The distribution of apolipoprotein A-I, apolipoprotein D, lecithin:cholesterol acyltransferase, and cholesteryl ester transfer protein in fasting normal human plasma was determined by two-dimensional electrophoresis followed by immunoblotting. The synthesis and transfer of labeled cholesteryl esters generated in plasma briefly incubated with $[^3H]$cholesterol-labeled fibroblasts was followed in terms of the lipoprotein species containing these antigens. Following the early appearance of labeled free cholesterol in two preβ-migrating apolipoprotein A-I species (Castro, G. R., and Fielding, C. J. (1988) Biochemistry 27, 25-29), labeled esters were first detected, after a 2-min delay, in a third preβ-migrating species which also contained apolipoprotein D, lecithin:cholesterol acyltransferase, and cholesteryl ester transfer protein. Pulse-chase experiments determined that label generated in this fraction was the precursor of at least a major part of labeled cholesteryl esters in the bulk of α-migrating high density lipoprotein. Over the maximum time course of these experiments (15 min, 37°C), <10% of labeled cholesteryl esters were recovered in low or very low density lipoproteins separated by electrophoresis, immunoaffinity, or heparin-agarose chromatography. These data suggest channeling of cell-derived cholesterol not only from circulating plasma lipoproteins but also from cell membranes to the plasma compartment.

The expected further metabolism of cell-derived cholesterol would involve its esterification by LCAT and a subsequent transfer of LCAT-derived cholesteryl esters by the cholesteryl ester transfer protein (CETP) (6, 7) to acceptors among the major plasma lipoprotein classes. Major acceptors in human plasma include LDL, HDL, and that part of very low density lipoprotein (VLDL) containing apolipoprotein E (apoE). In the present study, the synthesis and transfer of cholesteryl esters newly derived from $[^3H]$cholesterol-labeled cultured fibroblasts were determined. The results obtained suggest that a third preβ-migrating HDL species plays a key role in the subsequent processing (esterification and transfer) of a major fraction of cell-derived cholesterol. This lipoprotein complex contains LCAT and CETP, together with apoA-I, and apolipoprotein D, an analog of retinol binding protein.

**MATERIALS AND METHODS**

**Cultured Cells and Incubation Conditions**—Normal skin fibroblasts were cultured to late confluence at 37°C in 3.5-cm dishes in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (4.0 ± 0.5 × 10⁵ cell cholesterol/dish). Some dishes were prelabeled for 48 h with 0.5 μCi each of [1,2-3H]cholesterol (Du Pont-New England Nuclear) complexed with fetal calf serum. Final specific activity in the cells was 1.5-2.0 × 10⁶ cpm/μg of cholesterol.

Human blood was collected from fasting normal donors into tubes cooled in ice water, which contained as protease inhibitors 2-phenylalanyl-L-prolyl-arginine chloromethyl ketone (final concentration, 5 μg/ml) to inhibit thrombin (8), and aprotinin (8.5 μg/ml), benzamidine (2 mM), and 20 μg/ml of soybean and lima bean trypsin inhibitors to inhibit other proteases cleaving lipoprotein apoproteins (9). Plasma was then obtained by immediate centrifugation at 6°C (2000 × g, 30 min).

The labeled cells were washed (four times) with phosphate-buffered saline at 37°C and then incubated with phosphate-buffered saline containing tritiated cholesterol (50 μCi/ml) for 2 h at 37°C. The dishes were then rinsed twice with ice-cold phosphate-buffered saline.

The abbreviations used are: LCAT, lecithin:cholesterol acyltransferase; CETP cholesterol ester transfer protein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; DTNB, dithiobis(2-nitrobenzoic acid).

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1 The abbreviations used are: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; CETP, cholesteryl ester transfer protein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; DTNB, dithiobis(2-nitrobenzoic acid).

