Interaction of the high density lipoprotein conversion factor with recombinant discoidal complexes of egg phosphatidylcholine, free cholesterol, and apolipoprotein A-I

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Abstract An HDL conversion factor which promotes the conversion of HDL_3 to populations of larger and smaller particles has recently been identified in human plasma. In the present report a partially purified preparation of this factor has been used to examine the conversion of 79:0:1, 77:5:1, and 74:10:1 (mol:mol:mol) egg phosphatidylcholine–free cholesterol–apolipoprotein A-I (apoA-I) recombinant discoidal complexes. The study was carried out in order to ascertain whether the conversion process is regulated by the concentration of free cholesterol in the complexes. The complexes comprised one major and two minor populations of particles with respective Stokes' diameters of 96 Å, 84 Å, and 78 Å. The 74:10:1 complexes also contained a population of particles 112 Å in diameter. The 79:0:1 and 77:5:1 complexes contained two molecules of apoA-I per particle. The 74:10:1 complexes comprised two classes of particles with two or three molecules of apoA-I. When the 74:10:1 complexes were incubated with the conversion factor, the 96 Å and 84 Å particles were converted to a population of particles 78 Å in diameter that contained two apoA-I molecules. In the case of the 79:0:1 and 77:5:1 complexes, the 96 Å particles were converted to 78 Å particles but the concentration of 84 Å particles did not change. The rate of conversion of 96 Å particles to 78 Å particles was dependent on the concentration of free cholesterol in the complexes. When the 74:10:1 complexes were incubated for 24 hr with the conversion factor, the 96 Å particles were completely converted to particles 78 Å in diameter. In the case of the 77:5:1 complexes, complete conversion was achieved by 48 hr. Conversion of the 79:0:1 complexes did not proceed to completion, even when the incubation was extended beyond 48 hr. The rate of conversion of 96 Å particles to 78 Å particles was also dependent on the concentration of the conversion factor in the incubation mixtures. The previous incubations contained equivalent concentrations of apoA-I and conversion factor. When the concentration of the conversion factor relative to apoA-I was reduced, there was a concomitant decrease in the rate of conversion of 96 Å particles to 78 Å particles. Conversion was not evident when the concentration of the conversion factor was reduced to one-tenth that of apoA-I. It is concluded that *1*) egg phosphatidylcholine–free cholesterol–apoA-I discoidal complexes contain 96 Å particles that are converted to smaller particles 78 Å in diameter during incubation with the conversion factor; *2*) the rate of conversion is enhanced when the concentration of free cholesterol in the complexes is increased; *3*) the rate of conversion is dependent on the concentration of the conversion factor in the incubation mixtures; and *4*) conversion does not promote the redistribution of apolipoproteins. —**Rye, K-A.** Interaction of the high density lipoprotein conversion factor with recombinant discoidal complexes of egg phosphatidylcholine, free cholesterol, and apolipoprotein A-I. *J. Lipid Res.* 1989. **30:** 335–346.

Supplementary key words gradient gel electrophoresis • HDL subpopulations

Plasma high density lipoproteins (HDL) are a population of particles of heterogeneous size, density, and composition. They comprise two major subfractions, HDL_2 and HDL_3 (1). HDL_2, the larger and less dense subfraction, and HDL_3 are also heterogeneous and contain several discrete subpopulations of particles (2, 3). It has been reported that the HDL_2 and HDL_3 subfractions are interconvertible (4, 5). Although the identities of the subpopulations involved in the conversion processes are not known, it is important that this issue be addressed because elevated plasma concentrations of HDL_2 in human subjects are associated with a decreased risk of developing coronary artery disease (6). It has also been reported that HDL are involved in the transport of excess cholesterol from peripheral tissues to the liver for excretion, but

Abbreviations: apoA-I, apolipoprotein A-I; FC, free cholesterol; PC, phosphatidylcholine; HDL, high density lipoproteins; HDL_2, subfraction 2 of HDL; HDL_3, subfraction 3 of HDL; BS, bis(sulfosuccinimidyl)suberate; DPPC, dipalmitoyl phosphatidylcholine; LCAT, lecithin:cholesterol acyltransferase.

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again, the identities of the subpopulations involved in the process are not known (7).

Several in vitro studies have established that the subpopulation distribution of HDL is altered by incubation in the presence of plasma factors such as lecithin:cholesterol acyltransferase, lipid transfer protein, and lipoprotein lipase (4, 8, 9). Since these changes proceed only when additional lipoprotein classes (very low density lipoproteins, low density lipoproteins, or chylomicrons) are present, the resulting subpopulation interconversions have been attributed to *1*) transfers and exchanges of surface and core components between HDL and other lipoproteins and *2*) the direct interaction of the plasma factors with HDL. The relative contributions of these processes to the changes in the subpopulation distribution of HDL cannot, however, be determined. Furthermore, the technical problems associated with the isolation of HDL subpopulations which are of homogeneous size and composition make the study of precursor–product relationships between specific subpopulations extremely difficult.

These issues can now be addressed by taking advantage of the activity of the recently described HDL conversion factor (10, 11). This factor promotes the conversion of physiological concentrations of HDL_3 to particles the size and density of HDL_2, as well as to particles that are smaller and more dense than HDL_3 (10). Since these changes proceed in the absence of other lipoprotein classes and plasma factors, the conversion factor enables the interconversion of HDL subpopulations to be examined in a relatively simple system.

The present studies were carried out in order to ascertain whether the processes promoted by the conversion factor are influenced by the free cholesterol content of the substrate. Since it is not possible to identify individuals in whom free cholesterol is the sole compositional variable in the HDL_3 subfraction, recombinant discoidal complexes of egg phosphatidylcholine (egg PC) and apolipoprotein A-I (apoA-I) with various concentrations of free cholesterol (FC) were used throughout the study as substrates for the conversion factor. Since the morphology of discoidal complexes resembles nascent HDL rather than mature, spheroidal HDL (12), the conversion of the two classes of particles cannot be directly compared. Nevertheless, the results of this study provide the first evidence that the conversion process can be regulated by the free cholesterol content of the substrate.

Discoidal complexes also contain homogeneous populations of particles, which are ideal for studying precursor–product relationships between different classes of particles. Furthermore, since the composition of discoidal complexes can be strictly controlled, they offer a major advantage over native substrates for systematically examining the regulation of the conversion process in a simple model system.

MATERIALS AND METHODS

Plasma samples

For isolation of HDL_3, blood was obtained from a normal female volunteer. The anti-coagulant EDTA-Na_2 was immediately added to the samples at a final concentration of 1 mg/ml. The samples were stored on ice until the plasma was isolated by centrifugation at 4°C. The conversion factor was isolated from pooled, citrated plasma samples which were a gift from the Champaign County Blood Bank, Regional Health Resource Center.

