High density lipoprotein subfractions isolated by heparin-Sepharose affinity chromatography and their role in cholesteryl ester transfer to very low density lipoproteins

Yves L. Marcel, Camilla Vézina, Diane Emond, Roy B. Verdery, and Ross W. Milne

Laboratory of Lipoprotein Metabolism, Clinical Research Institute of Montreal, 110, Pine Avenue West, Montreal H2W 1R7, Quebec, Canada

Abstract Normal human plasma HDL was applied to a column of heparin-Sepharose in the presence of $MnCl_2$ and three fractions were obtained by stepwise elution with increasing NaCl concentrations: a non-retained fraction (NR, 78% of protein) and two retained fractions (R_1 and R_2, 18 and 2.5% of protein, respectively). Both unesterified and esterified cholesterol increased from NR to R_1 to R_2 but the increment was more pronounced for unesterified cholesterol. ApoA-II to apoA-I ratio was lower in R_1 compared to NR but R_1 contained more apoC than NR. ApoE increased from NR to R_1 to R_2 (0.07, 0.4, and 14% of protein in each fraction, respectively) while apoB was found only in R_2. Agarose gel electrophoresis and immunoadsorbers for apoB and apoE showed that R_2 consisted of two major lipoprotein populations, one containing apoB and some apoE and the other containing apoE and no apoB. Cholesteryl ester transfer between each HDL subfraction and VLDL in the presence of partially purified cholesterol ester transfer protein was studied. NR and R_1 gave the highest initial rates of transfer for labeled cholesteryl ester which were corroborated by significant mass transfer of cholesteryl esters. From these results, we concluded that there is no connection between cholesteryl ester transfer and apoE. On the other hand, transfer from R_2 to VLDL followed different kinetics with a high zero hour transfer but with subsequently lower rates when compared to NR and R_1. The cholesteryl ester transfer activity in R_2 was mainly due to the presence of apoE-containing lipoproteins whereas those containing apoB had minimal transfer activity. However, because this transfer of label was not translated into significant mass transfer of cholesteryl ester to VLDL, the apoE-containing lipoproteins appear involved mainly in the equilibration of cholesteryl esters.—**Marcel, Y. L., C. Vézina, D. Emond, R. B. Verdery, and R. W. Milne.** High density lipoprotein subfractions isolated by heparin-Sepharose affinity chromatography and their role in cholesteryl ester transfer to very low density lipoproteins. *J. Lipid Res.* 1981. **22:** 1198–1205.

Supplementary key words apoE · apoB · apoC · LCAT

Protein-mediated transfer of cholesteryl esters between lipoproteins (1) has recently been shown to be closely related to cholesteryl ester formation by LCAT (2, 3) and its transport by different lipoproteins. However, conflicting evidence has been presented on the characteristics of the cholesteryl ester transfer proteins (2, 4). In contrast to the equilibrium existing between HDL and LDL (5), we have shown net transfer of cholesteryl esters from HDL to triglyceride-rich lipoproteins at a rate comparable to the reaction rate of LCAT (6). We therefore proposed that this process supplies most of the cholesteryl esters found in plasma lipoproteins and that LDL cholesteryl esters are derived from those originally transferred to VLDL.

Because of the heterogeneity of HDL, we have studied the ability of HDL subfractions to serve as cholesteryl ester donors in the transfer process to VLDL. To this end, HDL subclasses have been isolated as a function of their apoE content by heparin-affinity chromatography according to methods previously described (7, 8). Using these subfractions, we investigated whether apoE-containing HDL played a special role in cholesteryl ester transfer, as previous studies had indirectly suggested (9, 10).

MATERIALS AND METHODS

Preparation of lipoproteins

Blood from normal volunteers was collected in blood packs containing citrate–phosphate–dextrose (Fenwall Laboratories, Deerfield, IL). Upon separation of the plasma, sodium azide was added (1 mg/ml) to all samples and this concentration was maintained throughout dialysis and handling.

Abbreviations: CETP, cholesteryl ester transfer protein: LCAT, lecithin:cholesterol acyltransferase.

Plasma lipoproteins were obtained by sequential preparative ultracentrifugation at 5°C in a Beckman 50.2 Ti rotor (11, 12). Chylomicrons were removed from the plasma of postprandial donors by flotation (1.1×10^6 g-min) through a layer of saline (0.15 M NaCl). Density for all other separations was adjusted by addition of solid KBr. VLDL was isolated at d 1.006 g/ml; HDL was isolated between the densities of 1.065 and 1.21 g/ml.

HDL cholesterol was labeled with $|1\alpha,2\alpha(n)^3H|$-cholesterol (Amersham, Arlington Heights, IL) as follows. The dialyzed plasma fraction of d > 1.065 (90–250 ml) was incubated for 20 hr at 37°C with 2–5 mCi of [^{3}H]cholesterol dissolved in 0.5 ml of ethanol. The density was then raised to 1.21 g/ml with solid KBr and the labeled HDL was isolated by preparative ultracentrifugation.