2 There is no agreed nomenclature for subfractions of HDL containing different apoproteins and metabolic factors. For convenience only, species containing apoA-I (apoA-I lipoproteins, LpA-I) among other factors are described here first on the basis of their migration rate on agarose gel electrophoresis ($\alpha$, $\beta$, $\prebb$), and second, on their relative migration rate in nonnadenaturing gradient gel electrophoresis, from fastest to slowest (e.g. $\text{LpA-1}_{\text{prebb}} > \text{LpA-1}_{\text{prebb}} > \text{LpA-1}_{\text{prebb}}$ in migration rate).
rapidly to 37°C for 1-15 min. During incubation, the plasma was mixed at 1.5 cpm on the plate of an orbital mixer (LabLine, Melrose Park, IL). Samples were taken at intervals for determination of medium radioactivity. The distribution of radioactivity among plasma lipoprotein fractions was determined by nondenaturing two-dimensional electrophoresis (5) or affinity chromatography as described below. In some experiments, after an initial incubation ("pulse") with medium radioactivity, the distribution of radioactivity among plasma lipoprotein fractions was determined by nondenaturing two-dimensional electrophoresis (5) or affinity chromatography. LCAT and CETP were purified from human plasma (1.21 g/ml) by phenyl-agarose and DEAE-cellulose chromatography (13, 15). LCAT by hydroxyapatite and subsequent preparative electrophoresis (7).

Preparation and Purification of Antibodies—Polyclonal antibodies against apoA-I, apoD, and apoB were prepared in rabbits (2). Antibodies were purified by ligand immunofinity chromatography (15) to isolate a reactive fraction reactive against the respective apolipoprotein antigen. 0.5-1.0 mg of each pure antigen protein in 0.1 M NaHCO₃ (pH 8.0) was coupled to 1-2 ml of Affi-Gel 10 (Bio-Rad). Unreacted sites were blocked with 0.1 M ethanolamine (pH 8.0). The gel was then washed with 10 mM Tris-HCl, 0.1 M NaCl (pH 7.5) to elute nonspecific bound protein, and finally eluted with the same buffer containing 0.1 M NaCl. Antiserum to the corresponding antigen was passed through the column, which was then washed with NaCl solution as above. Finally, bound antibody was eluted with 10 mM Tris-HCl (pH 7.5) containing either 0.5 M MgCl₂, 4 mM guanidinium HCl, or 0.1 M NaCl. The sample was injected with an equal volume of Freund's complete adjuvant (21). Antibody solutions were dialyzed against 0.15 M NaCl, 1 mM EDTA (pH 7.4) before use in the immunoblotting procedures described below.

Monoclonal antibodies against human CETP and LCAT were prepared from the corresponding isolated proteins (7, 13) injected into mice. The fusion of mouse spleen cells, and subsequent culture of hybridomas, was carried out as previously described (14). A high affinity IgG clone was isolated from each protein. The fusion with a carboxyl-terminal cysteine and to residues 165-183 of the mature protein (NH₂-terminal cysteine) (16) and residues 290-306 of hybridomas, was carried out as previously described (14). A high affinity IgG clone was purified for immunoblotting by antigen affinity chromatography as described above.

Affinity Chromatography of ApoB Lipoproteins—Rabbit anti-apoB polyclonal antisera was purified by affinity chromatography on columns of heparin-agarose (Pharmacia LKB Biotechnology Inc.) (18). Reacted sites were blocked with 0.1 M ethanolamine then the gel equilibrated in 0.15 M NaCl, 1 mM EDTA (pH 7.4). The gel was then washed with chromatographic buffer containing about 0.5 mg/ml packed gel. Plasma preincubated with [³H]cholesterol cells was passed through the columns at a flow rate of 0.1 ml/min at 37°C. The columns were then washed with 0.15 M NaCl, 1 mM EDTA to remove any plasma proteins bound nonspecifically. The free and esterified cholesterol radioactivity was recovered with 3 M NaSCN (pH 7.5). Total recovery of free and esterified cholesterol radioactivity from labeled plasma was >85%.

