The characterization of lipoproteins in the high density fraction obtained from patients with familial lecithin:cholesterol acyltransferase deficiency and their interaction with cultured human fibroblasts.

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Abstract Lipoproteins of density 1.063–1.21 g/ml were isolated from the plasma of three sisters of Irish origin with familial LCAT deficiency. Fractionation of the lipoproteins on the basis of particle size by chromatography on Sephacryl S-300 permitted partial separation of two major and at least three other minor components which differed in their lipid:protein ratio and their apolipoprotein content. One of the major components was a small spherical lipoprotein whose sole apolipoprotein was apoA-I; the second major component contained predominantly apoA-I, together with apoE, and in addition, an apolipoprotein of molecular weight 46,000 that was not cleaved by reduction of disulfide bonds, and which was identified as apoA-IV. This apoprotein has not previously been detected in the lipoproteins of LCAT-deficient patients. A second apoE-containing lipoprotein, which contained apoA-I and apoE in a ratio of approximately 2:1, was also present as a minor component, together with two or more minor components whose apoproteins were comprised of apoA-I and apoC. The apoE-containing lipoproteins competed efficiently with ^{125}I-labeled LDL for binding to high affinity LDL-receptor sites on the surface of cultured human skin fibroblasts. The ability to bind to the LDL-receptor was directly proportional to the apoE content of the lipoproteins, even when other apoproteins, with the exception of apoB, were present in relatively large proportions. ApoE-containing ^{125}I-labeled lipoproteins from an LCAT-deficient subject were also taken up and degraded by the cultured cells.—**Soutar, A. K., B. L. Knight, and N. B. Myant.** The characterization of lipoproteins in the high density fraction obtained from patients with familial lecithin:cholesterol acyltransferase deficiency and their interaction with cultured human fibroblasts. *J. Lipid Res.* 1982. **23:** 380–390.

Supplementary key words apoA-IV • apoE • HDL • LDL-receptor

The enzyme LCAT catalyzes the transfer of a fatty acyl group from the 2-position of lecithin to the 3-OH group of cholesterol, utilizing as substrate the lipids present on the surfaces of plasma lipoproteins (1). This reaction is accompanied by marked changes in the structure and composition of newly-secreted lipoproteins. If LCAT is not present in the plasma, as in familial LCAT deficiency, abnormal lipoproteins accumulate in the circulation (2). In particular, the lipoproteins isolated within the density range of HDL (1.063–1.21 g/ml) are heterogeneous and contain at least one population of disc-shaped particles composed of unesterified cholesterol and phospholipid, and with apoA-I and apoE as their major apoproteins (3). These abnormal particles are thought to represent newly-formed precursors of HDL that under normal conditions are very rapidly converted into mature HDL in the presence of LCAT. The interaction of "immature HDL" from LCAT-deficient patients with tissue cells in vitro may thus provide information as to what may occur in vivo when nascent HDL particles enter the blood circulation or the interstitial fluid. Of particular interest are the discoidal particles rich in apoE, since lipoproteins containing apoE are known to compete with LDL for binding to LDL receptors on human cells in culture (4).

In this study we describe the isolation and partial characterization of HDL from three recently described LCAT-deficient patients (5) and the interaction of these lipoproteins with the LDL receptors of cultured human skin fibroblasts.

METHODS

Subjects

The LCAT-deficient subjects were three sisters of Irish origin (K.B., T.C. and B.R.) whose clinical details

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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TABLE 1. Plasma lipids and LCAT activity in three LCAT-deficient subjects

	Total Cholesterol	% Esterified	Total Triglyceride	HDL-Cholesterol	Plasma LCAT: LCAT Activity[a]	Plasma LCAT: Esterification Rate[b]
	nmol/l		*mmol/l*			
B.R.	5.0	8.5	5.73	0.13	0	0
K.B.	5.5	16.2	2.46	0.13	0	0
T.C.	5.0	11.7	2.55	0.18	1.3	0
Control	5.0	77.3	1.0	1.1	121.0	110

[a] LCAT activity, μmol cholesterol esterified per liter plasma per hour (assayed by the method of Glomset and Wright (7)).
[b] Net esterification rate (assayed by the method of Stokke and Norum (6)).

are described elsewhere (5). B.R. had acute renal failure but there was no evidence of renal insufficiency in the other two sisters. The diagnosis of familial LCAT deficiency was based on the complete absence of plasma LCAT activity estimated by the methods of Stokke and Norum (6) and of Glomset and Wright (7). The plasma lipids and LCAT activity in the three subjects are shown in **Table 1.** Chylous urine was obtained from a patient with chyluria due to chronic filariasis.

Isolation and fractionation of lipoproteins

Plasma was isolated from freshly-drawn blood (obtained after an overnight fast) to which 0.01% EDTA (w/v) had been added as anticoagulant. The lipoproteins of density 1.063–1.21 g/ml (the HDL fraction) were isolated by differential density centrifugation in NaCl/KBr (8). To minimize loss of easily dissociated apoproteins, the lipoproteins were subjected to a single centrifugation at density 1.21 g/ml, although this resulted in some contamination with serum albumin (see Fig. 1). LDL (density 1.019–1.055 g/ml) and HDL (density 1.063–1.21 g/ml) were isolated from normal plasma by centrifugation in NaCl/KBr solutions, each lipoprotein being washed once by recentrifugation at the higher density (8). Lipoproteins of density less than 1.006 g/ml were isolated from chylous urine by a single centrifugation at background density for 16 hr at 53,000 rpm in a Beckman 60 Ti rotor.

Samples of the HDL fraction from LCAT-deficient subjects (4 ml from 20–50 ml of plasma) were applied to a column of Sephacryl S-300 (1.5 × 110 cm) equilibrated with 0.02 M Tris-HCl (pH 7.5) containing 0.15 M NaCl, 1 mM EDTA, and 0.01% azide, and the column was eluted with the same buffer at a flow rate of 12 ml/hr. Fractions of 2 ml were collected, monitored for protein by measurement of E_{280}, and stored at 4°C. Each fraction was assayed for protein, cholesterol, and phospholipid, and the lipoprotein and apoprotein content of each was determined by gradient PAGE and SDS-PAGE as described below.