Heparin-Sepharose affinity chromatography

Heparin-Sepharose was prepared by covalent linkage of 120 mg of pig mucosal heparin (Upjohn, Don Mills, Ontario) per 50 ml of CNBr-activated Sepharose-4B (Pharmacia) (13). Chromatography was done according to the method of Weisgraber and Mahley (8). Typically, HDL (60–120 mg of protein) was dialyzed against 5 mM Tris Buffer, pH 7.5, 50 mM NaCl, and made up to 25 mM $MnCl_2$ prior to application. The sample was applied to the column (1.6 × 25 cm) conditioned with the same buffer and the HDL was allowed to equilibrate with the column overnight. The non-retained fraction (NR) was eluted with the starting buffer, and two other fractions (R_1 and R_2) were eluted with the same buffer without $MnCl_2$, but with 70 mM and 300 mM NaCl, respectively.

Iodination of cholesteryl ester-labeled HDL subfractions

Cholesterol-labeled HDL was fractionated by heparin-Sepharose chromatography as described above and the HDL subfractions (NR, R_1 and R_2; 1 mg protein/ml) were dialyzed against 0.1 M borate buffer, pH 8.5. After iodination with ^{125}I by the Bolton-Hunter procedure (14), the doubly labeled lipoproteins were exhaustively dialyzed against 10 mM Tris, 1 mM EDTA, pH 7.4, and filtered before use. Distribution of ^{125}I among apoproteins of each HDL subfraction was determined by SDS polyacrylamide gel electrophoresis (15). After staining and identification of the various apoproteins by molecular weight, the gel was cut into 2-mm slices with a multiple blade gel slicer (BioRad, Richmond, CA) and the ^{125}I was measured.

Assay system for the study of cholesteryl ester transfer between lipoproteins

Cholesteryl ester transfer protein was partially purified as described by others (4) from the plasma fraction of d > 1.25 g/ml. The fraction retained by phenyl-Sepharose was used as the source of cholesteryl ester transfer protein in most experiments and in some experiments this was further purified by chromatography on Con A-Sepharose and CM-cellulose (4).

HDL or HDL subfractions labeled with radiocholesterol (15 μg of cholesteryl ester) were incubated with unlabeled VLDL (20 μg of cholesteryl ester, unless otherwise stated) in the presence of CETP (140 μg of protein) with or without albumin (3 mg/ml, human albumin, Pentex, Montreal, Quebec). LCAT activity was inhibited by the addition of DTNB (1.4 mM). Final volume was adjusted to 1 ml with 10 mM Tris, 0.15 M NaCl, 1 mM EDTA (pH 7.4) and the mixtures were incubated at 37°C for various times. In some control experiments, the VLDL was omitted from the incubations. In time course experiments, the zero hour value represents the mixing of incubation components at 4°C and their further processing for centrifugation at the same temperature. Immediately after incubation, the mixture was chilled to 4°C, overlayered with cold saline solution (0.9%), and centrifuged in a Beckman 40.3 rotor with 2-ml adaptors to float VLDL. The fractions d < 1.006 g/ml and d > 1.006 g/ml were quantitatively recovered and analyzed for cholesterol concentration and radioactivity. In some experiments, each centrifugal fraction was analyzed both for cholesterol and for distribution of apoprotein radioactivity. Separation and counting procedures for cholesterol and cholesteryl ester were as described previously (6). The amount of cholesteryl ester transferred to VLDL was calculated from the ratio of radioactivity in VLDL cholesteryl ester to the total radioactivity recovered in VLDL and HDL cholesteryl ester, and from the initial cholesteryl ester concentration in HDL.

In selected experiments, VLDL was linked to CNBr-activated Sepharose (13) and the appropriate weight of VLDL-Sepharose to supply the desired amount of VLDL cholesteryl esters was included in the incubation mixture instead of VLDL. After incubation, the supernatant was collected, the VLDL-Sepharose was washed 3 times with 10 ml of Tris-buffered saline containing 1% (w/v) bovine serum albumin and analyzed for cholesterol ester as described above.

Analyses

Protein levels were measured by the method of Lowry et al. (16) using bovine serum albumin as a

standard. Total cholesterol and triglycerides were measured enzymatically (17, 18) with an auto-analyzer (ABA-100 bichromatic analyzer, Abbott Laboratories). Unesterified cholesterol was determined by gas–liquid chromatography (19) and esterified cholesterol was calculated from the levels of total and unesterified cholesterol. Total phospholipids were measured as described previously (20).

Apolipoproteins were analyzed by SDS polyacrylamide gel electrophoresis (15) and by analytical isoelectric focusing on polyacrylamide gel (21). Lipoproteins were electrophoresed on agarose gel (Agarose C, Pharmacia) by the method of Noble (22). ApoE and apoB concentrations were measured by radioimmunoassays using monoclonal mouse anti-apoE antibody and rabbit anti-apoB IgG, respectively (23).

