Molecular species of cholesteryl esters formed in abetalipoproteinemia: effect of apoprotein B-containing lipoproteins

P. V. Subbaiah,[1] B. Banerji, R. E. Gregg, and J. D. Bagdade

Departments of Medicine and Biochemistry, Rush Medical College, Chicago, IL 60612, and National Institutes of Health, Bethesda, MD 20892

Abstract In order to study the effects of very low density (VLDL) and low density (LDL) lipoproteins on the acitivity and specificity of lecithin:cholesterol acyltransferase (LCAT), we determined the molecular species of cholesteryl esters (CE) synthesized in the plasma from three abetalipoproteinemic (ABL) patients, before and after supplementation with normal VLDL or LDL. The patients' plasma had significantly lower concentration of 18:2 CE and higher concentrations of 16:0 CE and 18:1 CE compared to normal plasma. Incubation of ABL plasma with [4-^{14}C]cholesterol at 37°C and the subsequent analysis of labeled CE formed by high performance liquid chromatography revealed that the major species formed was 16:0 CE (34% of total label), whereas similar incubation of the d > 1.063 g/ml fraction of normal plasma resulted in the formation of predominantly 18:2 CE (45% of total label). Addition of normal VLDL or LDL to ABL plasma stimulated the total LCAT activity by 30–80% and normalized the CE species synthesized. The LCAT activity of a normal d > 1.063 g/ml fraction also was stimulated by the normal VLDL or LDL, but there was no alteration in the species of CE formed. Most of the CE synthesized was found in the added VLDL or LDL with both ABL and normal plasma, indicating that the CE transfer (CET) activity was not affected in ABL plasma. These results suggest that while the VLDL and LDL are required for the maximal activity of LCAT, the species of CE formed are primarily determined by the molecular species composition of phosphatidylcholine in the plasma. — **Subbaiah, P. V., B. Banerji, R. E. Gregg, and J. D. Bagdade.** Molecular species of cholesteryl esters formed in abetalipoproteinemia: effect of apoprotein B-containing lipoproteins. *J. Lipid Res.* 1990. **31:** 927–932.

Supplementary key words cholesteryl ester transfer • lecithin:cholesteryl acyltransferase • molecular species of phosphatidylcholine • substrate specificity • LDL • VLDL

Most of the cholesteryl esters (CE) present in human plasma are believed to be derived from the lecithin:cholesterol acyltransferase (LCAT) reaction (1, 2). While the LCAT reaction takes place predominantly on HDL particles, the majority of the synthesized CE is transferred to the apoprotein B-containing lipoproteins, very low density lipoproteins (VLDL), and low density lipoproteins (LDL). In addition to serving as the acceptors for CE synthesized on HDL, VLDL and LDL also provide most of the free cholesterol and phosphatidylcholine (PC) utilized by the LCAT reaction (1). Furthermore, recent studies show that significant amounts of LCAT protein are associated with LDL (3–5) and that a small amount of CE formed in plasma normally is synthesized on the surface of LDL (4, 6). LDL also activates the lysolecithin acyltransferase reaction of plasma which alters the molecular species composition of PC utilized by LCAT reaction (7).

Therefore, one would predict that in the absence of VLDL and LDL in the plasma, the activity and the specificity of LCAT would be altered. In fact, in abetalipoproteinemic (ABL) patients, in whom both VLDL and LDL are absent, not only is the total amount of CE decreased (8–11), but the fatty acid composition of CE differs markedly from that present in normal plasma, showing relatively more 16:0 and 18:1 and less 18:2 (8). It is not known, however, whether these abnormalities in CE fatty acid composition are due to the altered composition of substrate PC, altered enzyme specificity, or due to the formation of CE by non-LCAT-mediated reactions, such as ACAT which preferentially forms 16:0 and 18:1 CE (12, 13). We have recently studied the molecular species composition of plasma PC and their utilization by LCAT in ABL subjects (14), and found that there were relatively more PC species containing 18:1 at the 2-position than those containing 18:2, and that the percentage contribution of various species to the LCAT reaction was altered accordingly. However, the selectivity of the enzyme to-