Preparation of antiserum to authentic apoA-IV

Apolipoprotein A-IV was isolated from lipoproteins of density less than 1.006 g/ml from chylous urine by preparative gel electrophoresis (9). Samples of gel containing 200–500 μg apoA-IV were homogenized with an equal volume (1–2 ml) of complete Freund's adjuvant and injected subcutaneously into four sites on the back of a male New Zealand White rabbit. This procedure was repeated twice at 2-week intervals and, after a further 2-week interval, blood was withdrawn from the rabbit to test for the presence of antibodies to apoA-IV in the serum. Double immunodiffusion on Ochterlony plates was performed on glass slides with 1.5% (w/v) agarose in 0.075 M barbitone buffer, pH 8.6. Samples of lipoproteins were delipidated and solubilized in 8 M urea, 10 mM Tris–HCl, pH 7.5, and applied to the wells. To test for the presence or absence of apoA-IV in samples, several 5-fold serial dilutions of antiserum were tested against each antigen, and several 5-fold serial dilutions of antigen were tested against the antiserum to apoA-IV and to a non-immune serum. After 48 hr, the plates were washed for 24 hr in several changes of phosphate-buffered saline, air-dried, and then stained for protein by immersion for 30 min in 0.1% Coomassie blue, 40% (v/v) aqueous methanol, 10% (v/v) acetic acid. Destaining was performed for 1 hr in 40% (v/v) aqueous methanol, 10% (v/v) acetic acid.

Labeling of lipoproteins

LDL was labeled by the iodine monochloride method (10) with the modification of Bilheimer et al. (11). A sample of the major chromatographic component of the HDL fraction of K.B. (see Results) was concentrated 5- to 10-fold by suspending a dialysis sac containing the lipoprotein solution at 4°C before a fan for 6–8 hr. The concentrated sample (1.0 mg of protein/ml) was dialyzed against 0.15 M NaCl, 1 mM EDTA (pH 7.4), containing 0.01% azide, to remove excess salt. A portion of the concentrated sample was labeled with ^{125}I essentially as

described above, but using 15 μg of ^{125}I per mg of protein. When the ^{125}I-labeled lipoprotein from K.B. was precipitated with 10% trichloroacetic acid (w/v, final concentration), 97% of the ^{125}I was associated with the protein pellet. After extraction with chloroform–methanol 2:1 (v/v) (12), 90% of the radioactivity was associated with the aqueous non-lipid phase. The apoprotein composition of the concentrated lipoprotein and of the labeled lipoprotein was compared with that of the dilute unlabeled lipoprotein by SDS-PAGE. Labeled lipoproteins were stored sterile at 4°C and used for experiments within 2 weeks, during which time no changes could be detected in their composition or biological activity. Before use, labeled lipoproteins were dialyzed against phosphate-buffered saline for 2 hr to remove free iodide, and the specific activity was determined.

Analytical methods

Protein concentrations in solutions of lipoproteins or cell protein were determined by the method of Lowry et al. (13) with bovine serum albumin as standard. The cholesterol content of aqueous solutions of lipoproteins was determined by the method of Heider and Boyett (14). Phospholipid was assayed in dilute lipoprotein solutions with the fluorimetric method of Jouanel et al. (15), with a sample of normal HDL as standard, in which the lipid phosphorus content had been determined previously by the modification of the Fiske-Subbarow method of Marinetti (16).

Gradient PAGE was carried out with a Uniscil-Gradipore system. The gels (4%–26% acrylamide, 7.5 cm^2) were pre-run for 20 min at 70 V in electrophoresis buffer (0.09 M Tris–HCl, 0.08 M borate, 3 mM EDTA, pH 8.35) before application of samples (50 μl, containing 2–10 μg protein in 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.01% azide, and 10% glycerol). Electrophoresis was carried out at 70 V until the bromophenol blue had entered the gel (about 20 min), followed by 17 hr at 125 V. Discontinuous SDS-PAGE was carried out on acrylamide slab gels (13% acrylamide, 16 cm × 16 cm × 1.5 mm) using the procedure of Laemmli (17). Samples of lipoprotein were lyophilized, delipidated with methanol–ether 1:2 (v/v), and washed with ether. The apoproteins were solubilized by heating for 1 min at 100°C in electrophoresis sample buffer (0.062 M Tris-HCl, pH 6.7, containing 1% SDS (w/v), 10% glycerol (w/v), and 0.1% 2-mercaptoethanol (v/v)). Recovery of protein during the delipidation process was essentially 100%. Furthermore, the pattern obtained from a sample that was delipidated before solubilization was indistinguishable from that obtained when the sample was lyophilized before solubilization, indicating that delipidation of the lipoprotein did not cause selective loss of apoproteins. On occasions, 2-mercaptoethanol was omitted from the sample buffer. Samples (50 μl containing 1–10 μg protein) were applied to the gel and electrophoresis was carried out for 3–4 hr at 30 ma per gel. Gradient gels and SDS-gels were stained by immersion in 0.05% (w/v) Coomassie blue in 40% methanol, 10% acetic acid (v/v). Apoproteins were identified as bands on SDS-gels by comparison of their apparent molecular weights with those of known standard proteins (Low MW Calibration Kit, Pharmacia Ltd.) and with apoproteins from normal human plasma lipoproteins. SDS-PAGE gels were scanned with a Joyce-Loebl gel-scanner and the relative amounts of apoA-I, apoA-IV, and apoE present were determined from the areas under the peaks. When different amounts of the same sample were applied to a gel, the area under the peak after scanning was proportional to the amount of sample applied to the gel in the range tested. No attempt was made to equate the area to an exact amount of apoprotein, and thus the content of a particular apoprotein in a sample was determined in "arbitrary units (of area)/ml" to allow comparison of the relative amounts of any one apoprotein in each type of particle.

Preparative SDS-gel electrophoresis was carried out essentially as described for the analytical procedure. After electrophoresis, the area of the gel containing apoA-IV was identified by immersing a small portion of the gel in a solution of 4 M sodium acetate (18) and this strip was removed. A small portion of the sample was eluted electrophoretically from the gel and run on an analytical gel to check its purity, and the remainder of the gel containing apoA-IV was used as an antigen.

Cell culture

Human fibroblasts derived from skin biopsies of normal subjects were maintained in monolayer culture as previously described (19). Cells between the tenth and fifteenth passage were seeded at a concentration of 10^5 cells/dish into 60-mm plastic petri dishes containing 3 ml of Eagle's minimal essential medium (MEM) with Earle's salts, 20 mM N-((trishydroxymethyl)methyl) glycine, 25 mM $NaHCO_3$, and 10% fetal calf serum. Cells were re-fed with fresh medium after 3 days, and on the sixth day after seeding the monolayer was washed with Dulbecco's phosphate-buffered saline and the cells were incubated with 2 ml of medium containing LPDS (2.5 mg/ml protein) in place of fetal calf serum. Experiments were performed 24 hr later when each dish contained approximately 300 μg of protein.