Ultracentrifugation

Sequential ultracentrifugation was carried out at 4°C in a Beckman L8-70 ultracentrifuge using a Beckman Ti50 rotor at a speed of 50,000 rpm. Density gradient ultracentrifugation was carried out at 10°C using a Beckman VTi65.1 rotor at a speed of 65,000 rpm (13). For density gradient ultracentrifugation, the samples (3.4 ml) were adjusted to a density of 1.35 g/ml and layered beneath a two-step gradient (6.6 ml, density 1.006 g/ml; 3.4 ml, density 1.20 g/ml). After ultracentrifugation (90 min) the samples were displaced upwards with a 1.35 g/ml KBr solution and 0.5-ml fractions were collected. Density adjustments for sequential and density gradient ultracentrifugation were made with solid KBr (14).

Isolation of HDL_3

HDL_3 were isolated by ultracentrifugation in the $1.14 < d < 1.21$ g/ml density range with a single 24 hr spin at the lower density and two 40 hr spins at the higher density. The isolated HDL_3 were exhaustively dialyzed against 0.01 M Tris-HCl, 0.15 M NaCl, 0.005% (w/v) EDTA-Na_2, 0.006% (w/v) NaN_3, pH 8.0, (Tris-buffered saline).

Isolation of the HDL conversion factor

The conversion factor was isolated from human plasma exactly as described previously (10). Briefly, ammonium sulfate was added to approximately 2 l of human plasma and the proteins that precipitated between 35% and 55% saturation were recovered and suspended in water. This solution was subjected to ultracentrifugation in the $1.21 < d < 1.25$ g/ml density range and the recovered proteins were applied to a column of CM-52 cellulose. The resulting fractions were assessed for the presence or absence of activity of the conversion factor by incubation with HDL_3 (10). For the experiments described in the current report, the conversion factor was further purified by anion exchange chromatography on a Mono Q HR 5/5 column attached to a Pharmacia FPLC system (Pharmacia Fine Chemicals, Uppsala, Sweden). The following details are for a single preparation, but are representative of several preparations of the conversion factor. The active fractions

from CM-cellulose were pooled and dialyzed against 0.02 M Tris-HCl, pH 7.4. A 12-ml (4 mg protein) aliquot of the resulting solution was applied to a pre-equilibrated Mono Q column and the bound proteins were eluted with a linear 0–0.25 M NaCl gradient at a flow rate of 1 ml/min. One-ml fractions were collected, dialyzed against Tris-buffered saline, and assessed for activity of the conversion factor by incubation with HDL_3. Following incubation, an aliquot from each sample was subjected to gradient gel electrophoresis. Representative laser densitometric scans of the gradient gels are shown in **Fig. 1.** The particle size distribution of HDL_3 (profile A) was not affected by incubation in the presence of Tris-buffered saline (profile B). When HDL_3 were incubated in the presence of the conversion factor, populations of particles larger and smaller than the original HDL_3 were formed (profile C). To ensure that the proteins that eluted from the Mono Q column did not co-migrate with HDL_3 or the conversion products, an aliquot from each Mono Q fraction was also subjected to gradient gel electrophoresis in the absence of HDL_3 (dotted line, profile C). The proteins in the conversion factor preparations appeared as sharply focused bands with Stokes' diameters less than that of bovine serum albumin (Stokes' diameter 71 Å). The fractions that displayed activity of the conversion factor were pooled, affording a 3-ml sample containing 0.5 mg protein.

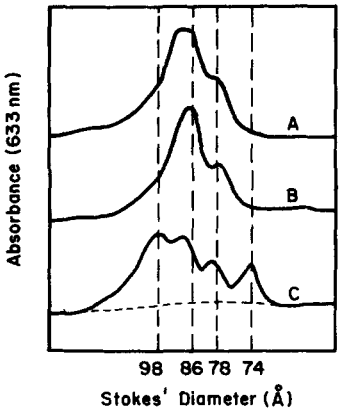

Fig. 1. Assay for the activity of the conversion factor. A 0.045-ml aliquot from each Mono Q fraction was incubated at 37°C for 24 hr with HDL_3 (0.005 ml). The final concentrations of HDL_3 protein and conversion factor in the incubation mixtures were 0.7 mg/ml and 0.18 mg/ml, respectively. Control samples containing HDL_3 (0.005 ml) and Tris-buffered saline (0.045 ml) were maintained at 4°C or incubated at 37°C for 24 hr. When the incubations were complete, a 0.01-ml aliquot from each sample was subjected to gradient gel electrophoresis. To ensure that the Mono Q fractions did not contain proteins that co-migrated with HDL_3 or the conversion products, a 0.01-ml aliquot from each fraction was subjected to gradient gel electrophoresis (---, profile C). Laser densitometric scans for nonincubated HDL_3 (profile A), HDL_3 incubated in buffer (profile B), and HDL_3 incubated in the presence of the conversion factor (profile C) are shown.

A typical elution profile obtained by chromatography on Mono Q has been reported elsewhere (15). Although a slightly different salt gradient was used to elute the conversion factor from Mono Q in the present study, the resulting profile was identical to that reported by Barter et al. (15). In addition, the activity of the factor was confined to the same area of the gradient as that reported previously (15). Barter et al. (15) also reported an additional purification step of chromatography on hydroxyapatite, to which the conversion factor was not subjected for the present studies.

When the active fractions from Mono Q were subjected to SDS-polyacrylamide gel electrophoresis, several proteins were evident by silver staining. Activity of the conversion factor was consistently associated with four partly resolved proteins with molecular weights ranging from 71,000 to 79,000 daltons. These four proteins comprised approximately 70% of the total protein content of the sample. At this stage it was not possible to determine which of these proteins promoted the conversion of HDL_3 or discoidal complexes. An SDS-polyacrylamide gel electrophoretogram of the conversion factor after chromatography on Mono Q and hydroxyapatite is shown elsewhere (15). Although the conversion factor in the present study was subjected to chromatography on Mono Q only, the proteins in the 71,000 to 79,000 dalton region remained essentially unchanged following chromatography on hydroxyapatite. Since, in the absence of a quantitative assay for the conversion factor, the percent recovery and fold-purification of the factor cannot be determined, the concentrations stated throughout this report refer to the total protein content of the active fractions obtained after chromatography on Mono Q.

