A study of the interaction of lecithin:cholesterol acyltransferase with subfractions of high density lipoproteins

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Abstract High density lipoproteins (HDL) were isolated by a chromatographic procedure and subsequently fractionated on a DEAE cellulose (DE-52) column. Four fractions were separated and analyzed for lipid and protein composition and molecular weight. During ion exchange chromatography, one of the four fractions consistently coincided with lecithin:cholesterol acyltransferase (LCAT) activity. When the HDL fractions were incubated with highly purified LCAT preparations, the LCAT activity showed a dependence on unesterified cholesterol concentrations. The HDL subfraction eluting at the highest ionic strength was found to be the best substrate for LCAT. This subfraction exhibited apoprotein and lipid composition similar to HDL_3 and contained 31% of the total apoprotein D present in all the subfractions. A positive correlation was found between LCAT activity and the cholesteryl ester/unesterified cholesterol ratio, and a negative correlation was found between LCAT substrate potential and apparent molecular weight of the HDL subfractions when these subfractions were incubated with LCAT. No correlation was apparent between LCAT activity, and the phospholipid/unesterified cholesterol ratio or with the apoA-I/apoA-II ratio.—**Jahani, M., and A. G. Lacko.** A study of the interaction of lecithin:cholesterol acyltransferase with subfractions of high density lipoproteins. *J. Lipid Res.* 1981. **22:** 1102–1110.

Supplementary key words HDL fractionation · LCAT mechanism of action

Lecithin:cholesterol acyltransferase (LCAT) is an enzyme of hepatic origin (1) that maintains the cholesteryl ester content of human plasma (2). In addition, LCAT has been implicated in key pathways of lipoprotein metabolism, particularly the catabolism of triglyceride-rich lipoproteins (3) and reverse cholesterol transport (2). Recently, several laboratories have reported procedures for the isolation of LCAT in the homogeneous state (4–7), and one laboratory demonstrated endogenous phospholipase activity in a homogeneous LCAT preparation (6). Although information concerning the molecular and catalytic properties of LCAT is gradually becoming available, very little is known concerning the nature of the substrate(s) for the LCAT reaction in vivo. High density lipoproteins (HDL) and very high density lipoproteins (VHDL) were reported to be preferred substrates for LCAT (8–10). In addition, experiments carried out with partially purified LCAT showed that HDL_3 (not HDL_2) was the preferred substrate for the enzyme (10, 11). Studies utilizing synthetic lipid mixtures and purified enzyme have shown that the major apoprotein of HDL (apoA-I) was an activator for the LCAT reaction (12). Other investigations utilized partially purified enzyme and showed that, with sonicated dispersions of lecithin and cholesterol as substrates, the rate of esterification was dependent on the molar ratio of lecithin to cholesterol and on the amount of apo-HDL_3 added to the reaction mixture (13). Thus, a variety of substrates, such as heat-denatured human plasma (14), and intact lipoproteins (15, 16) have been used in studies that have probed the enzymatic properties of LCAT. However, information concerning the interaction of highly purified LCAT preparations with subpopulations of HDL is not yet available.

The present investigation was designed to study the interaction of HDL subfractions (obtained by ion-exchange chromatography) with highly purified enzyme, and to investigate the properties of the subfractions as substrates for the enzyme. The results obtained correlate with earlier findings from other laboratories and suggest the possibility that the HDL/LCAT interaction may be of significance in vivo.

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; TLC, thin-layer chromatography; HSA, human serum albumin; DTNB, 5,5′dithiobis-2-nitrobenzoic acid; UC, unesterified cholesterol; CE, cholesteryl ester.

MATERIALS AND METHODS

Materials

[1,2-^{3}H]Cholesterol was purchased from Amersham Searle (sp act 9.3 Ci/mmol). Crystalline human serum albumin, from Sigma Chemical Co., St. Louis, MO was charcoal-treated according to Chen (17). Sephadex G-25, CNBr-activated Sepharose-4B, Sepharose-4B, and Sepharose CL-6B were obtained from Pharmacia Fine Chemicals, Inc. DE-52 (exchange capacity 1.1 meq/g) was obtained from Whatman Chemical Co. Dextran sulfate (analytical) was from Nutritional Biochemical Corp. 5,5′-Dithio-bis-nitrobenzoic acid (DTNB) was obtained from Boehringer-Mannheim. Cyanogen bromide, acrylamide, bis-acrylamide, riboflavin (vitamin B_2), ammonium persulfate, tetra-methylethylene diamine (TEMED), glycine, β-mercaptoethanol, and Coomassie Blue G-250 were obtained from Eastman Kodak Company. Dodecylamine was obtained from Aldrich Chemical Company. Antibodies against human HDL and LDL were from Miles-Yeda Ltd., Israel. Polygram Sil G Hy Plates were obtained from Brinkman Instruments, Inc. Protein assay kits were obtained from Bio-Rad Laboratories. All other chemicals were obtained from Fisher Scientific Company and were of reagent grade or better.