RESULTS

Separation of HDL subfractions by heparin-Sepharose affinity chromatography

With the stepwise elution system (10), a very reproducible series of HDL subfractions was obtained both within and between HDL samples. The nonretained fraction (NR) represented 78 ± 0.4% of HDL protein; the retained fraction eluted with 70 mM NaCl (R_1) represented 18 ± 0.2% and the retained fraction eluted with 0.3 M NaCl (R_2) represented 2.5 ± 0.3%. The recovery of protein was always greater than 95%.

Relative composition of these HDL subfractions was determined in four different HDL preparations (**Table 1**). Retained fractions, R_1 and R_2, were characterized by lower protein content compared to NR. This difference translated into significantly higher total cholesterol in R_1 and R_2. Both unesterified and esterified cholesterol increased from NR to R_1 to R_2 but the increment was more pronounced for unesterified cholesterol (12.5, 17.6, and 21.3% of total cholesterol in NR, R_1, and R_2, respectively).

TABLE 1. Relative composition of HDL subfractions isolated by heparin-Sepharose chromatography

Subfraction	Protein	Total Cholesterol	Triglycerides	Phospholipids
		%		
NR	57.5 ± 5.4[a]	13.3 ± 0.6	3.7 ± 1.0	25.1 ± 4.5
R_1	52.3 ± 3.3	15.9 ± 2.3[b]	3.5 ± 0.6	28.1 ± 2.4
R_2	51.6 ± 3.7	24.2 ± 3.2[c]	3.3 ± 2.3	20.8 ± 1.5

[a] Mean and S.D. (n = 4).
[b] Significantly higher compared to NR ($P < 0.05$).
[c] Significantly higher compared to NR ($P < 0.01$).

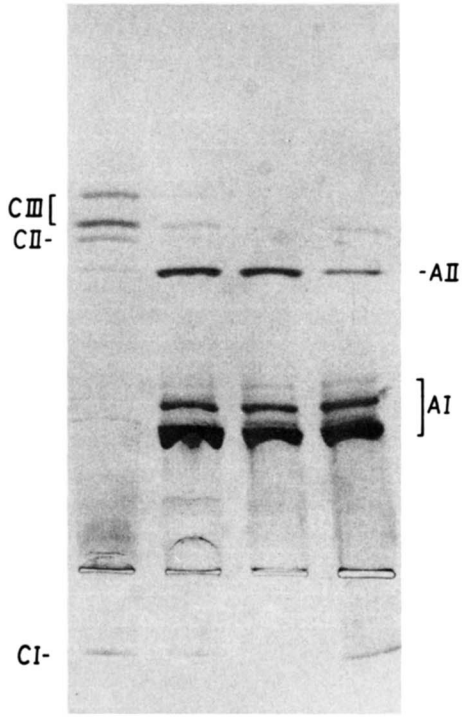

Fig. 1. Analytical isoelectric focusing on polyacrylamide gel of HDL subfractions isolated by heparin-Sepharose affinity chromatography. From left to right: apo VLDL, apo HDL, apo NR, and apo R_1 (120 μg protein each).

Apolipoproteins of each fraction were analyzed by analytical isoelectric focusing and electrophoresis on polyacrylamide. NR and R_1 had different ratios of apoA-I to apoA-II and R_1 contained more apoC than NR (**Fig. 1.**). Radioimmunoassays of apoB and apoE indicated that all apoB was present within the R_2 fraction while apoE concentration increased progressively from NR to R_1 to R_2 (**Table 2**). Radiolabeled apoproteins of each HDL subfraction were separated by SDS gel electrophoresis and the radioactivities of the various molecular weight zones were counted. The apoproteins of the NR and R_1 fractions had a similar distribution of radioactivity, with 50% present within the molecular weight range of apoA-I and 20% present within the molecular weight range of apoA-II and apoC (**Table 3**). The apoproteins of the R_2 fraction were clearly different, with 50% of the radioactivity present in the apoB region, 13% in the apoA-I region, and only 9% in the apoA-II-apoC region. In contrast, the distribution of radioactivity in the molecular weight range of apoE did not differ significantly between HDL subfractions.

Agarose-gel electrophoresis of the HDL subfractions clearly demonstrated the heterogeneity of the R_2 fraction which was resolved into two major components (**Fig. 2**): the slower component comigrated

TABLE 2. Concentration of apoE and apoB in HDL subfractions

HDL Subfractions	apoE	apoB
NR	0.7 ± 0.7[a]	0[b]
R_1	4.0 ± 5.7	0
R_2	140 ± 149	40 ± 30

[a] Values are expressed as the ratio of apoE or apoB protein to total protein × 10^3 (mean ± S.D., n = 11).
[b] Not detectable.

with LDL and could be removed by passage over an immunoadsorber column for apoB; the faster component migrated slightly ahead of VLDL and could represent Lp(a) and/or the apoE-containing HDL. Overloading allowed the observation of a third component which had an α-migration close to that of the R_1 fraction (not seen on Fig. 2). Agarose gel electrophoresis also indicated some degree of heterogeneity within the R_1 fraction, exhibiting two closely migrating bands in the α-lipoprotein region. The nature of this heterogeneity remains uncharacterized.

