lipoprotein components: a major peak of density approximately 1.095 to 1.125 g/ml and a smaller peak or shoulder at 1.070 to 1.095 g/ml (Fig. 6). The peaks represented two distinct lipoprotein products since they contained separate peaks of phosphatidylcholine specific radioactivity as well as particles of different morphology.

The increment in HDL lipoproteins was accompanied by disappearance of lipoprotein mass from d > 1.125 g/ml (Table I), indicating that the HDL were formed as a result of interaction of vesicle phosphatidylcholine with HDL. A dominant role of HDL in the uptake of phosphatidylcholine from vesicles was also suggested by finding that fractions within HDL had the greatest capacity for incorporation of phosphatidylcholine into lipoproteins of density > 1.063 g/ml (Fig. 4). The transfer of HDL protein and cholesterol esters into spherical particles within the HDL density range suggests that these phospholipid-rich lipoproteins were formed as a result of insertion of phosphatidylcholine into HDL. The discoidal particles within the HDL density range were probably formed as a result of displacement of apoprotein from HDL during uptake of phosphatidylcholine. In these respects, the interaction of phosphatidylcholine vesicles with HDL in plasma is similar to their interaction with isolated HDL.

The products of interaction of vesicles with isolated HDL were different from those formed as a result of interaction with HDL in whole plasma in two respects. First, the maximum amount of phosphatidylcholine incorporated into d > 1.063 g/ml was smaller for isolated HDL compared to whole plasma, and second, the discs were of greater diameter and were isolated at lower density following incubation with isolated HDL. The reduced capacity of isolated HDL to incorporate phosphatidylcholine into d > 1.063 g/ml was partly due to the fact that the discs were isolated at 1.04 to 1.06 g/ml. However, even the amount of phosphatidylcholine incorporated into the spherical lipoproteins of 1.100 to 1.125 g/ml was greater during incubation in plasma (0.4 mg of phosphatidylcholine/ml of HDL protein), compared to isolated HDL (0.2 mg/mg of HDL protein). The loss of phosphatidylcholine uptake capacity of isolated HDL was due to an effect of ultracentrifugation, since this effect depended on the period of ultracentrifugal preparation of HDL (Fig. 7) and since HDL isolated by column chromatography was at least as active in phosphatidylcholine uptake as whole plasma (Fig. 8). Ultracentrifugation causes loss of apoA-I from HDL (25). Decreased availability of apoA-I within HDL for exchange with vesicle phospholipids could result in diminished uptake of phosphatidylcholine by HDL, and would also account for the formation of larger, less dense discs of higher lipid/protein ratio (4). An alternative explanation for these findings is that phosphatidylcholine transfer into HDL is promoted by a plasma protein that is separated from HDL during ultracentrifugation.

With maximum phosphatidylcholine uptake, the phospholipid-enriched spherical HDL particles had similar size, density, lipid/protein ratio, and lipid composition to HDL. An analogy between HDL and phosphatidylcholine-enriched HDL was further suggested by their identical thermodynamic parameters of denaturation (Table III). These similarities suggest that physiological HDL formation might involve transfer of phosphatidylcholine into HDL, such as occurs during lipolysis of chylomicrons (26, 27). HDL is markedly increased and HDL is decreased in amount following administration of heparin to hypertriglyceridemic subjects (28), and also following ingestion of a fatty meal in normal subjects. Thus, HDL may arise from the interaction of chylomicron or VLDL surface constituents with HDL. The composition of particles formed during in vivo uptake of phosphatidylcholine by HDL would presumably also reflect apoprotein transfer, cholesterol uptake and the action of enzymes such as lecithin:cholesterol acyltransferase.

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REFERENCES

2 Unpublished results.