Experimental procedure

For studies of binding of lipoproteins to cultured cells at 4°C, the medium was replaced with 2 ml of medium, containing LPDS, from which $NaHCO_3$ was omitted and the cells were incubated for 1 hr at 4°C before the

addition of labeled lipoproteins. Labeled lipoprotein and competing unlabeled lipoproteins were added to the dish at concentrations indicated for each figure. Incubation at 4°C was continued for a further 2 hr, after which the medium was removed and discarded. The cell monolayers were extensively washed as described by Goldstein et al. (20) and solubilized in 2.0 ml of 0.1 M NaOH for determination of surface-bound radioactivity and cell protein. Specific surface-binding of ^{125}I-labeled LDL was expressed as the difference between radioactivity bound in the presence and absence of excess unlabeled LDL (1.0 mg/ml protein).

For experiments at 37°C, the labeled lipoprotein was added to the medium in which the cells had been incubated for the previous 24 hr. After incubation at 37°C for 5 hr, the medium was removed and assayed for trichloroacetic acid-soluble non-iodide radioactivity (21). The values obtained were corrected for breakdown that occurred in medium alone to give the amount of lipoprotein degraded by the cells. The cell monolayers were then washed extensively (21) and solubilized in 2.0 ml of 0.1 M NaOH for determination of total cell-associated radioactivity (that bound to cell surfaces plus that internalized but not degraded (20)). All determinations were carried out in duplicate.

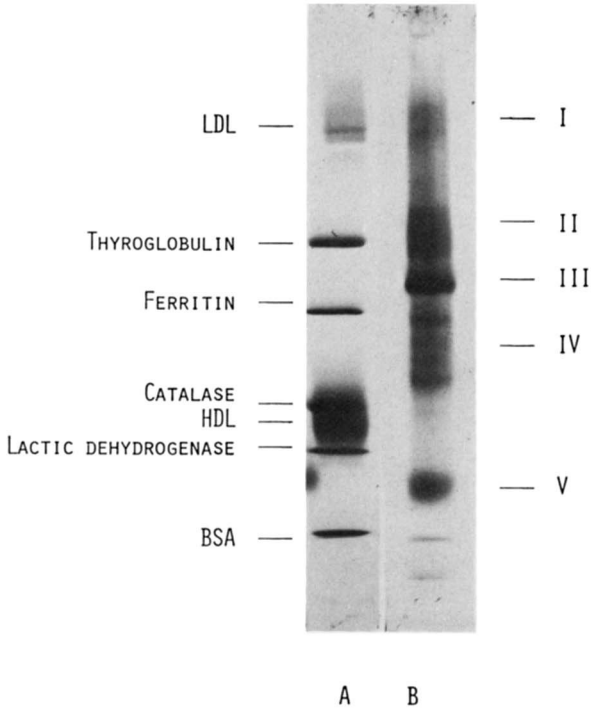

Fig. 1. Gradient polyacrylamide gel electrophoresis of the HDL fraction from a subject with familial LCAT deficiency. Samples of standard proteins A, approx. 1 μg of each protein; and B, the HDL fraction from T.C. (10 μg total protein) were applied to a slab gradient gel (4–26% acrylamide) and subjected to electrophoresis for 20 min at 70 V, followed by 17 hr at 125 V. The gel was stained for protein with Coomassie brilliant blue. BSA, bovine serum albumin.

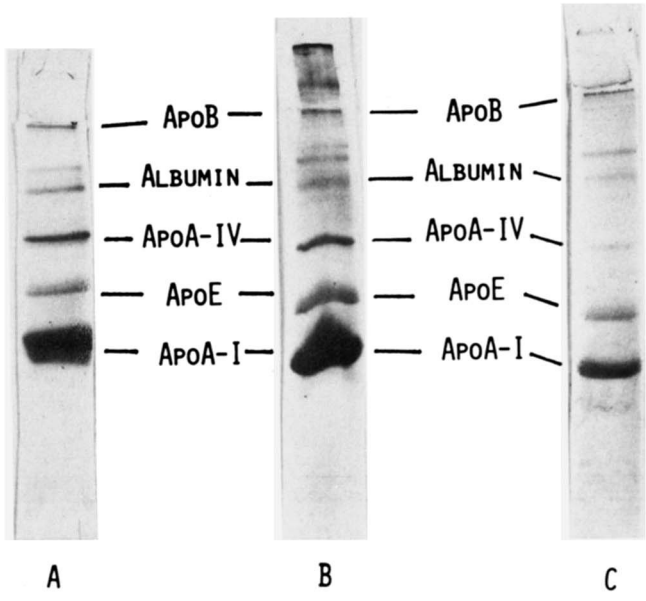

Fig. 2. SDS-polyacrylamide gel electrophoresis of delipidated HDL fractions from three subjects with familial LCAT deficiency. Samples of delipidated HDL fractions (approx. 5 μg total protein) were reduced with 2-mercaptoethanol and applied to slab SDS-polyacrylamide gels (13% acrylamide, 16 cm^2) and subjected to electrophoresis for 3–4 hr with 30 ma per gel. The gels were stained for protein with Coomassie brilliant blue. A, K.B.; B, T.C.; C, B.R.

RESULTS

Isolation and characterization of the HDL fraction from LCAT-deficient subjects

The HDL fraction was isolated from three LCAT-deficient subjects and subjected to gradient gel electrophoresis in a non-dissociating system. At least five separate lipoprotein bands could be detected, with apparent particle weights ranging from greater than 1×10^6 to 1×10^5, as shown in **Fig. 1.** There were two major components, designated III and V in Fig. 1, and other minor components including serum albumin. No band corresponding to normal HDL was visible in any of the samples. The distribution of bands obtained with the three subjects was similar although there were differences in the relative proportions of each band, especially between B.R., who had acute renal failure, and the other two subjects. Two samples obtained from T.C. on separate occasions gave virtually identical patterns. The apolipoprotein content of the total HDL fraction from the three subjects as assessed by SDS-PAGE is shown in **Fig. 2.** ApoA-I comprised a major proportion of the total apolipoproteins, while apoE was less abundant. Low molecular weight proteins, apoA-II and apoC, were also present. Of particular interest was the presence of an apoprotein with a molecular weight of 46,000. This apoprotein, which was totally resistant to cleavage by 2-mercaptoethanol, had identical mobility on the gel to that of the apoA-IV present in chylomicrons isolated from the