Role of HDL subfractions in cholesteryl ester transfer to VLDL

Normal plasma HDL was labeled in the cholesterol and cholesteryl ester moieties as described under Methods, applied to the heparin-Sepharose column, and eluted. The resulting NR, R_1, and R_2 subfractions all contained labeled cholesteryl esters with specific activities of 19, 18, and 12 × 10^6 cpm/μg cholesterol, respectively. Cholesterol-labeled HDL subfractions were used to study cholesteryl ester transfer to VLDL. The NR and R_1 fractions were equally effective cholesteryl ester donors to VLDL in the presence of CETP (**Fig. 3**), with an identical initial transfer velocity. The R_2 fraction exhibited very different properties (Fig. 3): it was characterized by a high initial transfer to VLDL, that is, a zero time value for cholesteryl ester transfer which varied from 5 to 30% of the R_2 cholesteryl ester and which may have been related to the unstability of the R_2 fraction causing its adsorption to VLDL. Subsequently, the transfer was a function of time and was linear for 2 hr. When VLDL was omitted from the incubation, the amount of cholesteryl ester from the R_2 fraction that floated at d 1.006 g/ml was markedly reduced and further decreased in the presence of albumin (**Table 4**). Therefore the R_2 fraction appeared to be stabilized by albumin and flotation of its labeled cholesteryl esters at 1.006 g/ml required the presence of VLDL. In addition, the R_2 fraction possessed some endogenous transfer activity whether alone or in the presence of albumin, but the transfer was significantly enhanced by addition of CETP (Table 4).

When the R_2 fraction was passed on an anti-apoE immunoadsorber column, cholesteryl ester transfer was significantly decreased, whereas there was no change caused by passage on an immunoadsorber column against normal mouse serum (**Fig. 4**). On the contrary, passage of R_2 over an anti-apoB immunoadsorber column, which removed most of the apoB but left most of the immuno-reactive apoE unretained, significantly increased the transfer rate to VLDL (Fig. 4). The simultaneous assay of the different HDL subfractions allowed us to compare them for cholesteryl ester transfer to VLDL: NR = R_1 > R_2-unretained on anti B > R_2. Similar results were obtained with each HDL subfraction when VLDL-Sepharose was substituted for VLDL as described under Methods (not shown).

Chemical determination of cholesteryl ester concentrations in VLDL before and after incubation with each HDL subfraction (**Table 5**) demonstrated that NR and R_1 could give significant net transfer of cholesteryl ester to VLDL whereas that measured with R_2 did not reach significance. In addition, the cholesteryl ester concentration in VLDL at 0 hr of the incubation with R_2 (34.0 ± 1.0 μg) was not significantly different from that of VLDL incubated without R_2 (33.7 ± 1.4 μg) and thus does not corroborate the high initial transfer of labeled cholesteryl ester found with R_2.

In an attempt to define the mechanism of cholesteryl ester transfer better, HDL subfractions were doubly labeled with [^{3}H]cholesterol and ^{125}I. These HDL subfractions were then incubated with VLDL in the presence of cholesteryl ester transfer protein. As a function of time, there was significant transfer of label for both cholesteryl ester and apoprotein, but the transfer of cholesteryl ester was faster than that of apoproteins (**Table 6**). Analysis of the apoprotein radioactivity in the VLDL fraction by SDS gel electrophoresis demonstrated that, in addition to the known equilibration of label of apoC between HDL and VLDL, other transfers or equilibration took place (**Table 7**). With NR, R_1, and R_2,

TABLE 3. Relative composition of apoproteins in ^{125}I-labeled HDL subfractions analyzed by SDS polyacrylamide gel electrophoresis

Fraction Analyzed	Percent Radioactivity in Various Molecular Weight Regions					
	>80[a]	70–50	40–32	31–25	22–18	<15
NR	2.0[b]	10.3	5.6	53.5	0.9	21.3
R_1	4.4	15.0	4.8	49.8	1.1	17.1
R_2	51.5	10.5	6.3	13.3	2.2	9.4

[a] Expressed as k daltons.
[b] Mean of three determinations (S.D. ≤ 10% of the mean).

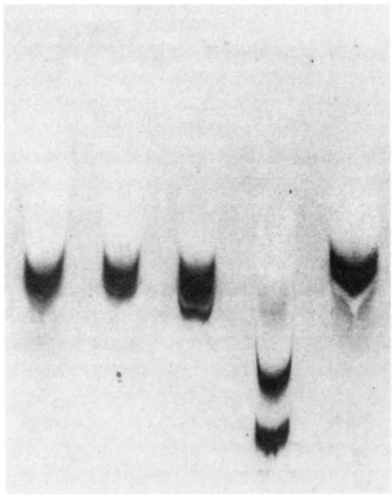

Fig. 2. Agarose gel electrophoresis of HDL subfractions isolated by heparin-Sepharose affinity chromatography. From left to right: HDL, NR, R_1, R_2, HDL.

there was a significant transfer of label within the molecular weight range of apoA-I; but only with R_2 was there a transfer of radioactivity in the molecular weight region of apoE and in the region of 22 to 18,000 daltons. When apoE levels were measured by radioimmunoasay in VLDL before and after incubation with HDL subfractions, there was no significant change although the variation in the assay (6%) may have been too important to measure an hypothetical transfer of apoE in the range of 10% of VLDL levels.