Abbreviations: ABL, abetalipoproteinemia; ACAT, acyl CoA:cholesterol acyltransferase; CE, cholesteryl esters; GLC, gas–liquid chromatography; HDL, high density lipoproteins; HPLC, high performance liquid chromatography; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoproteins; PC, phosphatidylcholine; TLC, thin-layer chromatography; VLDL, very low density lipoproteins.

[1]To whom correspondence should be addressed at: Rush-Presbyterian-St. Luke's Medical Center, 1653 West Congress Parkway, Chicago, IL 60612.

ward various PC species was not altered. Addition of VLDL or LDL prepared from normal plasma normalized the percentage contribution of various PC species for cholesterol esterification. In this communication we have studied the formation of cholesteryl esters in the plasma of the same ABL patients before and after in vitro addition of normal lipoproteins. Our results show that the species of CE formed in ABL plasma correlate closely with the PC species utilized, and that the apoprotein B-containing lipoproteins influence the amounts and types of CE formed.

MATERIALS AND METHODS

Plasma and plasma fractions

Three of the five ABL patients previously reported (14) were the subjects in the present study. One of the patients had homozygous familial hypobetalipoproteinemia, and the other two had homozygous familial abetalipoproteinemia. None had detectable levels of apoprotein B in their plasma. All subjects ingested self-selected low-fat diets containing 30–35% of total calories as fat. Informed consent was obtained from each patient. Fasting blood samples were drawn at the Clinical Center at N.I.H., Bethesda, Maryland in EDTA (1.0 mg/ml). The plasma was separated immediately, frozen, and sent on dry ice to Chicago. All plasma samples were kept frozen at −70°C until the day of the experiment and were analyzed within 3 weeks of blood drawing.

Plasma also was obtained from healthy male control subjects (ages 25–45) after an overnight fast, and centrifuged at a density of 1.063 g/ml for 18 h. The d > 1.063 g/ml infranatant fraction was obtained by tube-slicing, dialyzed extensively against 0.15 M NaCl–1 mM EDTA (pH 7.4), and used as a control for ABL plasma.

VLDL, LDL, and HDL were prepared from normal pooled plasma by preparative ultracentrifugation at d < 1.006, d 1.019–1.063, and d 1.063–1.210 g/ml, respectively. The lipoproteins were washed once at their isolation densities and extensively dialyzed against 0.15 M NaCl–1 mM EDTA, pH 7.4. They were concentrated by using Amicon Centriflo filters (25,000 mol wt cut off) to give a concentration of PC of 8–12 mM.

Analysis of CE fatty acids

Total lipids were extracted from the plasma by the Bligh and Dyer (15) procedure, and CE were separated on silica gel TLC plates using the solvent system of hexane–diethyl ether–acetic acid 70:30:1. The lipids were visualized by spraying the plates with 0.2% dichlorofluorescein in ethanol and viewing under UV light. The spot corresponding to CE was scraped and placed in an acid-washed tube with a Teflon-lined cap, and heated with 2 ml of BF_3-methanol reagent (Supelco) at 85°C for 90 min. After cooling the tube, the fatty acid methyl esters were extracted twice with 1.0 ml of petroleum ether. The pooled extracts were concentrated and injected into a Shimdazu GLC equipped with a flame ionization detector and a capillary column (Supelco Wax 10 fused silica column, 0.25 μ thickness, 30 meters) using hydrogen as carrier gas. The oven temperature was set initially at 172°C for 8 min, then raised to 220°C at the rate of 6°C/min and maintained at 220°C for 2 min. Each peak was identified from authentic standards run under identical conditions.