To determine whether the conversion factor, like the recently described phospholipid transfer protein (16), facilitated the transfer of phospholipid between phospholipid vesicles and HDL, a 0.05-ml aliquot (9 μg protein) of the conversion factor was incubated at 37°C for 30 min in the presence of bovine HDL and egg PC–FC vesicles which contained a trace amount of ^{14}C-labeled DPPC (17). A control sample containing 0.8 μg of partially purified human phospholipid transfer protein (18) instead of the conversion factor was also incubated under the same conditions. There was no evidence of facilitated transfer of phospholipid in the incubations that contained the conversion factor. Approximately 37% of the label was transferred from the vesicles to HDL in the incubations which contained the phospholipid transfer protein (results not shown).

Incubations

Prior to incubation, the HDL_3 samples, conversion factor preparations, and discoidal complexes were exhaustively dialyzed against 0.01 M Tris-HCl, 0.15 M NaCl,

0.005% (w/v) EDTA-Na_2, 0.006% (w/v) NaN_3, pH 8.0. All incubations were carried out in stoppered plastic tubes in a shaking water bath that was maintained at 37°C. Details of the individual experiments are described in the legends to the figures. Nonincubated control samples were stored at 4°C.

Polyacrylamide gradient gel electrophoresis

Electrophoresis was carried out in 0.09 M Tris/boric acid buffer, pH 8.4, for 19 hr at 150 V using PAA 4/30 nondenaturing gradient gels (Pharmacia Fine Chemicals, Uppsala, Sweden). When electrophoresis was complete, the gels were fixed with 10% (w/v) sulfosalicylic acid and stained with 0.04% (w/v) Coomassie Blue G-250, 3.5% (v/v) perchloric acid. The gels were destained with 5% (v/v) acetic acid and scanned with an Ultroscan XL laser densitometer (LKB, Bromma, Sweden). Hydrated Stokes' diameters of the discoidal complexes, HDL_3, and the conversion products were calculated by reference to the following standards: thyroglobulin (170 Å), ferritin (122 Å), lactate dehydrogenase (81.6 Å), and bovine serum albumin (71 Å) (High Molecular Weight Calibration Kit, Pharmacia Fine Chemicals, Uppsala, Sweden).

Preparations of discoidal complexes

Egg PC-FC-apoA-I discoidal complexes were prepared by the sodium cholate dialysis method of Matz and Jonas (19). Egg PC and free cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. ^{14}C-Labeled DPPC was obtained from New England Nuclear (Boston, MA). ApoA-I was isolated from human plasma by the method of Scanu (20). Purity of apoA-I was assessed by electrophoresis on a 10% SDS-polyacrylamide gel (21).

Cross-linking of apoA-I

The number of apoA-I molecules associated with the discoidal complexes was determined by cross-linking the intact particles with the membrane impermeant, bifunctional cross-linking reagent bis(sulfosuccinimidyl)suberate (BS) (Pierce Chemical Co., Rockford, IL) (22). The complexes were adjusted to a protein concentration of 0.5 mg/ml and dialyzed against 0.02 M phosphate-buffered saline, 0.15 M NaCl, 0.01% (w/v) EDTA-Na_2, 0.02% (w/v) NaN_3, pH 7.4. A stock solution of BS (0.01 M) was added to the complexes (0.2 ml) so that the final concentration of cross-linking reagent was 0.002 M. After standing at room temperature for 30 min, the reaction mixtures were dialyzed against 0.01 M Tris-HCl, 0.001 M EDTA-Na_2, 1% (w/v) SDS, pH 8.0, incubated at 37°C for 45 min, and subjected to SDS-polyacrylamide gel electrophoresis on a PAA 4/30 gradient gel (Pharmacia Fine Chemicals, Uppsala, Sweden). The number of molecules associated with the complexes was determined by reference to the oligomers produced by cross-linking free apoA-I with BS.

Other methods

Phospholipid concentrations were determined by the method of Chen, Toribara, and Warner (23). Free cholesterol was assayed enzymatically by the method of Heider and Boyett (24) as modified by Gamble et al. (25). Protein concentrations were determined by the method of Lowry et al. (26) using bovine serum albumin as a standard.

RESULTS

Characterization of egg PC-FC-apoA-I discoidal complexes (Figs. 2 and 3; Table 1)

Recombinant discoidal complexes containing egg PC, apoA-I, and free cholesterol were prepared by the sodium cholate dialysis method (19). The initial reaction mixtures contained egg PC-apoA-I molar ratios of 80:1 and 0 to 12 mol% free cholesterol (**Table 1**). The stoichiometries of the resulting complexes are shown in Table 1. Gradient gel

TABLE 1. Characterization of egg PC-FC-apoA-I discoidal complexes

Composition			
Initial Reaction Mixture Egg PC-FC-ApoA-I	Isolated Complexes Egg PC-FC-ApoA-I	Particle Size Distribution	Number of ApoA-I Molecules/Particle
mol:mol:mol	*mol:mol:mol*	*Å*	
80:0:1	79:0:1	96*, 84, 78	2
80:5:1	77:5:1	96*, 84, 78	2
80:10:1	74:10:1	112, 96*, 84, 78	2, 3

The compositional data represent the mean of duplicate determinations. Phospholipid (egg PC) concentrations were determined by the method of Chen et al. (23). Free cholesterol was determined enzymatically by the method of Heider and Boyett (24) as modified by Gamble et al. (25). ApoA-I concentrations were determined by the method of Lowry et al. (26). Particle size distributions were estimated by gradient gel electrophoresis and the sizes designated (*) represent the major population of particles for each complex preparation. The number of apoA-I molecules per particle was determined by cross-linking the complexes with BS as described by Staros (22).

electrophoretic profiles of the complexes are shown in **Fig. 2.** The 79:0:1 (profile A), 77:5:1 (profile B), and 74:10:1 (profile C) complexes all comprised a major population of particles 96 Å in diameter and two minor populations of particles, 84 Å and 78 Å in diameter. The 74:10:1 complexes (profile C) contained an additional population of particles 112 Å in diameter. These particle size distributions are consistent with those reported by other investigators (27). In order to ascertain the number of apoA-I molecules associated with each of the complexes, incubations were carried out with the cross-linking agent BS. The cross-linked complexes were subjected to gradient gel electrophoresis in the presence of SDS; laser densitometric scans of the stained gels are shown in **Fig. 3.** Cross-linked apoA-I was used as a molecular weight standard (profile D). Both the 79:0:1 (profile A) and the 77:5:1 (profile B) complexes contained two molecules of apoA-I per particle. The 74:10:1 complexes comprised two distinct classes of particles containing two or three molecules of apoA-I (profile C). Dimers of two sizes were formed when lipid-associated apoA-I was cross-linked with BS (profiles A–C). By contrast, only a single dimeric species was evident when free apoA-I was cross-linked (profile D). This observation suggests that the environments of the apoA-I functional groups involved in the cross-linking reaction become heterogeneous when the apolipoprotein is incorporated into discoidal complexes.