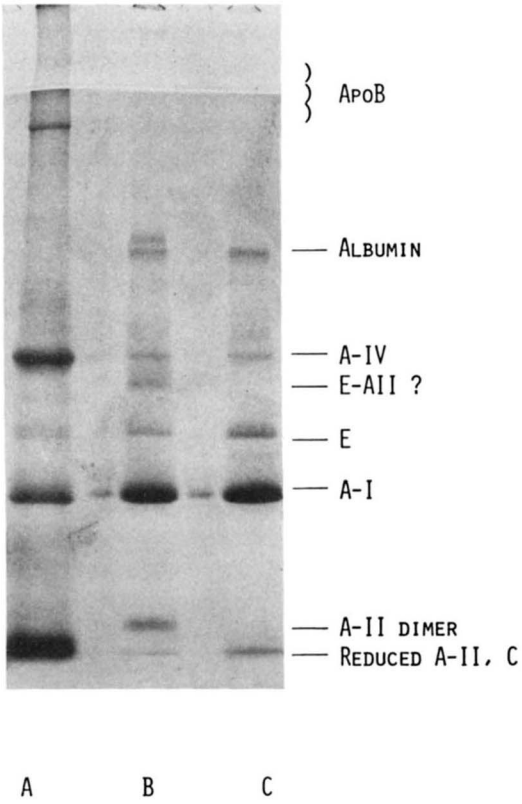

Fig. 3. Effect of reduction with 2-mercaptoethanol on the apoprotein content of the HDL fraction from an LCAT-deficient subject. Samples of delipidated protein were solubilized by heating to 100°C for 2 min in SDS-gel sample buffer with or without 2-mercaptoethanol (2 ME, 0.1% v/v) before application to an SDS gel (13% acrylamide). The gel was stained for protein with Coomassie brilliant blue. Samples were: A, chylous urine chylomicrons after reduction with 2 ME; B, a portion of the HDL fraction from LCAT-deficient subject T.C. (particle type III in Fig. 6) without prior reduction; and C, the same sample after reduction with 2 ME.

urine of a chyluric subject (**Figs. 3 and 4**). A protein with a molecular weight of 41,000 was visible on the gel of the non-reduced sample, but not when the sample was reduced with 2-mercaptoethanol prior to electrophoresis (Fig. 3). This apoprotein was tentatively identified as the apoA-II-apoE complex. Relatively small amounts of albumin could be detected, as well as a protein with a molecular weight of approximately 75,000, which was not identified. A large protein that barely entered the gel was presumed to be apoB.

The apoprotein of molecular weight 46,000 in the HDL-fraction of the LCAT-deficient subjects was immunochemically identical to apoA-IV in lipoproteins of density less than 1.006 g/ml isolated from chylous urine. The antiserum raised against purified apoA-IV from chylous urine did not give a precipitin line with normal plasma HDL, normal plasma VLDL, or purified apoA-I or apoA-II (a generous gift of Dr. D. Reichl), but gave an identical precipitin line with the delipidated apoproteins from lipoproteins of density less than 1.006 g/ml from chylous urine and with particles of type III isolated by gel filtration chromatography from the HDL fraction from LCAT-deficient subject T.C. (see below) (**Fig. 5**). This fraction contained apoA-I, apoC, apoE, and the apoprotein of molecular weight 46,000 as its sole apoprotein components. Thus, the failure of the antiserum to react with normal VLDL that contains apoE and apoC, or with normal HDL, that contains apoA-I, apoA-II and apoC, confirms the identity of the 46,000 molecular weight apoprotein with the 46,000 molecular weight apoprotein found in chylomicrons isolated from chylous urine previously designated apoA-IV (9, 22).

Partial separation of the different components in the d 1.063–1.21 g/ml fraction was effected by chromatography on Sephacryl S-300. The results obtained with a sample from T.C. are shown in **Fig. 6,** A and B, which shows the phospholipid, cholesterol, and protein content of each fraction, and the distribution of each lipoprotein

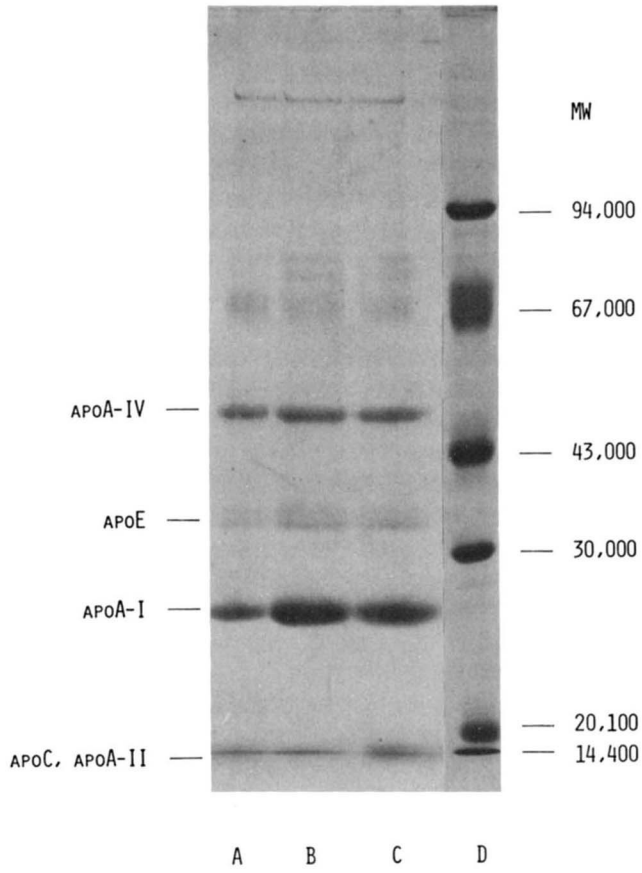

Fig. 4. Identity of the 46,000 molecular weight apoprotein in the HDL fraction of LCAT-deficient subjects with apoA-IV in lipoproteins of density less than 1.006 g/ml from chylous urine. SDS-PAGE of delipidated apoprotein of lipoproteins of density less than 1.006 g/ml from chylous urine (A), of total HDL fraction from K.B. (C), and a mixture of these two (B). Also shown is a mixture of standard proteins of known molecular weight (D).

component as assessed by gradient PAGE. The apolipoproteins present in each fraction were assessed from SDS-PAGE and the content of apoA-IV, apoE, and apoA-I were estimated semiquantitatively by scanning the gels as described in the Methods section. The content of apoB, apoA-II, and apoC apolipoproteins could not be determined quantitatively from these 13% gels, which were chosen to give a good separation of apoA-I and apoE. The distribution of lipoproteins and apolipoproteins in each fraction from the column suggested that several different particles with non-identical apoprotein compositions could be separated by gel filtration from the HDL fraction of these LCAT-deficient subjects. From the data in Fig. 6A and 6B, fractions were selected that contained essentially a single component whose properties are summarized in **Table 2.**