Analysis of molecular species of CE formed

To analyze the species of CE formed by the LCAT reaction, ABL plasma and the control d > 1.063 g/ml fraction were each incubated with [4-^{14}C]cholesterol for 24 h. The labeled CE were then separated by HPLC and their radioactivities were determined. To 1.0 ml of ABL plasma (containing 0.17 to 0.21 μmol of PC and 0.7 to 0.9 μmol of CE) or to 1.0 ml of control d > 1.063 g/ml fraction (containing 0.7 to 0.8 μmol PC and 0.7 to 0.9 μmol CE), 0.1 ml of a [4-^{14}C]cholesterol–albumin suspension (prepared by the procedure of Stokke and Norum (16) and containing 300,000 dpm), 0.1 ml of M mercaptoethanol, and 0.4 ml of Tris-NaCl buffer (10 mM Tris–0.15 M NaCl–1 mM EDTA, pH 7.4) were added and the reaction mixture was incubated in a shaking water bath at 37°C for 24 h. Where indicated, either LDL, VLDL, or HDL prepared from normal plasma and containing 1 μmol of PC were added to the reaction mixture. The lipoprotein preparations were heat-treated at 56°C for 15 min in order to inactivate any residual LCAT activity, before adding to the reaction mixture. The samples containing VLDL or LDL were subjected to heparin-Mn^{2+} precipitation after the incubation (17). The precipitated lipoproteins were then resuspended in 1.0 ml of Tris-NaCl buffer and both this suspension and the supernatant (containing the HDL) were extracted by the Bligh and Dyer procedure (15). The total lipid extract was subjected to chromatography on silica gel TLC plates with the solvent system hexane–diethyl ether–acetic acid 70:30:1, and the spot corresponding to CE was scraped, eluted (15), and analyzed by HPLC.

HPLC of CE

The labeled CE were separated into various molecular species by a modification of the procedure of Carroll and Rudel (18). A C-18 column (Ultrasphere ODS) was used with an isocratic solvent system of acetonitrile–tetrahydrofuran–water 65:35:1.5, at a flow rate of 2 ml/min and a temperature of 27°C. Separation of all the major CE species present in plasma was achieved within 25 min. Absorbance of the eluate was monitored at 213 nm, and each peak was identified by the analysis of fatty acid composition by GLC, as well as by running authentic stan-

dards under identical conditions. Fractions of 1.0 ml each (0.5 min) were collected, and the radioactivity was determined in a liquid scintillation counter after evaporation of the solvent and the addition of 5 ml of scintillation fluid. The total counts for each CE species were obtained by combining the counts of all fractions under the corresponding peak.

Analytical methods

Protein was estimated by the procedure of Lowry et al. (19) using bovine serum albumin as standard. Lipid phosphorus was determined by the modified Bartlett procedure (20).

RESULTS

Fatty acid composition of cholesteryl esters (CE) in ABL and normal plasma

Table 1 shows the fatty acid composition of plasma CE in three ABL and four normal subjects. As reported earlier (8), the major CE present in ABL plasma was 18:1, whereas 18:2 was the major species in normal plasma. The percentage of 18:2 in ABL plasma was even lower than that reported by Jones and Ways (8), possibly reflecting better separation of 18:2 and 18:1 (n-7) with our method. The CE of ABL plasma contained a significantly higher percentage of 16:0 compared to normal plasma.