Plasma containing [³H]cholesterol-labeled lipoproteins was also fractionated by heparin-agarose chromatography (19). LDL and HDL fractions containing VLDL were recovered from plasma by chromatography on columns (1 x 25 cm) of heparin-agarose (Pharmacia LKB Biotechnology Inc.) equilibrated in 0.15 M NaCl, 1 mM EDTA, pH 7.4. The adsorbed fraction was eluted with 3 M NaCl, 1 mM EDTA, pH 7.4. Recovery of free and ester cholesterol label from plasma under these conditions was >85%.

Two-dimensional Nondenaturing Electrophoresis and Antibody Blotting—First dimensional agarose gel electrophoresis and second dimension polycrylamide gradient gel electrophoresis were carried out at 0°C as previously described (5). Initial electrophoresis was in 0.75% agarose in 50 mM barbital buffer (pH 8.6) on Gelbond (FMZ, Rockville, ME). Duplicate agarose gel electrophoretic slabs were developed in the second dimension on each 14 x 16 cm-2 15% polycrylamide gradient slabs. In some experiments, electrophoretic transfer of the whole slab to nitrocellulose membrane (0.45 μm Sartorius, West Coast Scientific, Oakland, CA) (20) was carried out. Each half of the blot was then used for the visualization of different lipoprotein antigens. In other experiments, one-half of the double blot was used for transfer, to provide a template to identify cholesterol radioactivity in the other half, which was meanwhile kept overnight at 0-2°C in foil. To identify lipoprotein antigens, nitrocellulose blots were incubated with 5% bovine serum albumin in 50 mM Tris-HCl buffer (pH 7.4) (50 min, 37°C), and then with purified specific antibody against LCAT, apoA-I, apoD, HDL, and/or apoB, followed by goat anti-rabbit IgG preabsorbed against human IgG (Sigma), labeled with ¹²⁵I (21). In spite of preadsorption, in the visualization of low concentration antibodies, a residual reaction with the IgG of human plasma was sometimes detectable, migrating near the first dimension origin; however, this did not overlap in migration position with any of the lipoproteins studied in this research. Unbound antibody was removed with 2% bovine serum albumin containing 0.9% NaCl, 0.3 mM EDTA (pH 7.4), and extracted with methanol and chloroform (22). Free and ester cholesterol radioactivity was separated by thin layer chromatography on silicic acid layers developed in hexane/diethyl ether/acetic acid (83:161, v/v/v). Radioactivity in the other half, which was meanwhile kept overnight at 0-2°C in foil, to identify lipoprotein antigens, nitrocellulose blots were incubated with 5% bovine serum albumin in 50 mM Tris-HCl buffer (pH 7.4). Autoradiographs were made on Kodak XAR-2 film at ~70°C with two intensifier screens (Cronex, E. I. du Pont de Nemours & Co., Wilmington, DE). The autoradiographs were used as a template to trace the outline of individual labeled components on the nitrocellulose sheets. The distribution of antigen was then determined by counting the ¹²⁵I radioactivity in these areas. The migration rate of individual lipoproteins during agarose electrophoresis was standardized with reference to the major lipoproteins classes in plasma (LDL, VLDL, HDL, LDL visualized with Sudan black (5). The nomenclature of antigens following second dimension electrophoresis (β, preβ, and α) is referenced to these migration rates (e.g., LpA-IpreB, LpA-Io refer to lipoproteins containing apoA-I with preβ and α migrating specifically).