Preparation of HDL

The blood was obtained from a healthy donor after an overnight fast. Upon separation of plasma at 4°C by low speed centrifugation, DTNB was added to a final concentration of 1.2 mM to inhibit LCAT activity. The isolation of HDL has been described in detail in a separate communication (18). All chromatographic steps were done at 4°C. Briefly, HDL was isolated by first treating 100 ml of fresh human plasma with dextran sulfate and $CaCl_2$ according to Hatch and Lees (19). The supernatant solution was then subjected to a hydrophobic chromatography procedure (20). The HDL isolated by this method exhibited essentially identical chemical and morphological properties (18) to HDL isolated by established techniques (21) and showed no reaction against anti-LDL antiserum. Residual serum albumin was removed by antibody adsorption using specific immunoglobulins immobilized on Sepharose 4B (18).

Fractionation of HDL by ion exchange chromatography

Pre-swollen DE-52 was equilibrated with 20 mM sodium phosphate buffer, pH 7.4. The column (2.5 × 30 cm) was packed under gravity and was eluted with 20 mM sodium phosphate buffer until the pH and conductivity of the effluent equaled that of the equilibrating buffer. Twelve ml of HDL concentrate, collected from an anti-albumin column containing 220 mg of protein, was applied to the column in 20 mM sodium phosphate, pH 7.4. The column was then washed with 400 ml of the same buffer, followed by a gradient elution step. The gradient was generated by mixing 400 ml of 20 mM phosphate buffer, pH 7.4, with 300 ml of the same buffer containing 0.3 M NaCl. Fractions of 3.5 ml were collected at a flow rate of 32 ml/hr. Aliquots of fractions eluted from the DE-52 column were tested for the presence of LCAT activity using HDL as substrate in the presence of β-mercaptoethanol. The HDL substrate contained approximately 0.5 mg/ml of HDL and 2% of fatty acid-free albumin (17) in 0.01 M Tris buffer containing 0.005 M EDTA and 0.15 M NaCl, pH 7.4. Twenty-μl aliquots of each chromatographic fraction were added to 180 μl of [^{3}H]cholesterol-labeled HDL substrate (containing 5 mM β-mercaptoethanol) and incubated for 2 hr at 37°C. The reaction was stopped by addition of twenty volumes of chloroform–methanol 2:1 (v/v). The lipid extracts were analyzed for unesterified cholesterol (UC) and cholesteryl ester (CE) content as described by Lacko et al. (22). The LCAT activity was expressed as percent cholesterol esterified per hr.

Preparation of LCAT

LCAT preparations (free of apoA-I) of at least 95% purity (SDS-PAGE) were used as the source of enzyme. LCAT was purified by a procedure[1] consisting of three steps: dodecylamine-agarose chromatography (20), DEAE-agarose chromatography, and hydroxylapatite chromatography.

Delipidation of HDL subfractions

Butanol–diisopropylether was used in the extraction mixture as described by Cham and Knowles (23). Two volumes of butanol–diisopropylether 40:60 (v/v) were mixed with one volume of the HDL subfractions, and the mixture was rotated end-over-end at 28–30 rpm for 1.5 hr at room temperature. After the extraction, the mixture was centrifuged at 2,000 rpm for 2 min to separate the two phases. The organic phase was removed by careful aspiration with a needle and syringe, dried under nitrogen, and stored at 4°C for thin-layer chromatography.

[1] Chong, K. S., L. Davidson, R. G. Huttash, and A. G. Lacko. Unpublished observations.

Urea gel electrophoresis

Apoproteins of HDL subfractions were subjected to electrophoresis as described below. Polyacrylamide gel electrophoresis in the presence of 7 M urea was carried out in 7.5% gels according to Kane (24) at pH 9.1 without mercaptoacetic acid. Apoprotein samples of 50–100 μg from the butanol–diisopropylether lipid extraction were mixed with an equal volume of 10 M urea solution containing 0.01% bromphenol blue and were boiled for 5–10 min. The samples were then carefully layered beneath the upper buffer. A constant current of 1.25 mA per tube was applied until the tracking dye entered the lower gel. The current was then increased to 2.5 mA per tube. The gels were removed and stained in a Coomassie blue G-250 solution as described by Blakesley and Boezi (25) for 8 hr. The gels were destained by several changes of distilled water.

Lipid analysis by TLC

The organic phase of the lipid extraction step was analysed for lipid content as described by Wilson (26). The following solvent mixtures were used. Solvent I consisted of chloroform–methanol–acetic acid–water 25:15:4:2 (by volume); solvent II was a mixture of n-hexane–diethylether–acetic acid 40:10:0.5 (by volume); and solvent III consisted of n-hexane–diethylether 19:1 (by volume). The Polygram Sil G Hy plates (20 × 20 cm) were washed overnight in solvent I in the TLC chamber. The washed plates were dried at 37°C and stored at room temperature. Rectangles, 7 × 6 cm, were cut from 20 × 20 cm plates and washed again in solvent I for 30–45 min. These plates were dried again and activated in a 50°C oven for 15 min. Lipid samples of 2–4 μl were applied to the plate at 37°C, and the spots were dried at 37°C for 3 min. The plate was first developed with solvent I up to 2 cm from the origin. Then the plate was dried at 37°C for 3 min and transferred to a tank containing solvent II and developed up to 5 cm from the origin and dried at 37°C for 2 min. Finally, the plate was developed in Solvent III up to 5 cm from origin, and was dried at 37°C. The dried plate was then sprayed with a 20% ammonium bisulfate in 50% ethanol and dried at 37°C. Charring of the lipids was accomplished by heating the plate at 170°C for 2 hr. After cooling for 30 min, the plate was scanned on the Helena Quick Scan R & D equipped with a Quick Quant II computer.