Incorporation of [4-^{14}C]cholesterol into various CE species

In order to determine the species of CE formed in ABL plasma by the LCAT reaction, plasma from these patients was incubated with a [4-^{14}C]cholesterol-albumin complex (16) for 24 h at 37°C and the synthesized CE were separated by HPLC, as described under Methods. Fractions of 1.0 ml (0.5 min) were collected and counted for radioactivity in a scintillation counter. For comparison we subjected the d > 1.063 g/ml fraction of normal plasma to the same procedure. This fraction from controls is comparable to ABL plasma in that it lacks acceptor lipoproteins for the formed CE. As shown in **Fig. 1**, the composition of labeled CE formed in ABL plasma differed markedly from that found in normal plasma. While the total counts incorporated into CE were comparable in the two systems, a significantly higher percentage of label was incorporated into 16:0 and 18:1 species in ABL plasma than in the control d > 1.063 g/ml fraction (**Table 2**). In the normal d > 1.063 g/ml fraction 18:2 was the major CE species found, while in ABL plasma it was 16:0. Although the 18:1 CE constituted about 34.5% of total CE mass in ABL plasma, only 24% of the total label was incorporated into this CE species. Thus, the ratio of 18:1 CE/18:2 CE mass in ABL plasma was 2.85 (Table 1), but the ratio of label in the two species was only 0.97 (Table 2) indicating that a portion of the 18:1 CE present in ABL plasma may be derived from nonLCAT reactions. In contrast, in normal plasma corresponding ratios of mass (0.32) and of label (0.33) were comparable, indicating that the LCAT reaction produces most of CE present in normal plasma.

TABLE 1. Fatty acid composition of cholesteryl esters from abetalipoproteinemic (ABL) and normal plasma

Fatty Acid	ABL Plasma (n = 3)	Normal Plasma (n = 4)
14:0	0.58 ± 0.11	0.47 ± 0.24
16:0	22.58 ± 3.20	9.28 ± 1.57
16:1 (n-7)[a]	5.09 ± 1.48	1.97 ± 0.82
18:0	1.15 ± 0.27	0.73 ± 0.16
18:1 (n-9)	34.50 ± 4.22	14.20 ± 2.43
18:1 (n-7)	8.19 ± 2.58	3.01 ± 0.65
18:2 (n-6)	15.00 ± 2.61	54.44 ± 1.70
18:3 (n-6)	0.11 ± 0.11	0.47 ± 0.16
18.3 (n-3)	n.d.	0.38 ± 0.18
20:1 (n-9)	n.d.	0.51 ± 0.63
20:3 (n-6)	0.82 ± 0.26	0.43 ± 0.25
20:4 (n-6)	6.71 ± 2.29	8.97 ± 2.66
20:5 (n-3)	0.38 ± 0.14	0.77 ± 0.45
22:6 (n-3)	0.44 ± 0.36	0.38 ± 0.18

Values are given as mean ± SD; n.d., not detected.
[a]n-x Nomenclature: x represents the position of the first double bond from the methyl end of the fatty acid and n is the total number of carbon atoms in the fatty acid.

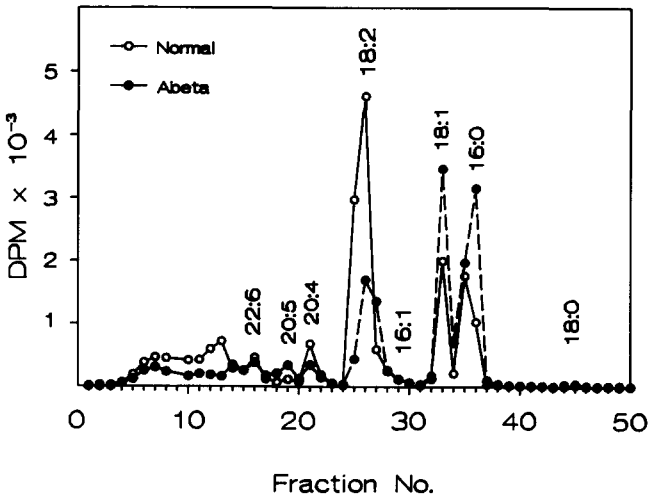

Fig. 1. Incorporation of [4-^{14}C]cholesterol into various molecular species of cholesteryl esters in ABL plasma and normal d > 1.063 g/ml fraction. The labeled cholesteryl esters from the reaction mixtures were first isolated on silica gel TLC plates and then subjected to HPLC on a C-18 reverse phase column with the solvent system of acetonitrile-tetrahydrofuran-water 65:35:1.5 at 27°C. Fractions of 1 ml each (0.5 min) were collected and the radioactivity was determined in a liquid scintillation counter.