The largest component (particle I in Fig. 1 and Table 2), which emerged at the void volume, was the most variable among the three subjects and probably represents contamination by apoB-containing lipoproteins more correctly considered as part of the LDL fraction. No attempts were made to characterize this fraction further. At least two apoE-containing species were found, the first being a minor component (II) with an apparent molecular weight of 650,000 with apoA-I and apoE in the ratio of approximately 2:1. The approximate ratio is based on the areas under the peaks after scanning SDS-PAGE gels, and does not take into account differences in chromogenicity of the two apoproteins. However, it does permit comparison of the ratio from one particle type to another. The second apoE-containing particle constituted approximately 40% of the total lipoprotein fraction in each subject and was slightly smaller, with an apparent molecular weight of 500,000 (III). This lipoprotein contained a much greater pro-

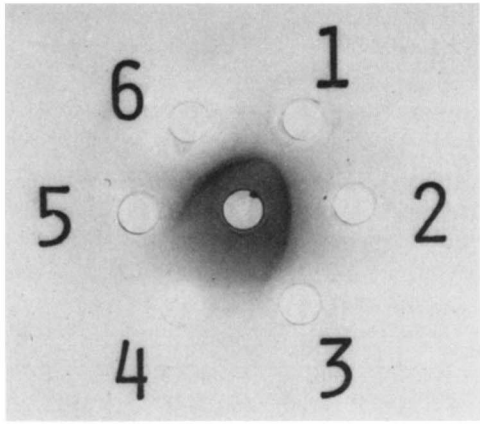

Fig. 5. Immunochemical identity of the 46,000 molecular weight apoprotein in the HDL fraction from LCAT-deficient subjects with apoA-IV in lipoproteins of density less than 1.006 g/ml from chylous urine. Immunodiffusion plate, with antiserum to apoA-IV in the center well. The outer wells contained normal plasma HDL (1), particles of type III from the HDL fraction of K.B. (2), normal plasma VLDL (3), apoA-I (4), apoA-II (5), and lipoproteins of density less than 1.006 g/ml from chylous urine (6). All lipoproteins were delipidated and these, together with the purified apoproteins, were solubilized in 8 M urea, 0.01 M Tris-HCl, pH 8.0, before application to the wells.

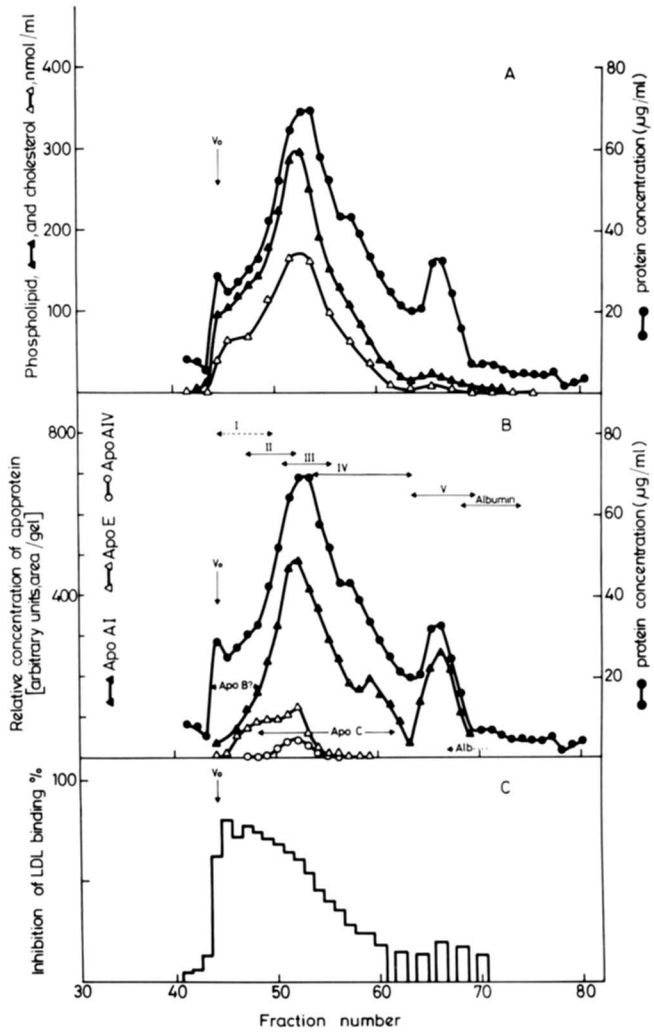

Fig. 6. Chromatography on Sephacryl S-300 of the HDL fraction from an LCAT-deficient subject. A sample of the HDL fraction from T.C. (4 ml containing approx. 10 mg total protein isolated from 45 ml of plasma) was applied to a column of Sephacryl S-300 (2.5 cm × 100 cm) equilibrated with 0.15 M NaCl, 0.001 M EDTA, 0.02 M Tris-HCl, pH 7.5; the column was eluted with the same buffer at a flow rate of 36 ml/hr and 4.5-ml fractions were collected. A, A sample from each fraction was assayed for protein, ● —— ●; phospholipid ▲ —— ▲; and free cholesterol, △ —— △. B, The relative content of apoA-I, ▲ —— ▲; apoE, △ - - - - - △, and apoA-IV, ○ —— ○, was determined from densitometric scans of SDS gels of 50 μl samples of each fraction. The presence of apoB and low molecular weight apoC proteins was determined qualitatively from the gels. The distribution of lipoproteins in fractions eluted from the column was assessed from gradient polyacrylamide gels of 50 μl samples of each fraction as described in Fig. 1. C, The ability of an equal volume (50 μl) of each fraction to compete with normal ^{125}I-labeled LDL at 5 μg/ml protein for binding at 4°C to the surface of human skin fibroblasts was determined. Inhibition is expressed as percentage inhibition of that portion of ^{125}I-labeled LDL bound that could be displaced by an excess of unlabeled normal LDL (1 mg/ml protein).