Determination of Free and Ester Cholesterol Radioactivity—Identification of lipoprotein-associated labeled free and esterified cholesterol on gels was carried out using an apolipoprotein autoradiogram as template, as described above. Reactive areas were cut out and eluted with 0.9% NaCl, 0.3 mM EDTA (pH 7.4), and extracted with methanol and chloroform (22). Free and ester cholesterol radioactivity was separated by thin layer chromatography on silicic acid layers developed in hexane/diethyl ether/acetic acid (83:16:1, v/v/v). Radioactivity was determined by liquid scintillation spectrometry. Since LCAT is inhibited by sulfhydryl reagents such as diethiothreitol (2-nitrobenzoic acid) (DTNB) (23), the LCAT dependence of esterification in these experiments was confirmed by including in all experiments control incubations of cultured cells, in which a final concentration of 1.5 mM DTNB was included in the plasma. It has been previously shown (2) that DTNB does not inhibit the efflux of cell-derived labeled cholesterol, or the transfer of preformed LCAT-derived cholesterol esters between plasma lipoproteins (24). The recovery of free cholesterol from plasma during the electrophoresis and extraction procedures was 81 ± 7 and 88 ± 5%, respectively.

Mass Assays of Cholesterol Transport and Cholesteryl Ester Transfer—Plasma-free cholesterol utilized for LCAT-mediated esterification was determined as the difference between the initial and final free cholesterol contents of plasma during the electrophoretic transfer. Plasma cholesterol was determined with cholesterol oxidase and homovanillic acid in the modified fluorimetric assay previously described (25).
RESULTS

The Distribution of Apolipoproteins A-I, B, D, LCAT, and CETP in Plasma—As shown in Fig. 1, apoA-I immunoreactivity consists of a major α-migrating band containing the bulk of this antigen (about 95%) while the remainder was present in several punctate regions with slower (preβ) migration. Two components identified as early acceptors of cell-derived free cholesterol radioactivity (LpA-Ipre1 and LpA-Ipre2) have been described previously (5). Two further minor fractions were identified by using extended autoradiography (up to 24 h) and affinity-purified antibodies to reduce nonspecific background radioactivity (Fig. 1). ApoA-I antigen was identified comigrating with the apoB of authentic LDL (0.63 ± 0.20% of total plasma apoA-I) (n = 3). ApoA-I was also present in a third preβ-migrating species (LpA-Ipre3, Fig. 1) which contained 0.24 ± 0.08% of total apoA-I (n = 5).

As shown in Fig. 2, plasma apoB entering the second dimensional electrophoresis gel was present as a single slowly migrating component. The properties of B-containing very low density lipoproteins which did not enter the gel on account of their high molecular weight (26), were studied separately by immunoaffinity chromatography, as described below.

The distribution of plasma LCAT immunoreactivity is shown in Fig. 2. LCAT protein was present in approximately equal proportions with α-migrating HDL, with the minor LpA-Ipre3 fraction, and with apoB in LDL (Fig. 3). Within bulk (α-migrating) HDL, LCAT immunoreactivity was present as three to five punctate areas on the preβ margin of α-migrating apoA-I. An identical distribution of LCAT antigenicity was obtained with anti-LCAT monoclonal antibody and with site-directed antibodies against the NH2-terminal and median regions of LCAT.

Apolipoprotein D preferentially associates with LCAT in native plasma (27–29). Most apoD immunoreactivity (85.5 ± 6.8%, n = 5) was present in α-migrating HDL (Fig. 2). Additional apoD immunoreactivity (7.0 ± 3.7%) was located with apoA-I in LpA-Ipre3, where it also comigrated with LCAT. The balance of apoD was detected in LDL.

FIG. 1. Distribution of apoA-I in normal plasma determined by two-dimensional gel electrophoresis. First dimensional agarose electrophoresis and second dimensional non-denaturing polyacrylamide gel electrophoresis were carried out as described under "Materials and Methods." Gel proteins were transferred to nitrocellulose membrane. Immunoblotting was carried out first with unlabeled affinity purified rabbit anti-human apoA-I antibody, then with 125I-labeled goat anti-rabbit IgG antibody. Visualization was by autoradiograph. The unlabeled reactive area (lower left) seen in some plasma samples comigrates with isolated delipidated apoA-I in this system.