Effect of addition of normal lipoproteins on CE species formed

To distinguish whether these abnormalities in CE synthesis were attributable to alterations in PC composition

TABLE 2. Incorporation of labeled cholesterol into various CE species in ABL and normal plasma

CE Species	% of Total Labled CE	
	ABL	Normal[a]
Ch-22:6	3.84	6.88
Ch-20:5	4.61	4.50
Ch-20:4	4.36	7.38
Ch-18:2	25.11	45.03
Ch-16:1	2.03	2.15
Ch-18:1	24.44	14.79
Ch-16:0	33.54	18.42
Ch-18:0	0.40	0.87

[a]Normal, d > 1.063 g/ml fraction.

or the absence of apoB lipoproteins, VLDL, LDL, and HDL prepared from normal plasma were added to ABL plasma and the d > 1.063 g/ml fraction of normal plasma, and the various CE species formed were quantitated. There was a 30% stimulation of total LCAT activity of ABL plasma by the addition of VLDL and about 80% stimulation after the addition of LDL, but no stimulation by normal HDL. Since the lipoproteins had all been heated-treated at 56°C for 15 min before being added to the reaction mixture, the stimulation observed could not be due to the presence of additional LCAT enzyme. The stimulatory effect, however, was not uniform for all the CE species. As shown in **Fig. 2**, VLDL and LDL stimulated primarily the formation of 18:2 and 20:4 CE species, and not 16:0 and 18:1 species. Although HDL did not stimulate LCAT activity, the species of CE formed were altered, with an increase observed in the synthesis of 18:2 CE at the expense of 18:1 and 16:0 species. These results suggest that while the types of CE formed are influenced by the molecular species of PC present, optimal activity of LCAT requires the presence of an acceptor lipoprotein.

The LCAT activity in the normal d > 1.063 g/ml fraction was stimulated fourfold by the addition of VLDL and LDL. In contrast to ABL plasma, normal HDL also stimulated the LCAT activity by twofold. In further contrast to ABL plasma, the addition of lipoproteins to the normal d > 1.063 g/ml fraction stimulated the synthesis of all CE species uniformly, not specifically 18:2 and 20:4 species (**Fig. 3**). These results support the conclusion that the species of CE synthesized by LCAT are influenced strongly by which PC species are available. Since the PC molecular species composition of added lipoproteins does not differ markedly from the normal d > 1.063 g/ml fraction, the composition of CE species formed was not altered by these lipoproteins.

The distribution of labeled CE species among the lipoproteins separated by heparin-Mn^{2+} precipitation is shown **Table 3**. In ABL plasma to which normal VLDL had been added, about 75% of the newly synthesized CE were present in VLDL. When LDL was added, an even larger percentage of CE was found in LDL (86.6%). All major species appeared to be transferred to the same extent, although some differences were seen in the presence of LDL. In the d > 1.063 g/ml fraction of normal plasma to which VLDL or LDL had been added, about 75% of the newly synthesized CE was present in the acceptor lipoprotein. There was no significant difference in the labeled CE composition of acceptor and donor lipoproteins indicating a lack of specificity of the transfer under the conditions used.

DISCUSSION

Although ABL plasma has been shown to have abnormal CE composition, it is not known whether this abnormality results from the altered PC substrate composition of plasma, altered LCAT specificity, or an increase in the contribution to plasma of tissue ACAT-derived CE. The results here show that in ABL plasma that LCAT reaction produces predominantly 16:0 and 18:1 species, while predominantly 18:2 CE are produced in normal plasma. Since the species of CE formed are not altered when the apoB-containing lipoproteins are removed from normal plasma (d > 1.063 g/ml fraction), it is unlikely that the specificity of the enzyme could have changed in ABL plasma. This is supported by the fact that when VLDL, LDL, or HDL from normal plasma were added to ABL plasma, the CE species formed resembled those present in normal plasma. Thus, it is the abnormal PC composition in ABL plasma and not the lack of VLDL or LDL that is the major contributor to the altered CE composition.