Interaction of egg PC–FC–apoA-I discoidal complexes and HDL_3 with the HDL conversion factor (Figs. 4 and 5)

The 79:0:1, 77:5:1, and 74:10:1 discoidal complexes and HDL_3 were mixed with Tris-buffered saline or the conversion factor. The samples containing Tris-buffered saline were either maintained at 4°C (**Fig. 4,** profile A) or incubated at 37°C for 24 hr (Fig. 4, profile B). The samples containing the conversion factor were also incubated at 37°C for 24 hr (Fig. 4, profile C). In order to directly compare the conversions, the same preparation of conversion factor was used for all the incubations. The particle size distributions of the 79:0:1 and 77:5:1 complexes changed slightly during incubation in the presence of Tris-buffered saline. In both cases the shoulder on the descending limb of the 96 Å peak was replaced by a discrete population of particles 84 Å in diameter. Similar, but more pronounced, changes were apparent in the 74:10:1 complexes. The particle size distribution of HDL_3 was not significantly affected by incubation in the presence of Tris-buffered saline. When the 79:0:1 complexes were incubated with the conversion factor, the concentration of the 96 Å particles decreased and there was a corresponding increase in the concentration of the particles 78 Å in diameter. Although the rate of conversion of 96 Å particles to 78 Å particles was more rapid in the case of the 77:5:1 complexes, conversion did not reach completion by 24 hr of incubation. When the 74:10:1 complexes were incubated with the conversion factor, the 96 Å particles were, by contrast, completely converted to particles 78 Å in diameter and a significant proportion of the 84 Å particles was also converted to 78 Å particles. When HDL_3 were incubated with the conversion factor, the changes in particle size distribution were similar to those described

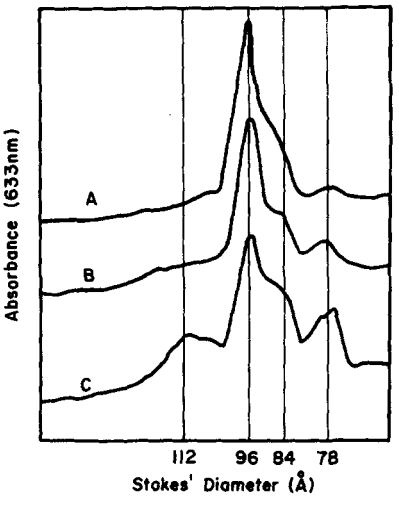

Fig. 2. Particle size distribution of egg PC–FC–apoA-I discoidal complexes. A 0.01-ml aliquot (0.03 mg apoA-I) of the 79:0:1 (profile A), 77:5:1 (profile B), and 74:10:1 (profile C) complexes was subjected to gradient gel electrophoresis as described in the Materials and Methods section. The profiles represent laser densitometric scans of the stained gels.

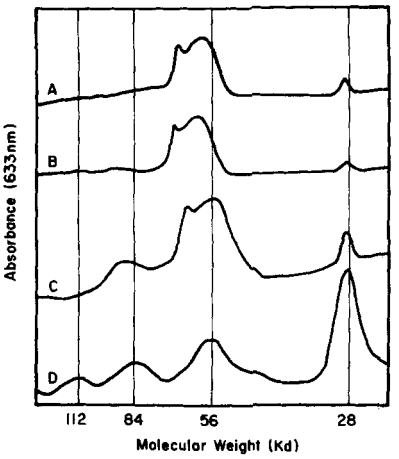

Fig. 3. Cross-linking of egg PC–FC–apoA-I complexes. A 0.2-ml aliquot (0.1 mg protein) of each complex and a comparable amount of free apoA-I were dialyzed against phosphate-buffered saline, mixed with BS (0.05 ml), and incubated at room temperature for 30 min. The final concentration of BS in the incubation mixture was 0.002 M. The samples were then dialyzed against 0.01 M Tris-HCl, 0.001 M EDTA-Na_2, 1% (w/v) SDS, pH 8.0, incubated at 37°C for 45 min and subjected to SDS-polyacrylamide gradient gel electrophoresis. The gels were stained with Coomassie Blue R-250 and scanned using a laser densitometer. Profiles A, B, and C represent the scans for the 79:0:1, 77:5:1, and 74:10:1 complexes, respectively. Profile D shows the scan for cross-linked apoA-I.

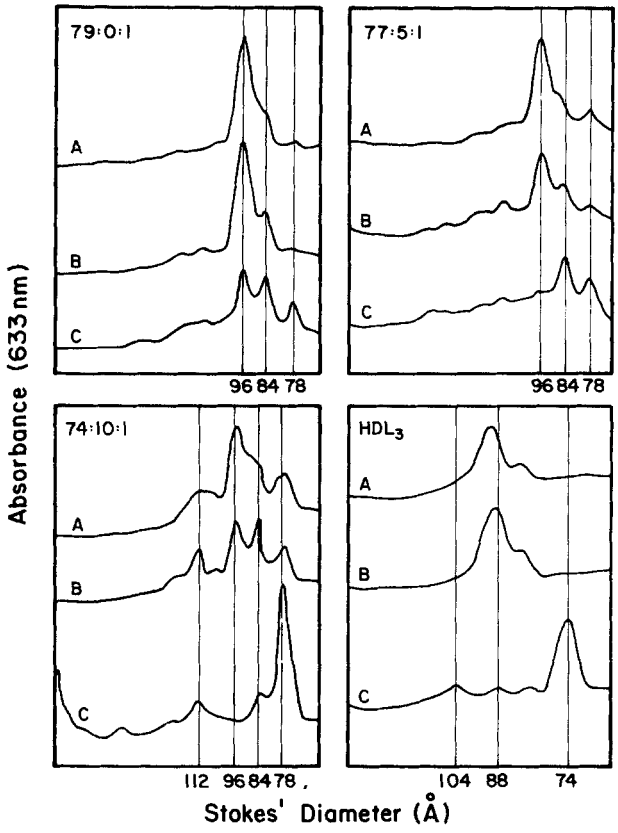

Fig. 4. Conversion of egg PC-FC-apoA-I discoidal complexes: comparison with HDL_3. The 79:0:1, 77:5:1, 74:10:1 complexes, and HDL_3 were adjusted to a protein concentration of 0.5 mg/ml with Tris-buffered saline. A 0.025-ml aliquot from each sample was mixed with 0.09 ml of the conversion factor. The final concentration of the conversion factor in the incubation mixture was 0.13 mg/ml. Control samples comprising the complexes or HDL_3 and Tris-buffered saline were maintained at 4°C (profile A) or incubated at 37°C for 24 hr (profile B). The samples containing the conversion factor were also incubated at 37°C for 24 hr (profile C). When the incubations were complete, a 0.02-ml aliquot from each sample was subjected to gradient gel electrophoresis. Laser densitometric scans of the gels are shown. To ensure that the conversion factor preparation did not contain proteins that co-migrated with the complexes, HDL_3 or the conversion products, a 0.09-ml aliquot of the preparation was mixed with Tris-buffered saline (0.025 ml) and a 0.02-ml aliquot of the resulting solution was subjected to gradient gel electrophoresis (result not shown).

for the 74:10:1 complexes. The original particles, 88 Å in diameter, were converted to a population of small particles 74 Å in diameter. A population of larger particles 104 Å in diameter also appeared.