TABLE 2. Composition of lipoproteins of d 1.063–1.21 g/ml from T.C., fractionated by gel filtration

Particle Type[a]	Fraction No.[b]	Approx Mol Wt[a]	Protein	Cholesterol	Phospholipid	ApoA-I[c]	ApoE[c]	ApoA-IV[c]	ApoB[d]	ApoC[d]
			μg/ml	*mmol/ml*		*arbitrary units/ml*[c]				
I	Void volume	$1–2 \times 10^6$	55.0	39.0	97.0				++	+
II	49	650,000	42.0	114.0	178.0	4.7	1.8	0.1		+
III	52	500,000	67.0	171.0	296.0	9.7	2.5	0.9		++
IV	55	400,000	51.0	78.0	152.0	5.8	0.2	trace		+++
	59	275–325,000	33.5	36.0	62.0	3.9	trace			+++
V	66	100,000	32.5	20.0	19.0	5.2				

[a] Identified from gradient PAGE (Fig. 1).
[b] Fractions eluted from Sephacryl S300 column (Fig. 6).
[c] Determined from areas under peaks after scanning SDS-PAGE gels (see Methods for further details).
[d] Not quantitative; proteins identified on SDS-PAGE.

portion of apoA-I to apoE and also contained the bulk of the apoA-IV.

The smallest lipoprotein (V) in the HDL fraction was the second major component, with a molecular weight of 100,000. This lipoprotein contained apoA-I as its sole apoprotein and had the highest protein:lipid ratio. Intermediate in size between the two major components were a variable number of discrete particles, depending on the subject, with molecular weights in the range 275,000–400,000 (IV) that were not well separated by the column. These lipoproteins contained apoA-I and apoC and were intermediate between the larger and smallest particles in their lipid:protein ratio. Electron microscopy of samples from the column showed that lipoproteins of type I were large spherical particles of diameter 300–400 Å. Lipoproteins of type II–IV appeared to be predominantly stacked discs, with diameters in the range 300–350 Å for type II, 160–240 Å for type III, and 110–180 Å for type IV particles. No stacked discs were visible in samples containing type V particles, but small spherical particles could be seen, some with diameters of less than 50 Å.

When lipoproteins of d 1.063–1.21 g/ml from K.B. and B.R. were fractionated by chromatography on Sephacryl S-300, essentially similar results were obtained to those shown for T.C. However, a major difference was that the larger of the two major components in B.R. (III) appeared to contain a much lower percentage of apoA-IV and apoE than the equivalent fractions from T.C. and K.B.

Interaction of lipoproteins in the HDL fraction from LCAT-deficient subjects with cultured cells

Lipoproteins in the HDL fraction isolated from the LCAT-deficient subjects competed effectively with normal ^{125}I-labeled LDL for binding to the surface of normal human skin fibroblasts, as shown in **Fig. 7.** The concentration of lipoprotein protein that gave 50% inhibition of binding of 5 μg/ml ^{125}I-labeled LDL was 5.2 μg/ml for normal unlabeled LDL and 1.3 μg/ml for the total HDL fraction from K.B.; normal HDL competed only poorly. With the total HDL fraction from each of the three LCAT-deficient subjects, in each case the concentration of protein required to give 50% inhibition of binding of labeled LDL was approximately one-third the concentration required of unlabeled LDL.

Since the total HDL fraction was a mixture of components and consisted of apoB- and apoE-containing particles, a preliminary experiment was performed to determine which components were responsible for the competition with ^{125}I-labeled LDL for binding sites. A constant volume of each fraction eluted from the Sephacryl S-300 column shown in Fig. 6A was added to dishes of cells that contained 5 μg of ^{125}I-labeled LDL per ml and the percent inhibition of LDL binding was determined (Fig. 6C). From these data it appeared as

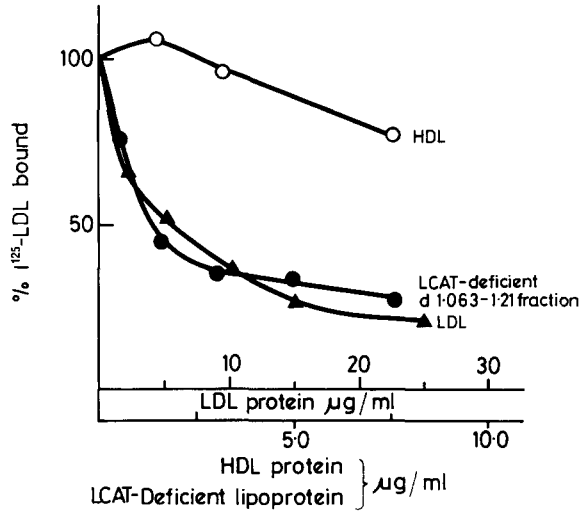

Fig. 7. Inhibition of binding of normal ^{125}I-labeled LDL to normal human skin fibroblasts at 4°C by the HDL fraction from an LCAT-deficient subject. Inhibition of binding of normal ^{125}I-labeled LDL (5 μg/ml protein) at 4°C by unlabeled normal LDL (▲), unlabeled normal HDL (○), and the total HDL fraction from LCAT-deficient subject K.B. (●) is expressed as percentage inhibition of that amount of ^{125}I-labeled LDL bound that was displaced by an excess of unlabeled normal LDL (1 mg/ml protein).

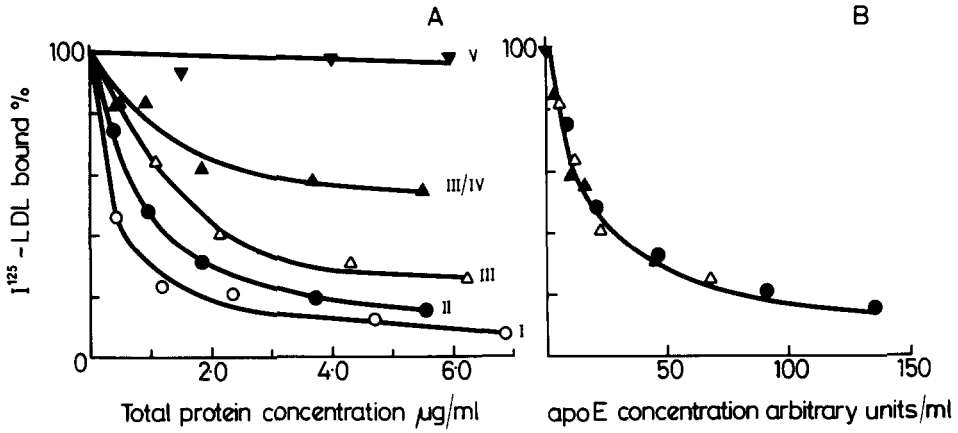

Fig. 8. Inhibition of binding of normal ^{125}I-labeled LDL to normal human skin fibroblasts by individual components of the HDL fraction of an LCAT-deficient subject separated by gel filtration chromatography. Normal human skin fibroblasts were incubated with normal ^{125}I-labeled LDL (5 μg/ml protein) at 4°C in the presence of different concentrations of fractions from the Sephacryl S-300 column shown in Fig. 6 containing particle I (○), II (●), III (△), a mixture of III and IV (▲), or V (▼). Inhibition of binding was expressed as percentage inhibition of that amount of bound ^{125}I-labeled LDL that was displaced by an excess of unlabeled normal LDL (1 mg/ml protein). A, Inhibition expressed as a function of total protein concentration. B, Inhibition expressed as a function of relative concentration of apoE, determined from densitometric scans of SDS-polyacrylamide gels.

if several of the components competed effectively with LDL. We therefore examined inhibition by each particle type at a range of concentrations (**Fig. 8A**). When the percentage inhibition of ^{125}I-labeled LDL binding by particle types II, III, and IV was expressed as a function of the relative content of apoE (Fig. 8B), all the points fell on a single curve, despite the variable proportions of other apoproteins in the different particle types. Type I particles, which contained apoB, were more effective than types II–IV when expressed in terms of total protein content. Particles of type V contained no detectable apoE and did not compete with ^{125}I-labeled LDL for binding.