Molecular weight determination of HDL subfractions

The molecular weights of HDL subfractions were estimated from elution volumes of a calibrated column (1.5 × 85 cm) packed with Sepharose CL-6B by plotting K_{av} against log mol wt where $K_{av} = (V_e - V_0)/(V_t - V_0)$. V_e is the elution volume of the material used (measured at the peak maximum); V_0 is the void volume (determined by the elution volume of Blue Dextran); and V_t is the total bed volume. The elution of all samples in this experiment was performed with 20 mM sodium phosphate buffer, pH 7.4. Bovine serum albumin (mol wt, 67,000), rabbit muscle Aldolase (mol wt, 158,000), beef liver catalase (mol wt, 240,000), and horse spleen apoferritin (mol wt, 460,000) were used as standards.

Substrate efficiency of HDL subfractions

Subfractions of HDL eluted from the DE-52 column were concentrated in a pressure dialysis cell (Amicon, Inc.), dialyzed against 20 mM sodium phosphate buffer, pH 7.4, and were subsequently used as substrates for the LCAT reaction. LCAT activity was determined by measuring the increase in the concentration of cholesteryl esters in the incubation mixture. The free cholesterol concentration of subfractions was determined enzymatically using kits from Boehringer-Mannheim. The HSA/[^{3}H]cholesterol mixture was prepared by adding 650 μl of [^{3}H]-cholesterol (0.005 mCi/ml in acetone, sp act 9 Ci/mmol) to 4 ml of charcoal-treated HSA (8%) slowly under nitrogen with continuous stirring (27). Fifty microliters of the prepared HSA/[^{3}H]cholesterol mixture was added to 20–200 μl of each of the subfractions and the substrate mixtures were incubated for 3 hr at 4°C (to allow equilibration of the tracer cholesterol with the unlabeled pool of the lipoproteins) (16). The assay mixtures contained approximately 740,000 dpm/ml, amounting to a cholesterol concentration of 0.036 nmol/ml. The tracer cholesterol therefore contributed less than 1.5% to the total unesterified cholesterol pool and thus was not expected to influence the rate of esterification by LCAT. At the end of the preincubation time, 25 μl of HSA was added to adjust the final concentration to 2%, and the final assay volume was adjusted to 275 μl by adding 20 mM sodium phosphate buffer, pH 7.4. The albumin preparation used in these assays was found to have less than 0.06% contamination by HDL apoproteins as indicated by radial immunodiffusion. The reaction was initiated by the addition of 20 μl of highly purified (apoA-I-free) LCAT (0.075 mg/ml). Assays were performed in duplicate with an average error of less than 5%. The assay tubes were incubated at 37°C for 3 hr which allowed for the measurement of linear esterification rates. β-Mercaptoethanol was not included in this assay mixture in order to avoid restoration of the inhibition of the endogenous

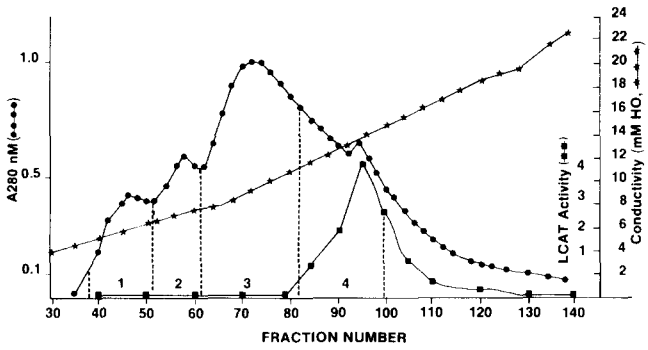

Fig. 1. Subfractionation of HDL by DE-52 chromatography. The albumin-free HDL pool was applied to a column (2.5 × 30 cm) of DE-52, which had been equilibrated with 20 mM phosphate buffer, pH 7.4. A gradient (0.0–0.3 M) of NaCl was applied, and 3.5-ml fractions were collected. The absorbance at 280 nm (— ● —), conductivity (— * —), and LCAT activity, in presence of β-mercaptoethanol, (— ■ —) were determined. The flow rate was 32 ml/hr.

LCAT activity which was found to be associated with subfraction 4 (Fig. 1). The reaction was stopped by the addition of 20 volumes of chloroform–methanol 2:1 (v/v). The lipid extracts were analyzed for unesterified cholesterol (UC) and cholesteryl ester (CE) content as described by Lacko et al. (22). The LCAT units (% cholesterol esterified per hr) were multiplied by the initial cholesterol (UC) concentration (nmol/ml) to give nmol of cholesterol esterified/ml per hr.

RESULTS

Fig. 1 shows the elution pattern of the DE-52 column. Four subfractions were pooled from the eluate and aliquots from each pool were subsequently analyzed for lipid and apoprotein, and reacted with purified LCAT. LCAT activity was consistently associated with fraction 4 indicating the specificity of the fractionation procedure. This interaction between LCAT and the indicated subfraction of HDL is probably due to the affinity of the enzyme for this unique lipid/protein complex, rather than to coincidental elution. The enzyme eluted from the DE-52 column at a considerably lower ionic strength than the delipidated fraction.[1] Additional chromatography on DE-52 did not appear to improve the homogeneity of the isolated subfractions. The four regions were pooled as shown in Fig. 1 and used for further studies. The LCAT assays used to analyze the fractions eluted from the DE-52 column were conducted in the presence of excess β-mercaptoethanol to reveal the endogenous LCAT activity of the respective subfractions.