FIG. 2. Distribution of apoB, LCAT, apoD, and CETP in the same normal plasma sample, determined by two-dimensional gel electrophoresis as described in the legend to Fig. 1. Anti-apoB antibody was raised against LDL and affinity purified. Anti-LCAT and anti-CETP antibodies were a site directed antibody raised in rabbits against residues 165-183 (LCAT) and 290-306 (CETP) of the mature proteins, respectively. Anti-apoD antibody was a polyclonal antibody raised against pure apoD in rabbits then affinity-purified. Labeling was with 125I-labeled goat anti-rabbit IgG antibody. Arrows in the upper right panel indicate multiple punctate reaction areas of LCAT in HDL.
accumulation of labeled esters in the plasma. However, as shown in Fig. 4, further incubation at 37°C then led to the appearance of a linear accumulation of labeled esters in the plasma.

After 5 min of incubation (and 2-3 min after the first appearance of labeled cholesteryl esters in the plasma), the major part of such esters (91 ± 5% of total cholesteryl ester radioactivity) was recovered along with LCAT in those gel regions identified with anti-LCAT antibody (Table I). This finding suggests that cholesteryl ester remains associated with this time period with those lipoproteins containing the active LCAT which generated it.

No detectable cholesteryl ester radioactivity was present along with apoB and LCAT in LDL under these conditions, although the apoB fraction did show considerable free cholesterol radiolabel (33.3 ± 4.1%, n = 4) of total plasma-free cholesterol label over the same time course.

These data suggested that the LCAT located in LpA-I subpopulations may have been metabolically active with cell-derived cholesterol, while that present in LDL was either inactive or reactive mainly with the unlabelled cholesterol of plasma lipoprotein origin. To further investigate this concept, the excised polyacrylamide gel LDL fraction containing LCAT, apoA-I, and free cholesterol radioactivity was further incubated (24 h) at 37°C. There was no detectable esterification of free cholesterol under these conditions, supporting the concept that this fraction of enzyme was not reactive with the labeled cell-derived cholesterol.

Evidence for the Preferential Utilization of Cell-derived Cholesterol by Plasma LCAT—The results described above indicated that cell-derived cholesterol was utilized by LCAT in native plasma, confirming mass data obtained earlier with endothelial cells and fibroblasts (2, 3). These data also suggested that when equivalent amounts of cellular and plasma lipoprotein cholesterol were present, there was a preferential utilization of the cellular cholesterol. Channeling of cell-derived cholesterol to preβ HDL species (LCAT-I subpopulations) and cholesterol esterified was metabolically active with cell-derived cholesterol. Evidence for the Preferential Utilization of Cell-derived Cholesterol by Plasma LCAT—The results described above indicated that cell-derived cholesterol was utilized by LCAT in native plasma, confirming mass data obtained earlier with endothelial cells and fibroblasts (2, 3). These data also suggested that when equivalent amounts of cellular and plasma lipoprotein cholesterol were present, there was a preferential utilization of the cellular cholesterol. Channeling of cell-derived cholesterol to preβ HDL species (LCAT-I subpopulations) and cholesterol esterified was metabolically active with cell-derived cholesterol.

Extending this concept, the preferred utilization of cell-derived cholesterol, to the LCAT reaction, was first examined by measuring the percent of total free cholesterol mass esterified in plasma, and the percent of labeled cholesterol esterified, when native plasma was incubated with the [3H]cholesterol-labeled cells. As shown in Table II, comparison of these rates consistently indicated a greater rate of esterification of cholesterol derived from the labeled cells than of pre-existing unlabeled plasma lipoprotein free cholesterol. Since two studies show that the supply of free cholesterol substrate for LCAT from other lipoproteins (mainly LDL) is not rate limiting (24, 30) this greater rate appeared to represent a true preferential utilization of cell-derived cholesterol. On average, the proportion of labeled cell-derived cholesterol