To ascertain whether conversion was associated with a rearrangement of apolipoproteins, the 77:5:1 conversion products were re-isolated by ultracentrifugation at a density of 1.25 g/ml and cross-linked with BS (**Fig. 5**). The ultracentrifugation step ensured that the free proteins in the conversion factor preparations did not cross-link to the conversion products. Following ultracentrifugation, the conversion products were recovered as the supernatant and cross-linked as described in the Methods section. The original complexes (profile A) and the conversion products (profile B) both contained two apoA-I molecules per particle. This suggests that the conversion factor does not affect the apolipoprotein distribution of discoidal complexes, and is in agreement with what has been previously reported for the conversion of HDL_3 (10).

Time course for the conversion of egg PC-FC-apoA-I discoidal complexes (Figs. 6 and 7)

The 79:0:1, 77:5:1, and 74:10:1 complexes were individually mixed with Tris-buffered saline or the conversion factor. The samples containing Tris-buffered saline were maintained at 4°C (nonincubated control) or incubated at 37°C for 96 hr (incubated control). The samples containing the conversion factor were incubated at 37°C for times ranging from 1 to 96 hr. In order to directly compare the conversion of the complexes, the same preparation of conversion factor was used for the entire experiment. Incubation for 96 hr in the presence of buffer did not alter the particle size distribution of the 79:0:1 complexes beyond what was described for 24 hr of incubation (**Fig. 6;** see also Fig. 4, profile B). The particle size distribution of the 77:5:1 complexes did, by contrast, change following incubation in buffer for 96 hr. The concentration of the 96 Å particles decreased and the concentrations of the 78 Å and 84 Å particles increased. Incubation in buffer for 96 hr resulted in even greater changes to the particle size distribution of the 74:10:1 complexes. The 96 Å particles almost disappeared and the concentrations of the 84 Å and 78 Å particles increased.

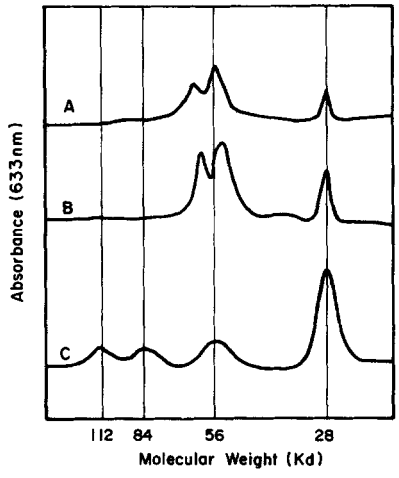

Fig. 5. Cross-linking of the 77:5:1 conversion products. The complexes were incubated with the conversion factor as described in the legend to Fig. 4, then isolated as the supernatant following ultracentrifugation for 40 hr at a density of 1.25 g/ml. The original complexes (profile A), the conversion products (profile B), and apoA-I (profile C) were cross-linked with BS (see legend to Fig. 3) and subjected to SDS-polyacrylamide gradient gel electrophoresis. Laser densitometric scans of the gels are shown.

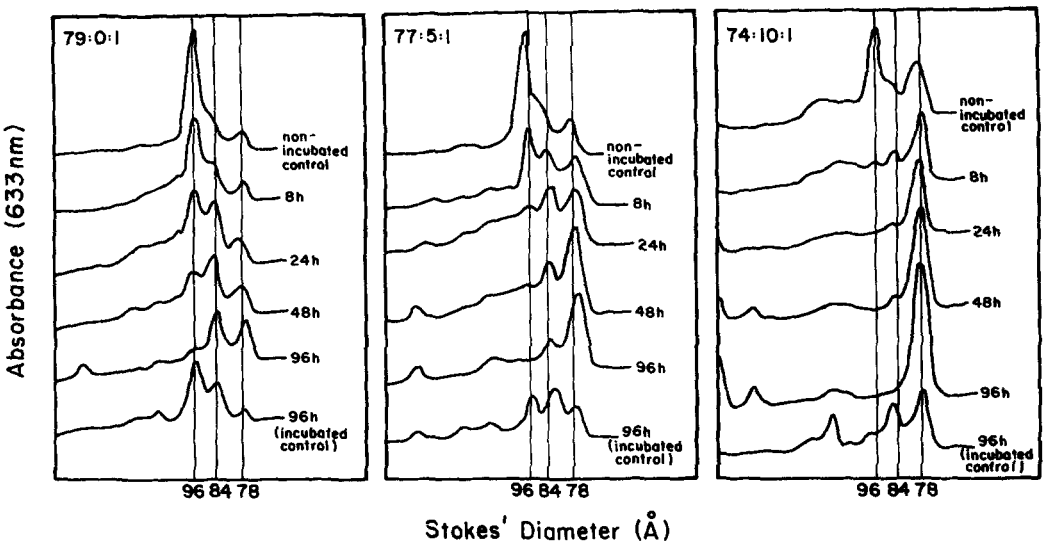

Fig. 6. Time course for conversion of egg PC–FC–apoA-I discoidal complexes. The 79:0:1, 77:5:1, and 74:10:1 complexes were incubated with the conversion factor as described in the legend to Fig. 4. Control samples were also prepared as described in Fig. 4. The controls were either maintained at 4°C for 96 hr (nonincubated control) or incubated at 37°C for 96 hr (incubated control). The samples containing the conversion factor were incubated for a range of times from 1 to 96 hr. Gradient gel electrophoretic profiles of the samples incubated with conversion factor for 8, 24, 48, and 96 hr as well as the profiles for the control samples are shown. A control sample comprising the conversion factor and Tris-buffered saline was also subjected to gradient gel electrophoresis (result not shown).

The population of particles 112 Å in diameter became more pronounced.

When the complexes were incubated in the presence of the conversion factor, the changes to the particle size distributions were much greater than those resulting from incubation in buffer alone. In the case of the 79:0:1 complexes, the concentration of 96 Å particles had significantly decreased by 48 hr and they had almost disappeared by 96 hr. There was a corresponding increase in the concentration of the 78 Å particles at 48 hr and 96 hr. In the case of the 77:5:1 complexes, the 96 Å particles were completely converted to 78 Å particles by 48 hr. Conversion of the 74:10:1 complexes proceeded even more rapidly. Most of the 96 Å particles disappeared by 8 hr of incubation and by 96 hr the 84 Å particles had also disappeared. The rate of formation of 78 Å particles was very rapid for the first 24 hr and reached equilibrium by 72 hr.