A comparison of the percentage composition of CE in ABL plasma (Table 1) with the percentage composition of labeled CE formed by the LCAT reaction (Table 2) indicated that not all the CE present in ABL plasma may be

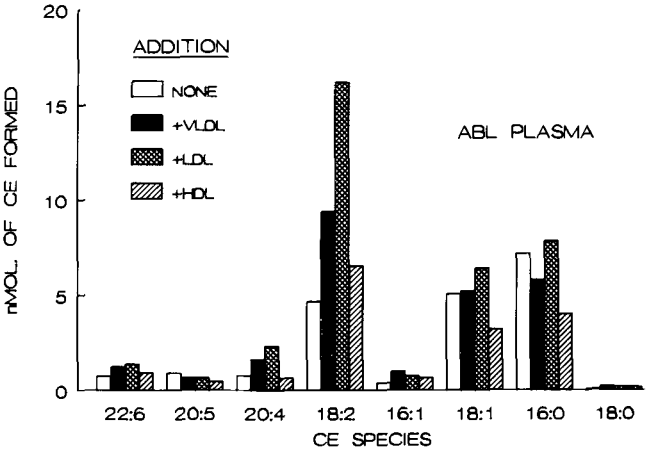

Fig. 2. Formation of labeled cholesteryl esters in ABL plasma in the absence and presence of various lipoproteins isolated from normal plasma.

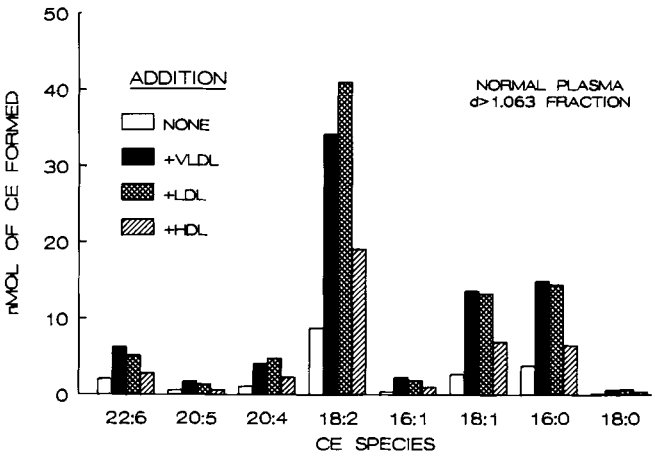

Fig. 3. Formation of labeled cholesteryl esters in the d > 1.063 g/ml fraction of normal plasma, in the absence and presence of various lipoproteins isolated from normal plasma.

derived from the LCAT reaction. For example, 18:1 (n-9) and 18:1 (n-7) species of CE together account for about 42% of the total mass of fatty acids in ABL CE. However, only about about 24% of the total labeled CE formed by LCAT is 18:1. Similarly, although only 15% of the total CE mass is 18:2, about 25% of the newly synthesized CE is 18:2. These results indicate that in the absence of apoB-containing lipoproteins, the relative contribution of tissue ACAT may be increased, since it is known to produce predominantly 18:1 CE (12, 13). On the other hand, in normal plasma the percentage composition of CE was closer to the percentage of labeled CE formed, with the exception of 16:0 CE, of which relatively more was formed. The disproportionate increase in the synthesis of 16:0 CE in ABL and normal plasma may result from the long incubation times used which may have resulted in the utilization of acyl groups from 1-position of PC. The utilization of 1-acyl group of PC for cholesterol esterification is also indicated by our data on the consumption of various PC species by LCAT in ABL plasma, which we have previously reported (14). Thus, we have shown that *sn*-2-18:1 and *sn*-2-18:2 PC species contributed about 47% and 34%, respectively, of the total acyl groups utilized by LCAT. However, the percentage of 18:1 and 18:2 CE formed under identical conditions in the present experiments is much lower than the above values (Table 1) and the percentage of 16:0 CE formed is greater than expected. These results can only be explained by the contribution of the 1-acyl group for CE formation. Nevertheless, the disparity between the composition of pre-existing CE species and that of newly formed CE species is greater in ABL plasma than in the normal d > 1.063 g/ml fraction, suggesting that ACAT is making a relatively larger contribution to plasma CE in ABL patients.