To determine whether or not skin fibroblasts were also able to internalize and degrade the lipoproteins in the HDL fraction of LCAT-deficient subjects, a sample of particle type III from K.B. was labeled with ^{125}I. As assessed by SDS-PAGE, it was found that the apoprotein content of the particle was apparently unaffected by labeling, but from a radioautograph of the dried gel it was found that the apoE moiety was labeled to only a small extent. From the radioautograph, the majority of the ^{125}I (approximately 80%) was associated with apoA-I while the remaining 20% was distributed equally between apoA-IV and an unidentified protein (apparent molecular weight 75,000) that was barely detectable on the gel stained for protein.

The labeled lipoprotein from the LCAT-deficient subject was incubated with cultured skin fibroblasts at 37°C for 5 hr in the presence and absence of an excess of unlabeled LDL. At the end of this time the amount of labeled lipoprotein that had been degraded and that which was associated with the cells were determined (**Fig. 9,** B and C). The lipoprotein was taken up by the cells and degraded by processes that appeared to be saturable, within the limited concentration range possible with the amount of lipoprotein available. Both uptake and degradation were inhibited strongly in the presence of unlabeled LDL at 1.0 mg/ml protein. In the same experiment, at 37°C, with ^{125}I-labeled LDL at 30 μg/ml protein, the fibroblasts degraded 1620 ng LDL protein/5 hr per mg of cell protein, while 950 μg LDL protein/mg of cell protein was cell-associated. In a separate experiment, the ^{125}I-labeled lipoprotein from the LCAT-deficient subject was incubated with cells at 4°C to determine surface binding (Fig. 9A). When the values were corrected for residual binding that occurred in the presence of an excess of unlabeled LDL, the cells bound the lipoprotein by a saturable process, with half-maximum binding occurring at a concentration of approximately 6 μg/ml protein. At a concentration of 30 μg/ml of ^{125}I-labeled LDL protein, these cells bound 39 ng LDL protein/mg of cell protein by the specific mechanism at 4°C, while the maximum amount of ^{125}I-labeled lipoprotein from the LCAT-deficient subject that was bound was approximately 12 ng protein/mg of cell protein.

DISCUSSION

In this study we have isolated and characterized the lipoproteins isolated in the density range of normal HDL (1.063–1.21 g/ml). Such an ultracentrifugal fraction is purely arbitrary since it is most unlikely that the different populations of abnormal lipoproteins in LCAT deficiency are confined to the same density classes as normal lipoproteins; however, isolation of such a fraction permits

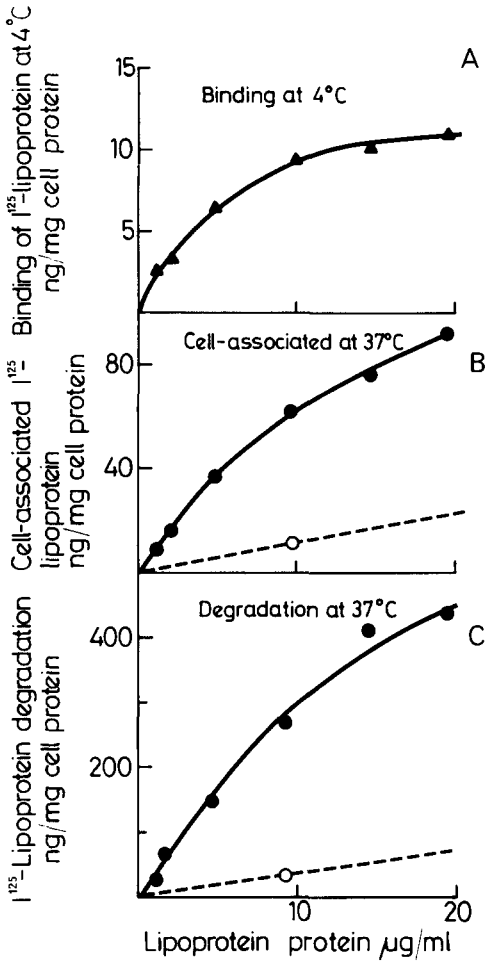

Fig. 9. Interaction of ^{125}I-labeled lipoprotein, separated by gel filtration chromatography from the HDL fraction of an LCAT-deficient subject, with normal human skin fibroblasts. One component (III in Fig. 1 and Table 2) of the HDL fraction from K.B. separated by gel filtration, labeled with ^{125}I, was incubated with normal skin fibroblasts. A, Specific binding of ^{125}I-labeled lipoprotein at 4°C was expressed as that amount of radioactivity bound that could be displaced by an excess of unlabeled normal LDL (1.0 mg/ml protein). B, Cell-associated radioactivity (●) at 37°C comprised ^{125}I-labeled lipoprotein that was bound to the cell surface or that had been taken up by the cells. The effect of adding an excess of unlabeled normal LDL (1 mg/ml protein) is shown (○). C, Degradation of ^{125}I-labeled lipoprotein (●) was assessed by determination of the release of TCA-soluble, non-iodide radioactivity into the medium. The effect of addition of an excess of unlabeled normal LDL (1 mg/ml protein) is shown (○). The values were corrected for apparent degradation in the absence of cells.

comparison with previously published data on lipoproteins of density 1.063–1.21 g/ml from LCAT-deficient plasma (3, 23, 24). Particular care was taken to minimize loss of components during isolation. With this in mind, the lipoproteins were subjected to a single centrifugation at the higher density and further fractionation was accomplished by gel filtration chromatography on Sephacryl S-300 rather than by any method involving binding of the lipoproteins to a solid matrix. Sephacryl S-300 provides greatly superior resolution of particles with molecular weights in the range 1×10^4–1.5×10^6 compared with other gel filtration media, with no increased risk of damage or loss of proteins as long as the pH is maintained above neutrality. At least five or six discrete populations of particles were isolated from the HDL fraction of plasma of the three subjects by this means, and although insufficient material was available to rechromatograph each component on the same gel filtration column, fractions were identified which contained essentially a single type of particle. In most respects, the various lipoproteins isolated in this study were comparable with fractions obtained by other workers utilizing the ability of apoE- and apoB-containing lipoproteins to bind to affinity-heparin-Sepharose (3, 24).