DISCUSSION

Application of the HDL to heparin-Sepharose in the presence of Mn^{2+} and stepwise elution as described by Weisgraber and Mahley (10) yielded three fractions which were different from those previously described. However, HDL subfractions retained by heparin always share common characteristics: as in previous works (9, 10), fractions retained on heparin-Sepharose were richer in total cholesterol compared to nonretained fractions. Also significant was the progressive increase in relative concentration of unesterified cholesterol with increasing heparin affinity of the fractions ($R_2 > R_1 > NR$). The relative concentration of apoE in the R_1 fractions, 0.4% of total protein, was far too low to explain the affinity of this fraction for heparin on the basis of one apoE molecule per particle. Both NR and R_1 fractions contained apoA-I and apoA-II as their major apoproteins, but the densitometric scanning of the isoelectric focusing gel electrophoretograms of their apoproteins indicated that the ratio of apoA-II to apoA-I was significantly lower in R_1 compared to NR, but that this decrease in apoA-II in R_1 was accompanied by an increase in apoC-I, apoC-II, and apoC-III in this fraction (Fig. 1). Since it could not be explained on the basis of its apoE content, the retention of R_1 on heparin-Sepharose might be related to a secondary affinity process: the original ligand, heparin, binds apoE and/or apoE-containing lipoproteins, which become a secondary ligand; these bound apoE molecules may have an affinity for certain HDL particles such as R_1 and thus cause their retention on the column in the presence of Mn^{2+}. Immunodiffusions with antiserum against apoD have shown the presence of apoD in all fractions from NR to R_1 to R_2, indicating that this apoprotein, which has been proposed as cholesteryl ester transfer protein (2), does not interact with heparin.

The R_2 subfraction was heterogeneous as evidenced by agarose gel electrophoresis (Fig. 2). The relative concentration of apoB and apoE varied greatly from preparation to preparation inasmuch as apoB levels in certain HDL were found to be very low. When R_2 was passed through an anti-apoB immunoadsorber column, the unretained fraction contained no apoB and half of the initial apoE concentration. When R_2 was passed through an anti-apoE immunoadsorber column, the unretained fraction contained no apoE and most of the initial apoB concentration. From these experiments, it can be concluded that the R_2 fraction is composed of at least two populations of lipoproteins, one containing both apoB and apoE and one containing apoE and no apoB.

When cholesteryl ester-labeled HDL was fraction-

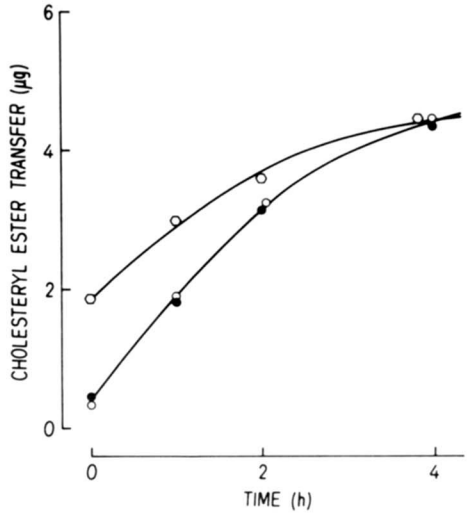

Fig. 3. Time course of cholesteryl ester transfer from cholesteryl ester-labeled HDL subfractions to VLDL: ○——○, NR; ●——●, R_1; ○——○, R_2. Each labeled HDL subfraction (15 μg cholesteryl ester) was incubated with VLDL (20 μg cholesteryl ester) as described under Methods.

TABLE 4. Flotation of cholesteryl esters at d 1.006 g/ml upon incubation of the R_2 fraction with or without VLDL and under various conditions

Additions to Incubation Mixture		Percent of labeled CE in d < 1.006 g/ml at	
VLDL	Protein	0 hr	4 hr
0	0	7.1	4.9
0	CETP[a] (140 μg)	5.0	3.6
0	Albumin (3 mg)	1.9	1.1
+	0	32.4	39.5
+	CETP[a] (140 μg)	26.6	55.7
+	Albumin (3 mg)	18.1	31.3

[a] Cholesteryl ester transfer protein.