The relative compositions of lipid constituents of the isolated subfractions are shown in **Table 1.** Subfractions 1 and 2 appear nearly identical in their lipid composition while subfractions 3 and 4 show markedly different patterns. Subfractions 3 and 4 exhibit a lower content of free cholesterol and triglyceride and a higher ratio of esterified cholesterol to free cholesterol than subfractions 1 and 2. Of particular interest, are the phospholipid to cholesterol (PL/UC) and the cholesteryl ester to free cholesterol (CE/UC) ratios (**Table 2**). In this regard the latter subfractions (3 and 4) showed considerably higher CE/UC ratios than the earlier ones, while fraction 3 had a much higher PL/UC ratio than the other fractions.

The polypeptide composition of the isolated subfractions was studied by two methods: urea gel electrophoresis and specific electroimmunoassays.[2] The urea gel electrophoresis patterns of the four subfractions revealed the presence of apoA-I, apoA-II, and the C apoproteins in all four subfractions, while apoE was most prominent in subfraction 3, and a protein component apparently corresponding to LCAT was essentially restricted to subfraction 4. Results of the electroimmunoassays are shown in **Table 3.** This method allowed the detection and quantitation of apolipoprotein D in addition to A-I and A-II. Fractions 3 and 4 contain the bulk of apolipoprotein D; fraction 3 having nearly half of all the apoD of the

[2] We thank Dr. Walter J. McConathy of the Oklahoma Medical Research Foundation for the performance of these analyses.

TABLE 1. Relative lipid composition of HDL subfractions[a]

HDL Subfractions	Phospholipids		Cholesteryl Ester		Unesterified (free) Cholesterol		Triglyceride	
	a	b	a	b	a	b	a	b
1	50.4	50.4	35.6	34.8	7.6	8.6	6.3	6.1
2	47.5	51.2	36.9	35.1	10.0	10.1	5.5	3.6
3	62.6	61.8	31.7	33.1	3.7	4.0	2.0	1.0
4	47.7	49.5	44.7	41.6	4.2	4.1	3.4	4.5

[a] The values represent the densitometric analysis (645 nm) of two separate TLC runs for each subfraction. The values reported are expressed as percentage total scan area units.

TABLE 2. Lipid[a] and apoprotein[b] ratios and molecular weight[c] of HDL subfractions isolated by DEAE cellulose chromatography

HDL Subfractions	Cholesteryl Ester / Free Cholesterol	Phospholipids / Free Cholesterol	ApoA-I / ApoA-II	Molecular Weight $\times 10^{-3}$
1	4.3	6.2	3.6	412.0
2	3.6	4.9	2.9	446.0
3	8.4	16.1	2.4	298.5
4	10.4	11.7	2.1	245.5

[a] Cholesteryl ester to free cholesterol and phospholipid to free cholesterol weight ratios were calculated from the lipid analyses shown in Table 1.

[b] Apoprotein A-I to apoprotein A-II ratios were calculated from the results shown in Table 3.

[c] Molecular weights of HDL subfractions were determined from elution volumes of a calibrated column of Sepharose CL-6B by plotting K_{av} against log molecular weight.

isolated HDL. The ratio of apoA-I/apoA-II shows a decrease progressing from fraction 1 to 4.

The molecular weights of the individual HDL subfractions were examined by gel chromatography on a calibrated sepharose CL-6B column. Subfraction 4, the one that associated with LCAT, was found to have the lowest molecular weight. The molecular weights of the respective subfractions are listed in **Fig. 2** and Table 2. A survey of the properties of the four isolated subfractions indicates that their characteristics correspond to similar fractions isolated by ultracentrifugation (28). For example, the subfractions isolated by increasing ionic strength during chromatography in this study correspond to the trend shown by HDL subfractions with increasing hydrated density upon ultracentrifugation.

Next, the reaction of the HDL subfractions with LCAT was investigated. In this experiment, each subfraction was first labeled with [^{3}H]cholesterol (16) and then incubated with highly purified LCAT preparations. The rate of cholesterol esterification was determined for free cholesterol concentrations

TABLE 3. Apoprotein composition of HDL subfractions isolated by DEAE-cellulose chromatography[a]

Fraction #	Apoprotein Content (mg) ApoA-I	ApoA-II	ApoD	A-I/A-II	% of Total ApoD in HDL
1	5.7	1.6	0.22	3.6	7
2	7.4	2.6	0.62	2.8	18
3	7.9	3.3	1.48	2.4	44
4	8.6	4.1	1.05	2.1	31

[a] Aliquots of the respective HDL subfractions were subjected to specific electroimmunoassays (48). The respective apoprotein contents of the subfraction were calculated based on the total volume of each subfraction pooled from the chromatogram (Fig. 1).