### Table I

Percent distribution of [3H]cholesterol ester radioactivity among LCAT species

| Experiment | LCAT-LpA-I | LCAT-LpA-Isubpop | LCAT-LpA-I |
|------------|------------|-----------------|------------|
| 1          | 10         | 58              | 30         |
| 2          | 2          | 13              | 85         |
| 3          | 2          | 31              | 69         |
| 4          | 0          | 20              | 80         |
| 5          | 3          | 38              | 59         |

Mean ± S.D. 3.4 ± 3.8 32.0 ± 17.4 64.6 ± 21.8
Plasma-free cholesterol used for LCAT-mediated esterification was determined as the rate of decrease in plasma-free cholesterol mass as a function of time; percent esterification/minute is expressed relative to the original mass of plasma free cholesterol. The percent of cell-derived cholesterol esterified as a function of time is expressed as the ratio of free and ester cholesterol radioactivity. Incubation of [3H]cholesterol-labeled cells with native plasma was carried out at 37 °C as described under "Materials and Methods."

The metabolism of labeled cholesteryl esters derived from LCAT was traced as cholesterol as a whole. The location and further metabolism might be further channeled from LpA-Iprepa, by a pathway in which LDL (on average <2%). In the pulse-chase experiments shown in Table III, even after further 15 min of incubation there was no increase in labeled cholesteryl esters associated with apoB in this fraction. This result provides additional support for the concept that at least in fasting normal plasma, LDL was not a preferred acceptor of cell-derived cholesteryl esters.

Fractionation of Plasma apoB Lipoprotein Label by Immunoaffinity Chromatography—In native plasma, choleseryl ester mass transfer takes place not only to LDL but also to larger apoB-containing lipoproteins (very low density lipoproteins) (24, 31, 32). One possible explanation of the preceding results was that transfer of labeled cholesteryl esters took place predominantly to the larger apoB particles (VLDL) that would not enter the second dimensional gradient electrophoresis gel. A further investigation was carried out in which these lipoproteins were separated instead by heparin-agarose or anti-apoB immunoaffinity chromatography. After 5 min of incubation, 10.9 ± 7.2% (n = 3) of cholesteryl ester radioactivity was detected in the retained fraction containing VLDL and LDL following heparin-agarose and an average of 5.1% (n = 2) of ester label after immunoaffinity chromatography. Fractionation by heparin-agarose chromatography following 15 min of incubation with [3H]cholesterol-labeled cells gave 4.2 ± 3.2% of label in the retained fraction containing VLDL and LDL. These data are compatible with those reported above in which LDL cholesteryl ester radioactivity was analyzed directly following electrophoresis, in suggesting that only a small proportion of cell-derived cholesterol esterified by LCAT is transferred directly to VLDL and LDL within 15 min of synthesis. While the longer term equilibration of cholesteryl esters from HDL to VLDL and LDL is not true.

### Table II

**Esterification of Cell-derived Cholesterol**

| Experiment | LCAT | Plasma free cholesterol esterified/minute (a) | Labeled cholesterol esterified/minute (b) | % | % | b/a |
|------------|------|------------------------------------------|-----------------------------------------|----|----|----|
| 1          | 65.5 | 0.125                                    | 0.910                                   | 7.3 |
| 2          | 11.9 | 0.026                                    | 0.237                                   | 9.1 |
| 3          | 32.4 | 0.074                                    | 0.667                                   | 9.0 |
| 4          | 36.3 | 0.084                                    | 0.447                                   | 3.3 |
| 5          | 33.1 | 0.066                                    | 0.777                                   | 11.8 |
| 6          | 30.9 | 0.064                                    | 0.413                                   | 6.5 |
| 7          | 50.3 | 0.086                                    | 0.717                                   | 8.3 |
| Mean ± S.D. | 36.9 ± 16.2 | 0.075 ± 0.050 | 0.595 ± 0.237 | 8.2 ± 2.1 |