For the 79:0:1 complexes, the laser densitometric profiles corresponding to 96 hr of incubation with buffer or 24 hr of incubation with the conversion factor were very similar. Likewise, the particle size distributions of the 77:5:1 and 74:10:1 complexes were similar following 8 hr or 96 hr of incubation with the conversion factor or Tris-buffered saline, respectively. These observations suggest that the process that is facilitated by the conversion factor proceeds spontaneously at 37°C and are consistent with the factor promoting a catalytic reaction.

The conversion of 96 Å and 84 Å particles to 78 Å particles was also examined in a semi-quantitative manner by plotting the changes in the concentrations of the 96 Å, 84 Å, and 78 Å particles as a function of time (**Fig. 7**). The concentrations of the particles were determined by calculating peak areas from laser densitometric scans of gradient gels. This approach assumes that the dye taken up by the complexes is proportional to the concentration of apoA-I. In the case of the 79:0:1 and 77:5:1 complexes, the concentration of the 84 Å particles remained constant for 72 hr. This suggests that the conversion of 96 Å particles to 78 Å particles proceeded in a single step, although the possibility cannot be ruled out that the conversion of 96 Å particles to 78 Å particles proceeded via 84 Å particles. Two distinct conversion processes were, by contrast, evident in the case of the 74:10:1 complexes. The concentration of the 78 Å particles increased as the concentrations of the 96 Å and 84 Å particles concomitantly decreased. The data in Fig. 7 also confirm that the rate of the conversion process is significantly enhanced as the concentration of free cholesterol in the complexes is increased.

Concentration dependence of the conversion of egg PC–FC–apoA-I discoidal complexes and HDL_3 (Figs. 8 and 9)

The discoidal complexes and HDL_3 were individually mixed with Tris-buffered saline or various concentrations

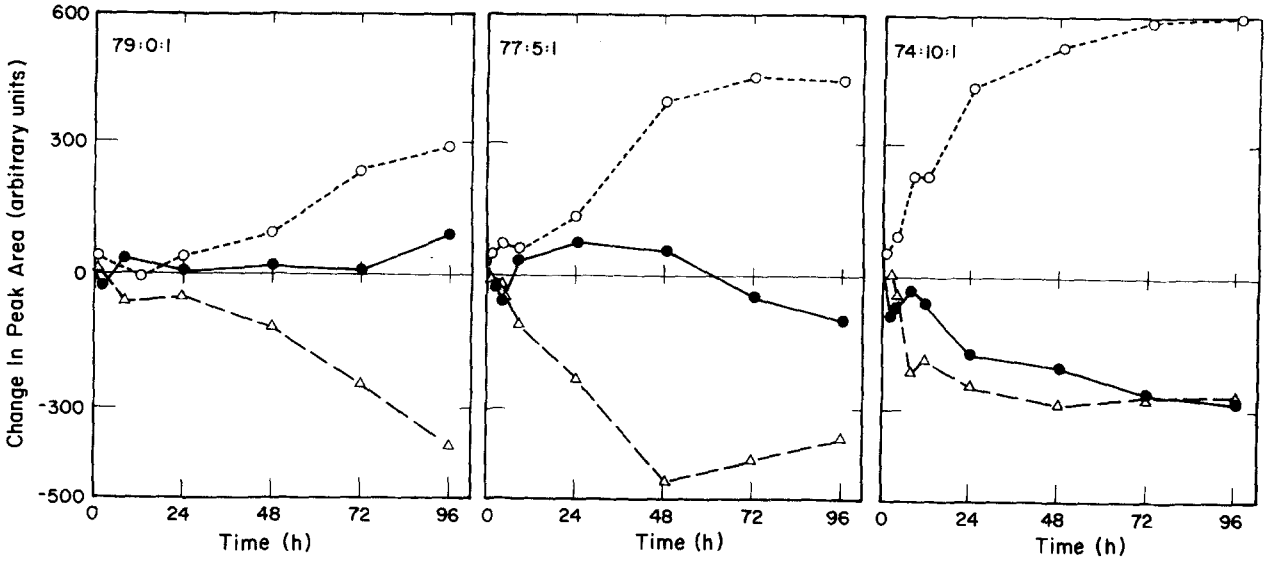

Fig. 7. Time course for conversion of egg PC-FC-apoA-I discoidal complexes as a function of change in peak area. The 79:0:1, 77:5:1, and 74:10:1 complexes were incubated with the conversion factor from 1 to 96 hr as described in the legend to Fig. 6. At each time point the peak areas corresponding to the 96 Å (△— —△), 84 Å (●—●), and 78 Å (○---○) particles were determined from the laser densitometric scans as the product of the peak height and the width of the peak at half-height. The areas were normalized and at each time point the areas of the incubated controls were subtracted from those corresponding to the samples incubated with the conversion factor. The peak areas for the 24 hr and 96 hr controls were obtained from Figs. 4 and 6, respectively. The areas for the other incubated controls were calculated by interpolation. The errors are ± 15%.

of the conversion factor so that the concentration of apoA-I or apoHDL relative to the conversion factor ranged from 1:1 to 20:1. The samples containing buffer were maintained at 4°C (nonincubated control) or incubated at 37°C (incubated control) for 24 hr. The samples that contained the conversion factor were also incubated at 37°C for 24 hr. Since the results for all the complexes and HDL_3 were similar, only the scans for the 77:5:1 complexes and HDL_3 are shown (**Fig. 8**). When the concentrations of apoA-I or apoHDL and conversion factor were equivalent, major changes to the particle size distributions of the complexes and HDL_3 were evident by 24 hr of incubation. These changes were described previously (Fig. 4; profile C). When the concentration of the conversion factor in the incubation mixtures was reduced relative to apoA-I or apoHDL, there was a concomitant decrease in the rate of conversion. Conversion did not proceed when the concentration of the conversion factor was reduced to one-tenth that of apoA-I or apoHDL. The results for the conversion of the 77:5:1 complexes were also examined in a semi-quantitative manner by calculating, from the laser densitometric scans, the areas corresponding to the 78 Å and 96 Å particles at each concentration of the conversion factor (**Fig. 9**). Examination of Fig. 9 also shows that the conversion of 96 Å particles to 78 Å particles was incomplete after 24 hr of incubation, confirming the results obtained for the 77:5:1 complexes in the extended time course study (Fig. 7).