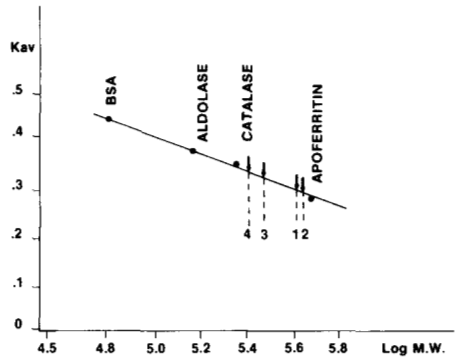

Fig. 2 Molecular weights of the standard proteins and the HDL subfractions presented as a function of K_{av}. A column (1.5 × 85 cm) was packed with Sepharose CL-6B and equilibrated with 0.02 M sodium phosphate buffer, pH 7.4. Solutions (10 mg/ml) of bovine serum albumin (67,000), rabbit muscle aldolase (158,000), beef liver catalase (240,000), and horse spleen apoferritin (460,000) were used as standards.

of 25 to 340 nmol/ml (contained by each HDL subfraction). The results in **Fig. 3** indicate that subfractions 1 and 2 reacted poorly, while subfractions 3 and 4 showed a considerably higher substrate potential for LCAT. Subfraction 4, which was consistently found to be associated with endogenous LCAT activity, showed the highest reactivity with the enzyme. In these experiments, *no β-mercaptoethanol* was used; thus the endogenous activity contained in subfraction 4 remained almost totally inhibited by the original administration of DTNB. In addition, blank assays were performed for each subfraction and the small amount of residual activity of subfraction 4 was subtracted from the total to yield a net LCAT activity

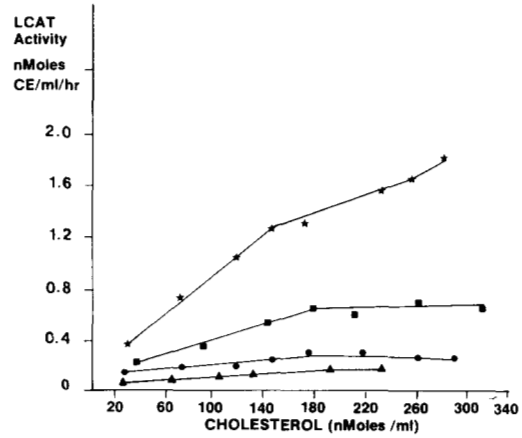

Fig. 3. Reactivity of the HDL subfractions with LCAT as a function of unesterified cholesterol concentrations. Aliquots (20–200 μl) of the HDL subfractions were labeled with [^{3}H]cholesterol and incubated with purified LCAT at 37°C for 3 hr. The LCAT activity was determined as nmol of cholesterol esterified per ml per hr for subfraction 1 (— ● —), subfraction 2 (— ▲ —), subfraction 3 (— ■ —), and subfraction 4 (— * —).

representing the esterification carried out by the added purified enzyme.

A number of parameters, previously related to LCAT activity (12, 13, 29, 30) are listed in Table 2. Of the four parameters considered, the CE/UC ratio and the apparent molecular weight showed some correlation with LCAT activity, since the subfraction with the highest CE/UC and lowest apparent molecular weight turned out to be the best substrate for the enzyme.

DISCUSSION

The studies presented here were designed to provide information regarding the effects of lipid and protein components of HDL on the LCAT reaction and to aid in the characterization of the in vivo substrates of the enzyme. Hamilton *et al.* (31) have shown that discoidal particles isolated from perfused rat liver (called "nascent HDL") were much better substrates for LCAT than the circulating spherical macromolecules referred to as "plasma HDL" (32). While plasma HDL appears to be an inferior substrate to nascent HDL in vitro, it is much more available to LCAT than nascent HDL, due to its much longer half life in vivo. Thus the investigation of the interaction between LCAT and the HDL subfractions appears relevant to the identification of the in vivo substrate(s) for the enzyme.

Human plasma HDL has been known for some time to be heterogeneous with respect to electrophoretic mobility and immunochemical behavior (33). Initially, two main subfractions were isolated by preparative ultracentrifugation and designated as HDL_2 (d 1.067–1.125 g/ml) and HDL_3 (d 1.125–1.21 g/ml) (34). Kostner and Alaupovic (35) have reported the isolation of high density lipoprotein subfamilies with different immunochemical and chemical composition. Olofsson and Gustafson (36) showed that HDL, isolated by ultracentrifugation, can be separated into three subfractions by chromatography and additional HDL subfamilies were identified upon the reaction of these HDL subfractions with LCAT (37). More recently, HDL isolated by ultracentrifugation, has been separated into three fractions with different apoA-I/apoA-II ratios using ion exchange chromatography (38). Glomset has suggested that a large number of HDL subfractions may exist in vivo. These may arise as the nascent particles leave the liver and subsequently interact with triglyceride-rich lipoproteins and LCAT (39).

The aim of the studies reported here, was to isolate HDL using a relatively mild procedure and to characterize the subfractions separated by anion exchange chromatography. Levy and Fredrickson (33) drew attention to the fact that preparative ultracentrifugation might result in the alteration of high density lipoprotein structure and composition. Cheung and Albers (40) have provided evidence that even a single step density gradient procedure altered the polypeptide distribution of high density lipoproteins and that apoA-I is readily dissociated from HDL during the centrifugation process (41, 42). In addition, studies with rat lipoproteins revealed that more than 50% of the total apoE might be dissociated from lipoproteins during sequential ultracentrifugation (43). These findings by other investigators prompted us to utilize a procedure, consisting of a precipitation step and a chromatographic step (18) for the isolation of HDL, which nevertheless yields HDL particles with the overall characteristics of those isolated by ultracentrifugation (21). The HDL preparation was then subjected to chromatography on DEAE cellulose (Fig. 1), giving rise to the data that suggest the presence of at least four subfractions of HDL with apparently unique lipid and/or apoprotein compositions, and molecular weights. During normal HDL catabolism from nascent particles to "plasma HDL" (32), there might be many more subspecies that exist in the circulation in vivo (44).