### Table III

**Effects of an unlabeled plasma chase on the distribution of [3H]cholesterol ester radioactivity among LCAT species**

Plasma was incubated with [3H]cholesterol-labeled fibroblasts for 5 min at 37 °C to generate labeled cholesteryl esters (pulse), and a sample was taken for determination of the distribution of cholesteryl ester radioactivity following two-dimensional electrophoresis. Further cholesteryl ester synthesis was inhibited by addition of a final concentration of 1.5 mM DTNB, and the plasma immediately transferred to an equivalent monolayer of unlabeled cells. A further incubation was then carried out at 37 °C for 15 min (chase), and a second sample was taken for separation by two-dimensional electrophoresis as described under "Materials and Methods." Lipoprotein lipid radioactivity was extracted and cholesteryl ester radioactivity determined by thin layer chromatography on silicic acid. –, no detectable radioactivity.

| Experiment | LCAT-LpA-Ip | LCAT-LpA-Ipm | LCAT-LpA-Ipre | LCAT-LpA-Isp |
|------------|-------------|--------------|---------------|--------------|
| 1          | 700         | 4,400        | 28,700        | –            | 150          | 37,250       |
| 2          | 400         | 6,000        | 12,950        | –            | 250          | 22,800       |
| 3          | –           | 2,100        | 8,400         | –            | –            | 14,100       |
| 4          | 450         | 5,450        | 8,500         | 150          | –            | 13,200       |
| Mean ± S.D. | 388 ± 290 | 4,488 ± 1,745 | 14,638 ± 9,612 | 38 ± 75 | 100 ± 122 | 21,836 ± 11,150 |
out, the data do suggest that most of cholesteryl esters generated from cell-derived cholesterol are initially transferred to α-migrating HDL.

**DISCUSSION**

The data above provide evidence that the preβ-migrating fraction of HDL and in particular the minor fraction here designated LpA-1preB, plays a major role in the transport, esterification, and transfer of cell-derived cholesterol. Evidence has also been obtained for a channeling of cell-derived free cholesterol through the LCAT reaction in plasma. This suggests that cell- and lipoprotein-derived free cholesterol may be metabolized differently in plasma. As one aspect of this, data from several types of fractionation experiments suggested that, at least in the plasma of normal fasting donors, cell-derived free cholesterol after esterification was selectively transferred to α-migrating HDL.

Several earlier studies from this laboratory and others have shown that preβ-migrating HDL, which contains apoA-I but not apoA-II, is relatively enriched in LCAT and CETP and is required for the transport of cholesterol from cell membranes into plasma (2, 29, 33). The same fraction was shown previously to include two early acceptors of a major part of cell-derived cholesterol (5). Improved visualization of apoA-I here permits the identification of three subfractions of preβ HDL, which differ in migration rate during nondenaturing polyacrylamide gel electrophoresis, together with an additional small proportion of apoA-I comigrating with apoB in LDL. The two major fractions of preβ HDL (LpA-1preA and LpA-1preB) (each containing 1–2% of total apoA-I) (5) included no LCAT or CETP and contained no labeled cholesteryl esters when plasma was incubated with [3H]cholesterol-labeled cells (5). The third (here termed LpA-1preC), which contains about 0.2% of total plasma apoA-I, also included a major part of total plasma LCAT immunoreactivity, and substantial proportions of CETP and apoD.

We considered the possibility that the distribution of LCAT in HDL had been modified during electrophoresis. However, when plasma was preincubated with cellular [3H]cholesterol, about one-third of total plasma-labeled cholesteryl ester was recovered along with the small amount of apoA-I in LpA-1preA, while the remainder was recovered with LCAT in α-migrating HDL. Since the early distribution of labeled cholesteryl esters in HDL is very similar to the distribution of LCAT in the same lipoprotein, it seems likely that the distribution of LCAT observed after two-dimensional electrophoresis reflects that originally present in plasma before electrophoresis. The remainder of LCAT in plasma (about one-third of total immunoreactivity) was associated with apoB in the β-migrating LDL region. This was not associated with any detectable cholesteryl ester radioactivity, neither was the labeled free cholesterol which was transferred there early in incubation esterified when the isolated gel section containing LDL and LCAT was subsequently incubated at 37 °C. It has been suggested that LCAT associated with LDL may be active not in cholesterol esterification but in the exchange of acyl groups among LDL phospholipids (34). It is also possible that this fraction of LCAT reflects a catabolic compartment. In any case, it seems clear that LDL-associated LCAT is not a significant component of the mechanism which esterifies cell-derived cholesterol in plasma.