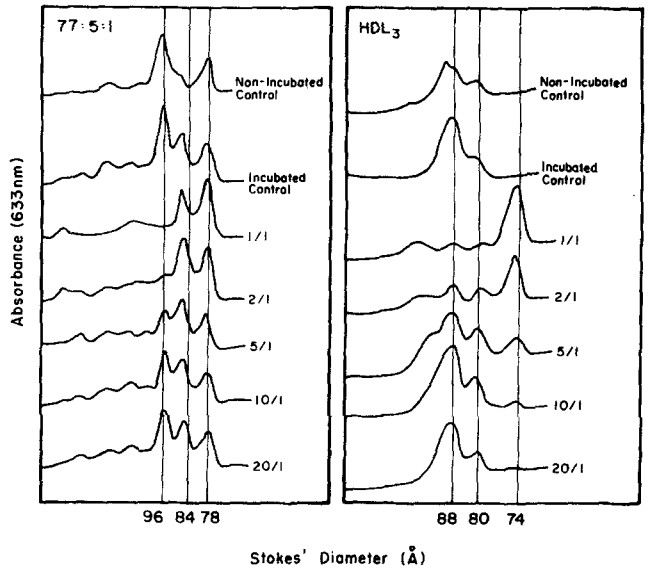

Fig. 8. Concentration dependence of the conversion of egg PC-FC-apoA-I discoidal complexes: comparison with HDL_3. The complexes and HDL_3 were adjusted to a protein concentration of 0.5 mg/ml with Tris-buffered saline and mixed with the conversion factor. The final concentration of conversion factor in the incubation mixtures ranged from 0.142 mg/ml to 0.007 mg/ml. The ratio of the concentrations of apoA-I or apoHDL to conversion factor ranged from 1:1 to 20:1. Buffer was added where necessary to adjust the volumes of the solutions to 0.087 ml. Control samples were prepared as described in the legend to Fig. 4. Incubations were carried out at 37°C for 24 hr. Laser densitometric scans for the 77:5:1 complexes and HDL_3 are shown.

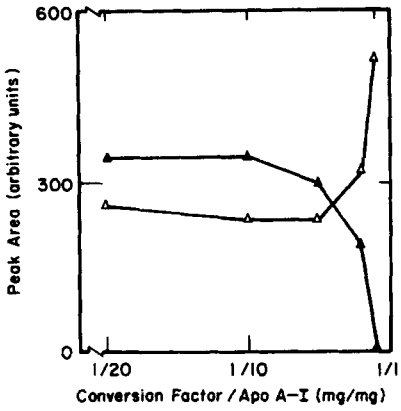

Fig. 9. Concentration dependence of the conversion of 77:5:1 complexes. The 77:5:1 complexes were incubated with a range of concentrations of the conversion factor as described in the legend to Fig. 8. The areas of the peaks corresponding to the 96 Å (▲—▲) and the 78 Å (△—△) particles were obtained from laser densitometric scans of gradient gels and plotted as a function of the relative concentrations of the conversion factor and apoA-I in the incubation mixture.

DISCUSSION

It has previously been reported that human plasma contains a specific HDL conversion factor that promotes the conversion of physiological concentrations of HDL_3 to particles the size and density of HDL_2 as well as to particles that are smaller and more dense than HDL_3 (10). In the current report a partially purified preparation of the conversion factor has been used to examine the conversion of egg PC-FC-apoA-I discoidal complexes.

The study was carried out in order to ascertain whether the conversion process is regulated by the concentration of free cholesterol in the discoidal complexes. Three complex preparations which contained similar molar ratios of egg PC and apoA-I, and 0, 6, and 12 mol% free cholesterol were prepared. Most of the particles in the resulting complexes were 96 Å in diameter. Two minor populations of particles with Stokes' diameters of 84 Å and 78 Å were also apparent.

When the complexes were incubated for 24 hr in the presence of the conversion factor, the 96 Å particles were converted to particles 78 Å in diameter. The rate of conversion was enhanced as the concentration of free cholesterol in the complexes increased. The conversion of the complexes was also studied in an extended time course experiment, where the complexes were incubated with the conversion factor for times ranging from 1 hr to 96 hr. In the case of the 77:5:1 and 74:10:1 complexes, the conversion of 96 Å particles to 78 Å particles reached equilibrium by 72 hr of incubation. Since the concentration of the 78 Å conversion products did not change when the incubation was extended from 72 hr to 96 hr, this result suggests that particles 78 Å in diameter are not substrates for the conversion factor. The possibility that the accumulation of 78 Å particles in the incubation mixture was due to the inactivation of the conversion factor was ruled out because the conversion of 96 Å particles to 78 Å particles was on-going at 96 hr in the incubation containing the 79:0:1 complexes.

Although a precursor-product relationship clearly exists between 96 Å and 78 Å particles, it does not necessarily follow that conversion proceeds in a single step. Even though the concentration of 84 Å particles did not change in the incubations that contained 79:0:1 and 77:5:1 complexes, it is possible that 84 Å particles are intermediate products in the conversion of 96 Å particles to 78 Å particles. Indeed, in the case of the 74:10:1 complexes, both 96 Å and 84 Å particles were converted to particles 78 Å in diameter.

Irrespective of whether the 96 Å particles are directly converted to 78 Å particles or whether the conversion proceeds by way of particles 84 Å in diameter, this precursor-product relationship can be exploited in order to address the mechanism of the conversion process. The following approach assumes that the conversion of 96 Å egg PC-FC-apoA-I discoidal complexes to a population of smaller particles 78 Å in diameter is associated with the loss of one or more of the components. Since the cross-linking studies demonstrated that the 96 Å precursors and 78 Å conversion products both contain two molecules of apoA-I per particle, it is evident that conversion cannot be attributed to the loss of this component. Furthermore, since the rate of desorption of free cholesterol from recombinant discoidal complexes is more rapid than the rate of the conversion process (28), it follows that conversion is not a consequence of the redistribution of this component. It therefore seems probable that the conversion of 96 Å particles to 78 Å particles is due to the removal of phospholipid molecules from the larger particles. When the volumes of the 96 Å particles and 78 Å particles were calculated, the difference was found to be equal to the volume of 78 molecules of phospholipid (**Table 2**). Furthermore, since the discoidal complexes 96 Å in diameter contain approximately 161 to 173 molecules of egg PC (**Table 3**), it follows that conversion to particles 78 Å in diameter may be associated with the loss of 45% to 48% of the total phospholipid.