Thus the findings in this communication most likely describe a cross section of the many HDL subspecies that have been proposed to circulate in plasma from a fasting subject. The subfractions eluted at lower salt concentration (1 and 2) were found to have higher free cholesterol and triglyceride content and a lower CE/UC ratio than subfractions 3 and 4. Subfractions 1 and 2 thus resembled the subfractions isolated from HDL_2 by chromatography on hydroxylapatite (28). On the other hand, the subfractions that eluted at higher salt concentration (with relatively lower apoA-I/A-II ratio and higher CE/UC ratio) were similar to HDL_3 subfractions reported by the same investigators (28). The data from gel filtration experiments further support this possibility since subfractions 1 and 2 had a considerably higher molecular weight than subfractions 3 and 4 (Table 2). The association of endogenous LCAT activity with subfraction 4 (found to be similar to HDL_3) also supports the idea that LCAT circulates in the plasma in an HDL complex (44). Earlier reports have concluded that most of the enzyme activity was associated with HDL_3 and VHDL fractions (10, 45). Recent findings by Fielding and Fielding (46) suggested the existence of a complex consisting of one molecule of apoA-I, two molecules of apoD, and one molecule of LCAT. This complex apparently accounted for all the LCAT

and 20% of the apoD in the plasma. Subfraction 4, which contained all of the endogenous LCAT activity and about 30% of the total apoD (Fig. 1 and Table 3), may be similar to the transfer complex that was described by Fielding and Fielding (46). However, the transfer complex does not contain any apoA-II and the lipid composition of it has not yet been determined.

In order to study the LCAT substrate-potential of the isolated HDL substrate fractions, aliquots of purified enzyme were incubated with the isolated HDL fractions and the LCAT activity was assessed by measuring the rate of esterification of the free cholesterol in these subfractions. The results of these experiments are shown in Fig. 3. Subfraction 4 was found to react most efficiently with the purified LCAT preparation. This subfraction had the highest esterified to free cholesterol ratio, the lowest A-I to A-II ratio, the second highest phospholipid to unesterified cholesterol ratio, and the lowest molecular weight among the isolated subfractions. The last parameter may be of significance. Indeed, results from Glomset's laboratory (39) indicated earlier that the "small" HDL may be the best substrate for the enzyme and Fielding and Fielding (10) also found the smaller HDL_3 particles to be better substrates for LCAT than fractionated HDL or HDL_2. It remains to be established whether the favorable arrangement of lipid substrate(s) or the presence of some yet unrecognized cofactor(s) enabled the smaller particles (HDL_3) to serve as superior substrates for LCAT.

Indeed, numerous studies have been conducted with artificial substrates in order to elucidate the factors that might control the rate of the LCAT reaction in vivo. These investigations have established some of the factors that control the specificity of the enzyme in vitro, although the relevance of these studies to cholesterol esterification in vivo is yet to be established. Fielding, Shore, and Fielding (12) found that a lecithin to cholesterol molar ratio of approximately 3:1 was most favorable for the LCAT reaction and that excess amounts of triglycerides and cholesteryl esters were inhibitory in a lecithin–cholesterol–apoA-I liposome system. Using single bilayer vesicles as substrates, Chung et al. (29) showed that apoA-II, due to its greater affinity for the lipid surface, inhibited the LCAT reaction in the presence of phospholipids, cholesterol, and apoA-I. Furthermore, they reasoned that the apoA-I/A-II ratio at the vesicle surface could thus control the rate of the LCAT reaction in vitro. In more recent experiments, Chung et al. (30) prepared hybrid particles, by incubating dog HDL with human apoA-II. These particles had apoA-II/A-I ratios varying from 0.05 to 2.0. The LCAT activity, when tested against these lipoprotein hybrids, was inversely proportional to the apoA-II/apoA-I ratio.

Fielding and Fielding on the other hand showed (10) that HDL_3 was a considerably better substrate than HDL_2, even though the apoA-I/A-II ratio is higher in HDL_2 than in HDL_3 (42). Further, Pinon, Bridoux, and Laudat (11) found the relatively apoA-I-rich HDL_2 particles to be inhibitors of LCAT. Our data showed no correlation between LCAT activity and the phospholipid/cholesterol or the apoA-I/apoA-II ratios (Table 2). However, two of the studied parameters, the CE/UC ratio (positively) and the molecular weight of the HDL fractions (negatively) showed some correlation with LCAT activity. Of these two latter relationships, the correlation between the CE/UC ratio and LCAT activity might be fortuitous. The enrichment in cholesteryl esters may reflect the particles' prior association with LCAT. Alternatively, the relatively low ester content of the high molecular weight particles could be the result of preceeding transfer of cholesteryl esters to other classes of lipoproteins (47). There appears to be some discrepancy in the correlation between LCAT activity and PL/UC, CE/UC, and apoA-I/apoA-II ratios. However, this can be best explained by differences that exist between the substrates used for those studies. Therefore, it must be emphasized that physiochemical properties and overall morphologies of the artificial lipid/protein complexes are likely to be quite different from plasma HDL.