A lipoprotein complex ("cholesteryl ester transfer complex") containing apoA-I and apoD and active in the esterification and transfer of cholesterol was earlier defined on the basis of data obtained by immunoaffinity chromatography (29). At that time apoD itself, a component of partially purified CETP (35) was suggested as the candidate transfer protein. Our subsequent isolation and sequencing of both CETP and apoD (16, 36) have shown these to be distinct proteins. ApoD is structurally closely related to a protein family (which includes retinol binding protein) binding hydrophobic organic ligands (37); its exact role in the esterification and transfer of cholesterol remains to be defined. However, the presence of both proteins, with LCAT and apoA-I, in LpA-1preA, obviously suggests the identity of this fraction with the functional complex esterifying and transferring cholesterol in plasma that was described earlier (29). Although cell-derived free cholesterol was found as early as 1 min in preβ HDL, there was a lag of approximately 2 min before the first appearance of detectable cholesteryl esters. On further incubation, a major proportion of total labeled cholesteryl esters appeared in LpA-1preB. Furthermore the addition of DTNB after the first appearance of cholesteryl esters prevented the accumulation of labeled esters there. These data strongly suggest that the labeled cholesteryl ester present in LpA-1preA, under these conditions was synthesized there by the LCAT identified in this fraction. The same lipoprotein also contained considerable CETP immunoreactivity. Pulse-chase experiments were carried out to follow the further metabolism of labeled cholesteryl esters in LpA-1preA. These demonstrated that labeled cholesteryl esters in this lipoprotein were rapidly turned over, and that the label was recovered in the "bulk" or α-migrating HDL. In the absence of a specific inhibitor of CETP, it cannot be proven that the CETP in LpA-1preA was functional in this transfer. However, this seems likely, in view of the relative lack of specificity of this factor in the transfer of cholesteryl esters between different lipoproteins (6, 7). The cholesteryl esters already present in α-migrating HDL after 5 min of incubation with labeled cells might represent either free cholesterol esterified there by resident LCAT; or the products of earlier transfer of esters synthesized in LpA-1preB. What does appear evident, however, is that LpA-1preA contains very little (0.2%) of total apoA-I, and relatively a major proportion (30%) of LCAT and of CETD-derived cholesteryl ester (30%) that are rapidly generated and transferred in plasma. On the basis of these data, we suggest that LpA-1preA, represents the structural equivalent of the transfer complex earlier proposed on functional grounds (29).

Finally, the structural and biochemical data in this study have instructive parallels with observations of plasma cholesteryl metabolism made in vivo. A major part of plasma cholesteryl esters may be derived from a small, rapidly recycling pool (38). These data are compatible with the role suggested for LpA-1preA, on the basis of the present short term, in vitro experiments. Computerized analysis of human cholesterol metabolism has also suggested compartmentalization of HDL cholesterol (39). The present data suggest a structural basis that might account for this type of metabolic channeling. It is important to emphasize that the present data, obtained with normal fasting plasma, may not be representative of cholesterol transport postprandially, when the transfer of cholesteryl ester mass from LCAT to VLDL and LDL is more important (32) or in hyperlipidemic or other abnormal plasma samples, where the distribution of preβ- and α-migrating HDL is known to be abnormal (40). However, the techniques described here may be useful in further studies of these conditions. What appears clear is that plasma cholesterol metabolism is much more complex than previously conceived, and that a detailed analysis not only of total LCAT, CETP, and other factors, but of the distribution and activity of these within HDL subfractions, will be required to understand what
regulates the flow of cholesterol through the plasma.

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