To test this hypothesis, 77:5:1 egg PC-FC-apoA-I discoidal complexes which contained a trace amount of ^{14}C-labeled DPPC were prepared. The complexes were maintained at 4°C or incubated at 37°C for 24 hr with Tris-buffered saline or the conversion factor. When the incubations were complete, the samples were subjected to density gradient ultracentrifugation. The nonincubated and incubated control samples were both recovered at a density of 1.12 g/ml. This corresponds closely to the calculated densities of the complexes (Table 3). When the labeled 77:5:1 complexes were incubated with the conversion fac-

TABLE 2. Estimated properties of the conversion products from 96 Å discoidal complexes

Volume 96 Å particles	290,000 Å^3
Volume 78 Å particles	191,000 Å^3
Number of egg PC molecules liberated from 96 Å particles during conversion to 78 Å particles	78
Calculated (experimental) density of free egg PC	1.03 (1.06) g/ml
Calculated (experimental) density of 78 Å conversion products	1.18 (1.14) g/ml

Densities and particle volumes were calculated as described in Table 3. The contribution of water was not considered when calculating the density of the free egg PC. The effect of phospholipid depletion on the volumes of the remaining components was not considered when calculating the density of the 78 Å conversion products.

tor, 41% of the radioactivity was recovered at a density of 1.06 g/ml. The remainder of the label was recovered at a density of 1.14 g/ml (Table 2). These densities correspond closely to the values for free egg PC (1.03 g/ml, Table 2) and egg PC-FC-apoA-I complexes 78 Å in diameter (1.18 g/ml, Table 2). It should be noted that the densities of the 78 Å conversion products and liberated egg PC molecules differ slightly from the calculated values (Table 2). These discrepancies are not unexpected because the conditions of density gradient ultracentrifugation were such that equilibrium was not attained. Nevertheless, the results support the hypothesis that the conversion factor promotes the liberation of phospholipid molecules from discoidal complexes and does not influence the distribution of apolipoproteins. Indeed, when 96 Å discoidal complexes are converted to particles 78 Å in diameter, the apoA-I molecules which were originally present in the 96 Å particles become incorporated into the 78 Å conversion products and the liberated egg PC molecules, which are possibly present as liposomes, are essentially free of protein. Attempts to quantitate the particle size distribution of the liberated egg PC by pre-staining the incubated samples with Sudan Black and subjecting them to electrophoresis on a 2-16% polyacrylamide gradient gel were unsuccessful.

Although the interaction of the conversion factor with discoidal complexes does not promote the redistribution of free cholesterol, the mechanism by which this component regulates the conversion process raises several questions. Since the rate of desorption of this molecule from lipid-water interfaces is very rapid (28), its presence, per se, is unlikely to affect the rate of the conversion process. It has, however, been reported that the incorporation of free cholesterol into egg PC-FC-apoA-I discoidal complexes increases the packing order of the phospholipid acyl chains, thus altering the interfacial properties of the particles (29, 30). The present results suggest that the activity of the conversion factor may be sensitive to such changes. Evidence consistent with this hypothesis was obtained when discoidal complexes containing DPPC, apoA-I, and 5-10% free cholesterol were incubated in the presence of the conversion factor. The particle size distribution of these complexes did not alter during incubation under conditions that promoted conversion of egg PC-FC-apoA-I complexes (Rye, K-A., and A. Jonas, unpublished observations). Although the particle size distributions of egg PC and DPPC complexes are similar, they have quite different lipid-water interfaces (31). The ability of the conversion factor to discriminate between these complexes suggests that this property of the substrate may influence the conversion process.

TABLE 3. Estimated composition and properties of egg PC-FC-apoA-I discoidal complexes

Stoichiometry	Component	Component Mole Percent	Component Volume Percent	Number Component Molecules per Particle	Calculated Density	Experimental Density
					g/ml	
79:0:1	Egg PC	98.8	75.8	173	1.12	n.d.
	Cholesterol	0	0	0		
	ApoA-I	1.2	24.2	2		
77:5:1	Egg PC	92.8	73.2	167	1.13	1.12
	Cholesterol	6.0	2.3	11		
	ApoA-I	1.2	24.7	2		
74:10:1	Egg PC	87.1	70.2	161	1.13	n.d.
	Cholesterol	11.8	4.7	21		
	ApoA-I	1.2	25.5	2		

Stoichiometries represent the mean composition of the 96 Å, 86 Å, and 78 Å particles (Table 1). Since the contribution of the 78 Å and 84 Å particles to the stoichiometries of the complexes was minor, the values for the number of component molecules are approximately equal to the values expected for particles 96 Å in diameter. Component volumes were calculated from the following partial specific volumes: egg PC, 0.970; free cholesterol, 0.968; apoA-I, 0.705 (33). Particle mass was determined as the sum of the individual components. The volumes of the discoidal particles were calculated using a hydrated Stokes' radius of 48 Å, assuming a bilayer thickness of 40 Å (34). Densities were determined experimentally by density gradient ultracentrifugation; n.d., not determined.

In the absence of a conversion factor that has been purified to homogeneity, the possibility that the preparations used in this study may contain more than one active factor cannot be ruled out. Thus, the conversion of discoidal complexes may be promoted by a factor that is distinct from the one that acts on HDL_3. Furthermore, it is also possible that the conversion of HDL_3 and discoidal complexes may be promoted by more than a single factor. Nevertheless, the results in the present report reveal several qualitative parallels between the conversion of discoidal complexes and native HDL_3. Firstly, similar concentrations of conversion factor are required to promote conversion of both substrates. Furthermore, when HDL_3 are diluted to a protein concentration equivalent to that used for the incubation of discoidal complexes, the major conversion product comprises a population of particles 74 Å in diameter. Finally, if the 74 Å particles, like the 78 Å particles, represent equilibrium products of the conversion of HDL_3, it follows that the large particles that appear when physiological concentrations of HDL_3 are incubated with the conversion factor may be intermediate products of the conversion process. These observations suggest that studies designed to examine the interaction of the conversion factor with HDL_3 enriched with free cholesterol may provide further insight into the ways by which the particle size distribution of these lipoproteins is regulated.

It is interesting that HDL particles 74 Å in diameter are rarely found in the plasma of normal subjects. It has, however, been reported that the plasma of LCAT-deficient subjects contains small, spheroidal particles in this size range. These particles are converted to larger particles, of a size and density similar to that of native HDL, during incubation in vitro in the presence of LCAT and a source of free cholesterol (32). Although a physiological role for the conversion factor has yet to be determined, it is interesting to speculate that the conversion factor may interact with HDL_3 in vivo to promote the formation of small particles which are substrates for LCAT.

This work was carried out in the laboratory of Dr. Ana Jonas under the support of the National Institutes of Health grant, HL-16059. The phospholipid transfer protein was a gift from Mr. M. I. Williams. The author also acknowledges the expert technical assistance of Ms. K. Kézdy.

Manuscript received 23 March 1988 and in revised form 28 June 1988.

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