The study presented here provides, for the first time, information on the LCAT reaction with HDL subfractions isolated by column chromatography. However, additional and more definitive experiments will have to be performed to explain the precise nature of the interaction of LCAT with HDL subfractions and its possible significance in vivo.

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REFERENCES

1. Simon, J. B., and J. L. Boyer. 1970. Production of lecithin:cholesterol acyltransferase by the isolated perfused rat liver. *Biochim. Biophys. Acta.* **218:** 549–551.
2. Glomset, J. A. 1970. Physiological role of lecithin: cholesterol acyltransferase. *Am. J. Clin. Nutr.* **23:** 1129–1136.
3. Marcel, Y. L., and C. Vezina. 1973. Lecithin: cholesterol acyltransferase of human plasma. Role of chylomicrons, very low, and high density lipoproteins in the reaction. *J. Biol. Chem.* **248:** 8254–8259.
4. Varma, K. G., and L. A. Soloff. 1976. A method for

purification of milligram quantities of stable human phosphatidylcholine cholesterol acyltransferase. *Biochem. J.* **155:** 583–588.
5. Albers, J. J., V. G. Cabana, and Y. D. B. Stahl. 1976. Purification and characterization of human plasma lecithin:cholesterol acyltransferase. *Biochemistry.* **15:** 1084–1087.
6. Aron, L., S. Jones, and C. J. Fielding. 1978. Human plasma lecithin:cholesterol acyltransferase: characterization of cofactor-dependent phospholipase activity. *J. Biol. Chem.* **253:** 7220–7226.
7. Kitabatake, K., U. Piran, Y. Kamio, Y. Doi, and T. Nishida. 1979. Purification of human plasma lecithin: cholesterol acyltransferase and its specificity toward the acyl acceptor. *Biochim. Biophys. Acta.* **573:** 145–154.
8. Glomset, J. A., E. T. Janssen, R. Kennedy, and J. Dobbins. 1966. Role of plasma lecithin:cholesterol acyltransferase in the metabolism of high density lipoproteins. *J. Lipid Res.* **7:** 638–648.
9. Akanuma, Y., and J. Glomset. 1968. In vitro incorporation of cholesterol-^{14}C into very low density lipoprotein cholesteryl esters. *J. Lipid Res.* **9:** 620–626.
10. Fielding, C. J., and P. E. Fielding. 1971. Purification and substrate specificity of lecithin:cholesterol acyltransferase from human plasma. *FEBS Lett.* **15:** 355–358.
11. Pinon, J. C., A. M. Bridoux, and M. H. Laudat. 1980. Initial rate of cholesterol esterification associated with high density lipoproteins in human plasma. *J. Lipid Res.* **21:** 406–414.
12. Fielding, C. J., V. G. Shore, and P. E. Fielding. 1972. Lecithin:cholesterol acyltransferase: effects of substrate composition upon enzyme activity. *Biochim. Biophys. Acta.* **270:** 513–518.
13. Nakagawa, M., and T. Nishida. 1973. Role of high density lipoproteins in the lecithin:cholesterol acyltransferase activity with sonicated lecithin:cholesterol dispersions as substrate. *Biochim. Biophys. Acta.* **296:** 577–585.
14. Glomset, J. A. 1963. Further studies of the mechanism of the plasma cholesterol esterification reaction. *Biochim. Biophys. Acta.* **70:** 389–395.
15. Kostner, G. M. 1978. The influence of various lipoproteins and apolipoproteins on the in vitro esterification of cholesterol in human plasma by the enzyme lecithin:cholesterol acyltransferase. *Scand. J. Clin. Lab. Invest.* **38: Suppl. 150:** 66–71.
16. David, J. S. K., L. A. Soloff, and A. G. Lacko. 1976. Factors affecting the esterification of lipoprotein cholesterol by lecithin:cholesterol acyltransferase. *Life Sci.* **18:** 701–706.
17. Chen, R. F. 1966. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* **242:** 173–181.
18. Jahani, M., R. Huttash, and A. G. Lacko. 1980. A novel chromatographic method for the isolation preparation of high density lipoproteins. *Prep. Biochem.* **10:** 431–444.
19. Hatch, F. T., and R. S. Lees. 1968. Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* **6:** 1–68.
20. Lacko, A. G., and T. F. Chen. 1977. Enzyme purification by affinity chromatography using a non-covalently bound adsorbent. *J. Chromatogr.* **130:** 446–450.
21. Rudel, L. L., J. A. Lee, M. D. Morris, and J. M. Felts. 1974. Characterization of plasma lipoproteins separated and purified by agarose-column chromatography. *Biochem. J.* **139:** 89–95.
22. Lacko, A. G., H. L. Rutenberg, and L. A. Soloff. 1972. Recovery of labeled cholesterol and cholesterol esters from thin-layer chromatograms. *Clin. Chim. Acta.* **39:** 506–511.
23. Cham, B. E., and B. R. Knowles. 1976. A solvent system for delipidation of plasma or serum without protein precipitation. *J. Lipid Res.* **17:** 176–181.
24. Kane, J. P. 1973. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. *Anal. Biochem.* **53:** 350–364.
25. Blakesley, R. W., and J. A. Boezi. 1977. A new staining technique for proteins in polyacrylamide gels using Coomassie brilliant blue G-250. *Anal. Biochem.* **82:** 580–582.
26. Wilson, C. 1979. M.Sc. Thesis, North Texas State University.
27. Stokke, K. T., and K. R. Norum. 1971. Determination of lecithin:cholesterol acyltransfer in human blood plasma. *Scand. J. Clin. Lab. Invest.* **27:** 21–27.
28. Kostner, G., and A. Holasek. 1977. The separation of human serum high density lipoproteins by hydroxylapatite column chromatography. *Biochim. Biophys. Acta.* **488:** 417–431.
29. Chung, J., D. A. Abano, G. M. Fless, and A. M. Scanu. 1979. Isolation, properties, and mechanism of in vitro action of lecithin:cholesterol acyltransferase from human plasma. *J. Biol. Chem.* **254:** 7456–7464.
30. Chung, J., P. Lagocki, D. Albano, and A. M. Scanu. 1979. Activity in vitro of lecithin:cholesterol acyltransferase (LCAT) on high density lipoproteins (HDL) of varying ApoA-I:ApoA-II ratios. *Circulation.* **60:** 11–85.
31. Hamilton, R. L., M. C. Williams, C. J. Fielding, and R. J. Havel. 1976. Discoidal bilayer structure of nascent high density lipoproteins from perfused rat liver. *J. Clin. Invest.* **58:** 667–680.
32. Hamilton, R. L. 1978. *In* Disturbances in Lipid and Lipoprotein Metabolism. J. M. Dietchy, A. M. Gotto, Jr., and J. A. Ontko, editors. American Physiology Society. Williams and Wilkins, Baltimore. 155–173.
33. Levy, R. I., and D. Fredrickson. 1965. Heterogeneity of plasma high density lipoproteins. *J. Clin. Invest.* **44:** 426–440.
34. DeLalla, O., and J. W. Gofman. 1954. Ultracentrifugal analysis of serum lipoproteins. *In* Methods of Biochemical Analysis. Vol. 1. D. Glick, editor. Interscience, New York. 459–478.
35. Kostner, G. M., and P. Alaupovic. 1972. Studies of the composition and structure of plasma lipoprotein, separation, and quantification of lipoprotein families occurring in high density lipoproteins of human plasma. *Biochemistry.* **11:** 3419–3428.
36. Olofsson, S. O., and A. Gustafson. 1974. Studies of human serum high density lipoproteins 2. Isolation of subfractions in the cold. *Scand. J. Clin. Lab. Invest.* **34:** 257–263.
37. Olofsson, S. O., and A. Gustafson. 1974. Degradation of high density lipoproteins (HDL) in vitro. *Scand. J. Clin. Lab. Invest.* **33: Suppl. 137:** 57–62.