The R_2 fraction (14 μg cholesteryl ester) was incubated with or without VLDL (24 μg cholesteryl ester) as described under Methods.

ated on heparin-Sepharose, each subfraction was cholesteryl ester-labeled, but the specific activity calculated for the R_2 fraction was always lower than in NR and R_1 and this difference persisted in the R_2 fraction unretained on anti-B-Sepharose. These results indicate that cholesteryl esters are formed or equilibrate at the same rates in NR and R_1 but not in R_2. These findings corroborate our earlier observations on the lower reaction rates of LCAT with apoE-containing HDL (9), although another interpretation might be that newly formed labeled cholesteryl esters are transferred from R_2 to NR and R_1 during the incubation of d > 1.065 g/ml.

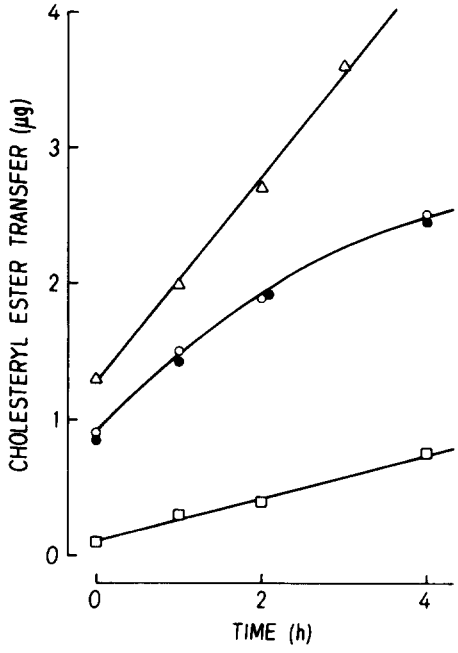

Fig. 4. Time course of cholesteryl ester transfer from cholesteryl ester-labeled R_2 fraction to VLDL: ○——○, original R_2 fraction; ●——●, R_2 unretained on anti-normal mouse serum-Sepharose; □——□, R_2 unretained on anti-apoE-Sepharose; △——△, R_2 unretained on anti-apoB-Sepharose. The various R_2 fractions (15 μg cholesteryl ester each) were incubated with VLDL (20 μg cholesteryl ester) as described under Methods.

NR and R_1 fractions gave the highest rates of cholesteryl ester transfer to VLDL and there was no difference between these two fractions. Cholesteryl ester transfer from NR and R_1 to VLDL was dependent upon the presence of cholesteryl ester transfer protein and was a function of time. As the NR fraction has negligible apoE, it can be concluded that cholesteryl ester transfer between HDL and VLDL can take place independently of apoE. Experiments with HDL subfractions labeled in both cholesteryl ester and apoproteins showed that there were small but significant transfers or equilibration of labeled apoproteins concommitant with cholesteryl ester transfer. With NR and R_1, the transfer to VLDL of apoprotein label was mainly within the molecular weight range of apoA-I in addition to the known transfer and equilibration of apoC. Since VLDL does not contain any significant level of apoA-I, the process of cholesteryl ester transfer from the NR and R_1 subfractions of HDL to VLDL may involve a temporary fusion of the particles which results in a net increase in VLDL cholesteryl esters and in a probable transient transfer of some HDL apoproteins to VLDL. However the definitive demonstration of such a process would require the demonstration by immunoassays that apoA-I increases in VLDL.

The kinetics of cholesteryl ester transfer from R_2 to VLDL were quite different from those of NR and R_1, and were characterized by a high initial transfer at 0 h to VLDL. Although this initial transfer could be decreased in the presence of physiological concentrations of albumin, it could not be eliminated altogether. Experiments have shown that this transfer is not due to the flotation of R_2 fraction at d 1.006 g/ml and that it does require the presence of VLDL (Table 4). R_2 also contains some endogenous cholesteryl ester transfer activity but the process is further enhanced by addition of a partially purified cholesteryl ester

TABLE 5. Chemical determination of cholesteryl ester transfer from individual HDL subfractions to VLDL

HDL Subfraction Added	Cholesteryl Esters (μg) Recovered in VLDL at	
	0 hr	4 hr
NR	34.2 ± 1.7[a]	37.0 ± 1.5 ($P < 0.02$)[b]
R_1	35.7 ± 2.6	38.9 ± 1.9 ($P < 0.05$)[b]
R_2	34.0 ± 1.9	35.0 ± 2.9 (NS)[b]

[a] Mean ± S.D., n = 6.

[b] Significantly higher than 0-hr value or not significantly higher (NS).

VLDL and each HDL subfraction were incubated separately in the presence of the cholesteryl ester transfer protein fraction for 0 and 4 hr and the VLDL was isolated by centrifugation at d 1.006 g/ml for subsequent determination of cholesteryl ester concentrations.