In the plasma of normal subjects, VLDL and LDL not only provide the free cholesterol and PC substrates for LCAT reaction, but they also serve as acceptors for most of the CE synthesized. Therefore, in their absence the activity of LCAT is decreased considerably. We have previously shown that the mass of LCAT protein is decreased in ABL plasma (21). The present results showing that LCAT activity of ABL plasma increases 30-80% after addition of normal VLDL and LDL suggest that the reduced LCAT activity in this disease is not only due to reduced enzyme concentration, but also results from the absence of stimulation by VLDL and LDL. It is of interest to note that HDL failed to stimulate LCAT activity in ABL plasma, indicating that an acceptor of the synthesized CE is required for optimal activity of the enzyme, as Fielding and Fielding (22) have suggested.

TABLE 3. Distribution of cholesteryl ester species between supernatant and precipitate

	ABL Plasma				Normal d > 1.063 g/ml Fraction			
	+ VLDL		+ LDL		+ VLDL		+ LDL	
Species	Supt	PPT	Supt	PPT	Supt	PPT	Supt	PPT
	% of total				*% of total*			
Ch-22:6	35.17	64.83	23.30	76.70	17.02	82.98	17.40	82.60
CH-20:5	33.71	66.29	21.64	78.36	20.38	79.62	23.59	76.41
CH-20:4	28:87	71.13	12.13	87.87	29.10	70.90	31.05	68.95
CH-18:2	20.74	79.26	11.89	88.11	26.85	73.15	36.13	73.87
CH-16:1	39.77	60.23	25.16	74.84	23.54	76.46	27.92	72.08
CH-18:1	21.15	78.85	13.17	86.83	20.41	79.59	19.96	80.04
CH-16:0	25.59	74.41	13.30	86.70	26.25	73.75	26.80	73.20
CH-18:0	32.73	67.27	8.11	91.89	17.51	82.49	21.77	78.23
Total	24.38	75.62	13.33	86.67	24.60	75.40	24.95	75.05

After incubation of ABL plasma or normal d > 1.063 g/ml fraction with VLDL or LDL, the reaction mixture was subjected to heparin-Mn^{2+} precipitation and the radioactivity of CE species was determined by HPLC as described in Methods. The percentage of each labeled CE species present in precipitate and supernatant was calculated from these values.

The fact that most of the CE synthesized in ABL plasma after addition of VLDL or LDL was present in the acceptor lipoproteins is evidence that the CE transfer activity was normal under the conditions used. This finding conflicts with the report of Pappu and Illingworth (23) who found a heat-stable protease-sensitive inhibitor of neutral lipid transfer between HDL and VLDL in ABL plasma. While our results do not indicate that the transfer of newly synthesized CE is impaired in ABL, it is possible that the long period of incubation (24 h) we used here obscures possible changes in CE transfer activity. The composition of the labeled CE transferred indicates that transfer protein has no specificity for particular CE species.

In conclusion, our results show that composition of the plasma CE is largely determined by the PC molecular species composition, but the optimal activity of LCAT and its relative contribution to CE composition are dependent upon the presence of apoB-containing lipoproteins.

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