38. Rubenstein, B. 1979. Protein content and composition of human high density lipoprotein subfractions. *Atherosclerosis.* **33:** 415–423.
39. Glomset, J. A. 1972. *In* Blood Lipids and Lipoproteins. G. J. Nelson, Editor. Wiley-Interscience. 745–788.
40. Cheung, M. C., and J. J. Albers. 1979. Distribution of cholesterol and apolipoprotein A-1 and A-11 in human high density lipoprotein subfractions separated by CsCl equilibrium gradient centrifugation: evidence for HDL subpopulations with differing A-1/A-11 molar ratios. *J. Lipid Res.* **20:** 200–207.
41. Albers, J. J., and F. Aladjem. 1971. Precipitation of ^{125}I-labeled lipoproteins with specific polypeptide antisera. Evidence for two populations with differing polypeptide composition in human high density lipoproteins. *Biochemistry.* **10:** 3436– 3442.
42. Kostner, G. M., J. R. Patch, S. Sailer, H. Braunsteiner, and A. Holasek. 1974. Polypeptide distribution of main lipoprotein density classes separated from human plasma by rate zonal ultracentrifugation. *Eur. J. Biochem.* **15:** 611–621.
43. Mahley, R. W., and K. S. Holcombe. 1977. Alterations of plasma lipoproteins and apoproteins following cholesterol feeding in the rat. *J. Lipid Res.* **18:** 314–324.
44. Glomset, J. A. 1980. Lecithin:cholesterol acyltransferase. An exercise in comparative biology. *Prog. Biochem. Pharmcol.* **15:** 41–66.
45. Lacko, A. G., K. G. Varma, H. L. Rutenberg, and L. A. Soloff. 1974. Studies on the enzymatic and molecular properties of LCAT. *Scand. J. Clin. Lab. Invest.* **33: Suppl 137:** 29–34.
46. Fielding, P. E., and C. J. Fielding. 1980. A cholesteryl ester complex in human plasma. *Proc. Natl. Acad. Sci. USA.* **77:** 3327–3330.
47. Rose, H. G. 1978. Transfer and equilibration of LCAT-generated cholesteryl esters in human lipoproteins. *Scand. J. Clin. Lab. Invest.* **38: Suppl. 150:** 91–97.
48. Alaupovic, P., M. D. Curry, and W. J. McConathy. 1978. *In* International Conference on Atherosclerosis. L. A. Carlson, R. Paoletti, and G. Weber, editors. Raven Press. 109–121.