TABLE 6. Transfer of ^{125}I-labeled apoprotein and [^{3}H]cholesteryl ester from HDL subfractions to VLDL in the presence of cholesteryl ester transfer protein

Doubly Labeled HDL Subfraction	Percent of Radioactivity Recovered in VLDL at			
	0 hr		4 hr	
	Cholesteryl Ester	Protein	Cholesteryl Ester	Protein
NR	3.3[a]	1.8	11.5	3.1
R_1	4.2	3.4	15.7	5.1
R_2	8.7	3.5	21.9	6.7

[a] Each value is the mean of three determinations (S.D. ≤ 10% of the mean).

transfer protein fraction. Most importantly, we have shown that within the R_2 fraction, the labeled cholesteryl esters of apoE-containing lipoproteins could transfer to or equilibrate with VLDL whereas those of apoB-containing lipoproteins could not. It should be emphasized that the apoE-containing lipoproteins (R_2 fraction unretained on anti-B-Sepharose) did not react with antisera against apoB and therefore were not contaminated with LDL or Lp(a).

When VLDL-Sepharose was used as a cholesteryl ester acceptor, the transfers of labeled cholesteryl ester from each HDL subfraction were similar to those noted with VLDL as an acceptor, confirming that the transfers do not result from a centrifugal artifact. Determinations of VLDL cholesteryl ester concentrations before and after incubation with various HDL subfractions and after reisolation by centrifugation demonstrated that NR and R_1 could give significant net transfer of cholesteryl ester to VLDL during a 4-hr incubation (Table 5) and corroborated the results obtained with labeled cholesteryl esters. However, when cholesteryl ester levels in VLDL incubated with cholesteryl ester transfer protein and albumin with or without R_2 at 4°C were compared, these levels were the same and therefore do not corroborate the high initial transfer of labeled cholesteryl esters that was found with R_2 (Table 4). This discrepancy may be only apparent and related to an active equilibration of cholesteryl esters between R_2 and VLDL in the absence of mass transfer, but it could also result from artifacts such as intrinsic unstability of R_2 caused perhaps by the incubation of d 1.065 g/ml infranatant or to a loss of VLDL cholesteryl esters upon centrifugation. It should be recognized that the ultracentrifugation used to separate VLDL after incubation was a self-defeating, but necessary, step which may have obscured certain transfer taking place physiologically. Precipitation methods were not practical because they precipitate apoE-containing lipoproteins while the variations observed with VLDL-Sepharose were too important to allow the study of cholesteryl ester mass transfer.

TABLE 7. Transfer of apolipoproteins from ^{125}I-labeled HDL subfractions to VLDL[a] in the presence of cholesteryl ester transfer protein

Fraction Analyzed	Percent of Radioactivity in Various Molecular Weight Regions					
	>80[b]	70–50	40–32	30–23	22–18	<15
Donor NR[c]	2.0	10.3	5.6	53.5	0.9	21.3
VLDL (0 hr)	6.3	4.7	4.5	25.0	2.9	47.0
VLDL (3 hr)	3.7	3.0	3.8	30.6	1.4	51.9
Donor R_1[c]	4.4	15.0	4.8	49.8	1.1	17.1
VLDL (0 hr)	3.9	6.2	2.7	28.2	0.8	49.2
VLDL (3 hr)	1.8	6.0	4.2	35.6	3.0	41.2
Donor R_2[c]	51.5	10.5	6.3	13.3	2.2	9.4
VLDL (0 hr)	18.4	3.3	7.0	8.7	6.4	46.1
VLDL (3 hr)	10.1	1.9	9.9	13.4	11.7	40.6

[a] The values for VLDL (0 and 3 hr) are the mean of two determinations.

[b] Expressed as K daltons.

[c] In each experiment, the first line represents the distribution of radioactive apoproteins in the donor HDL subfraction before incubation, while lines 2 and 3 represent the distribution of radioactive apoproteins recovered in the VLDL fraction after incubation for 0 and 3 hr, respectively.

Earlier experiments had shown that incubation of an LCAT-deficient serum with a partially purified LCAT caused a shift of apoE from HDL to VLDL and IDL which occurred together with the increase in cholesteryl esters in these lipoproteins (9). These results have recently been confirmed by the same group (24) but Utermann et al. (25), who used a highly purified LCAT and an electroimmunoassay for apoE, did not corroborate these findings. Our experiments clearly demonstrate that cholesteryl ester transfer between HDL and VLDL isolated from normal plasma can take place independently of apoE. However, R_2 nonretained on anti apoB-Sepharose, i.e., the apoE-containing HDL, can also transfer cholesteryl ester to VLDL, although through a mechanism which involves equilibration rather than net transfer.

Further experiments with VLDL isolated from LCAT-deficient plasma or with nascent VLDL isolated from perfused animal liver will be necessary to define the role of apoE-containing HDL in cholesteryl ester transfer to triglyceride-rich lipoproteins.

This research was supported in part by grants from the Medical Research Council of Canada (MT-4011), from the Quebec Heart Foundation, and from the Conseil de la Recherche en Santé du Québec (R. W. M.). We acknowledge the excellent secretarial assistance of Ms. Louise Lalonde.

Manuscript received 8 January 1981 and in revised form 5 May 1981.

REFERENCES

1. Zilversmit, D. B., L. B. Hughes, and J. Balmer, 1975. Stimulation of cholesterol ester exchange by lipoprotein-free rabbit plasma. *Biochim. Biophys. Acta.* **409:** 393–398.
2. Chajek, T., and C. J. Fielding. 1978. Isolation and characterization of a human serum cholesteryl ester transfer protein. *Proc. Natl. Acad. Sci. USA.* **75:** 3445–3449.
3. Fielding, P. E., and C. J. Fielding. 1980. A cholesteryl ester transfer complex in human plasma. *Proc. Natl. Acad. Sci. USA.* **77:** 3327–3330.
4. Pattnaik, N. M., A. Montes, L. B. Hughes, and D. B. Zilversmit. 1978. Cholesteryl ester exchange protein in human plasma. Isolation and characterization. *Biochim. Biophys. Acta.* **530:** 428–438.
5. Sniderman, A., B. Teng, C. Vézina, and Y. L. Marcel. 1978. Cholesterol ester exchange between human plasma high and low density lipoproteins mediated by a plasma protein factor. *Atherosclerosis.* **31:** 327–333.
6. Marcel, Y. L., C. Vézina, B. Teng, and A. Sniderman. 1980. Transfer of cholesterol esters between human high density lipoproteins and triglyceride-rich lipoproteins controlled by a plasma protein factor. *Atherosclerosis.* **35:** 127–133.
7. Marcel, Y. L., C. Vézina, D. Emond, and G. Suzue. 1980. Heterogeneity of human high density lipoproteins: presence of lipoproteins with and without apoE and their roles as substrates for lecithin:cholesterol acyltransferase reaction. *Proc. Natl. Acad. Sci. USA* **77:** 2969–2973.
8. Weisgraber, K. H., and R. W. Mahley. 1980. Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* **21:** 316–325.
9. Norum, K. R., J. A. Glomset, A. V. Nichols, T. Forte, J. J. Albers, W. C. King, C. D. Mitchell, K. R. Applegate, E. L. Gong, V. Cabana, and E. Gjone. 1975. Plasma lipoproteins in familial lecithin:cholesterol acyltransferase deficiency: effects of incubation with lecithin: cholesterol acyltransferase in vitro. *Scand. J. Clin. Lab. Invest.* **35 Suppl. 142:** 31–55.
10. Ragland, J. B., P. D. Bertram, and S. M. Sabesin. 1978. Identification of nascent high density lipoproteins containing arginine-rich protein in human plasma. *Biochem. Biophys. Res. Commun.* **80:** 81–88.
11. Hatch, F. T., and R. S. Lees. 1968. Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* **6:** 1–68.
12. Scanu, A. M. 1966. Forms of human serum high density lipoprotein protein. *J. Lipid Res.* **7:** 295–306.
13. Iverius, P. H. 1971. Coupling of glycosaminoglycans to agarose beads (sepharose 4B). *Biochem. J.* **124:** 677–683.
14. Bolton, A. E., and W. N. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Application to the radioimmunoassay. *Biochem. J.* **133:** 529–539.
15. Weber, K., and M. Osborne. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406–4412.
16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
17. Allain, C. C., L. S. Poon, C. S. G. Chan, W. Richmond, and P. C. Fu. 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:** 470–475.
18. Sampson, E. J., L. M. Demers, and A. F. Krieg. 1975. Faster enzymatic procedure for serum triglycerides. *Clin. Chem.* **21:** 1983–1985.
19. Marcel, Y. L., and C. Vézina. 1973. A method for the determination of the initial rate of reaction of lecithin: cholesterol acyltransferase in human plasma. *Biochem. Biophys. Acta.* **306:** 497–504.
20. Noel, C., Y. L. Marcel, and J. Davignon. 1972. Plasma phospholipids in the different types of primary hyperlipoproteinemia. *J. Lab. Clin. Med.* **79:** 611–621.
21. Marcel, Y. L., M. Bergseth, and A. C. Nestruck. 1979. Preparative isoelectric focussing of apolipoproteins C and E from human very low density lipoproteins. *Biochim. Biophys. Acta.* **573:** 175–183.
22. Noble, R. P. 1968. Electrophoretic separation of plasma lipoproteins in agarose gel. *J. Lipid Res.* **9:** 693–700.
23. Milne, R. W., P. Douste-Blazy, L. Retegui, and Y. L. Marcel. 1981. Characterization of monoclonal antibodies against human apolipoprotein. *J. Clin. Invest.* **68:** 111–117.
24. Glomset, J. A., C. D. Mitchell, W. C. King, K. A. Applegate, T. Forte, K. R. Norum, and E. Gjone. 1980. In vitro effects of lecithin:cholesterol acyltransferase on apolipoprotein distribution in familial lecithin:cholesterol acyltransferase deficiency. *Ann N.Y. Acad. Sci.* **348:** 224–243.
25. Utermann, G., H. J. Menzel, G. Adler, P. Dieker, and W. Weber. 1980. Substitution in vitro of lecithin:cholesterol acyltransferase. Analyses of changes in plasma lipoproteins. *Eur. J. Biochem.* **107:** 225–241.