Marcel et al. (24) and Mitchell et al. (3) separated the total density 1.063–1.21 g/ml fraction from LCAT-deficient plasma into an apoE-containing fraction, which bound to heparin-Sepharose, and a non-apoE-containing fraction, which was not bound, following the original observation by Utermann et al. (23) that lipoproteins in the density 1.063–1.125 g/ml fractions from an LCAT-deficient subject contained an apoE-rich fraction. Mitchell et al. (3) removed apoB-containing particles from the apoE-containing material by chromatography on Con-A-Sepharose, which apparently binds apoB but not apoE.

By gel filtration, we found that the apoE-containing lipoproteins were not homogeneous, but consisted of one minor population of particles whose apoproteins comprised apoA-I and apoE in the ratio of approximately 2:1 and another, a major component of the total HDL fraction, which contained predominantly apoA-I but also contained apoE and apoA-IV. No fraction was obtained that contained apoE as the predominant apoprotein, in contrast to the findings of Utermann et al. (25), Marcel et al. (24) and Mitchell et al. (3). We also found marked heterogeneity amongst the non-apoE-containing lipoproteins, in that particles ranging from molecular weight 400,000–250,000 (IV) which gave discrete bands on gradient PAGE and which contained apoA-I and apoC were obtained; a further fraction (V), containing only apoA-I, with a molecular weight of 100,000 was also obtained. Whether these lipoproteins exist as such in the plasma of the LCAT-deficient subjects or whether they are generated as artefacts of the centrifugation in KBr solutions is not known.

The major difference in composition between the lipoproteins isolated in the present work and those reported in other LCAT-deficient families is the presence of apoA-IV in the HDL fraction of the Irish patients. This apoprotein has hitherto only been demonstrated in normal human plasma in the lipoprotein-free fraction of density greater than 1.21 g/ml and, to a small extent, in circulating chylomicrons (22), although its presence in triglyceride-rich lipoproteins of intestinal origin that have not yet entered the plasma is well-documented (9).

However, brief reports suggest that it has been observed in the HDL of subjects with acute alcoholic hepatitis (22) and the HDL_c of cholesterol-fed dogs (26). Furthermore, chylomicrons and HDL of rat plasma contain an analogous apoprotein (27, 28). Apoproteins of molecular weight greater than that of apoE have been observed on SDS-PAGE in the HDL fraction of LCAT-deficient plasma, but these have either remained unidentified (23) or have been identified as the apoE-apoA-II complex (29) by virtue of their cleavage to smaller components after disulfide reduction (3, 24). In our studies, the 46,000 molecular weight protein was totally resistant to cleavage by 2-mercaptoethanol and was immunologically identical with apoA-IV obtained from chylous urine (9). Furthermore, in our studies, an additional apoprotein of molecular weight 41,000 was also detected on gels of samples of the HDL fraction that had not been reduced with 2-mercaptoethanol prior to electrophoresis. Since reduction of the same sample before electrophoresis resulted in the disappearance of this apoprotein with a concomitant increase in apoE and apoA-II, it was probably the apoE-apoA-II complex.

Whether or not the failure to find apoA-IV in the lipoproteins of density 1.063–1.21 g/ml in other LCAT-deficient subjects constitutes a real difference between the families or is a result of different experimental procedures is difficult to judge at this stage. In any event, the presence of apoA-IV in the disc-shaped lipoproteins in the HDL fraction of LCAT-deficient subjects is of particular interest because it provides a further clue to their origin. Since this apoprotein appears to be a constituent of the surface of newly-synthesized chylomicrons, and is lost during hydrolysis of the triglyceride core (22), it provides further evidence that the discoidal lipoproteins in LCAT deficiency are derived in part from chylomicron surface material. Since these particles are known to be converted, on incubation with LCAT, to spherical, cholesteryl ester-filled lipoproteins that resemble normal HDL (25, 30), our data support the view that in the presence of LCAT, surface components of chylomicrons released during their lipolysis contribute to normal HDL (31). However, Fidge (32) has shown that when rat chylomicrons carrying ^{125}I-labeled apoA-IV are injected into a rat, apoA-IV is lost from the chylomicrons as the triglyceride core is hydrolyzed but that it appears to enter the lipoprotein-free $d > 1.21$ g/ml fraction before becoming associated with HDL. Thus the alternative possibility exists that the composition of the discoidal particles in LCAT-deficiency is such that they have an affinity for, and therefore bind, apoA-IV that has been released into the lipoprotein-free fraction during chylomicron lipolysis.

The interaction of the HDL fraction with the LDL-receptor is not unexpected, since apoB and apoE were both present; the ability of the different particles to compete with ^{125}I-labeled LDL for binding to sites on skin fibroblasts correlated well with the apoE-content when the particles contained no apoB, although the most efficient competitor, when expressed in terms of mg of total protein, was the apoB-containing material eluted at the void volume of the column. Presumably this was because the apoE-containing particles also contained a relatively high proportion of other apoproteins. Lipoproteins containing only apoE, for example apoE-HDL_c, are known to have a higher affinity for the LDL-binding sites on skin fibroblasts than apoB-containing lipoproteins, with half-maximum binding of HDL_c occurring at approximately one-twentieth the concentration of LDL required (33).

In the present study it was difficult to compare binding affinities in terms of particle numbers, as sufficiently accurate values for the molecular weight and percentage composition of the lipoproteins from the LCAT-deficient subjects were not determined. However, when the particle number/mg protein was calculated from the data in Table 1, type III particles which contained apoA-I, apoE, and apoA-IV, and normal LDL particles were required in equal numbers to displace 50% of the ^{125}I-labeled LDL from the binding sites; however, only half the number of type II particles, which contain relatively more apoE, were required. From a comparison, in terms of particle numbers, of the ability of HDL_c to compete with LDL for binding to the LDL receptor (33) with that of the two apoE-containing fractions in HDL of LCAT-deficient subjects (II and III), one can conclude either that the apoE is not distributed evenly amongst the particles within either fraction from the LCAT-deficient subject, or that the presence of other apoproteins modulates the ability of apoE to interact with the receptor. Since the concentration of apoE in any fraction appeared to be directly proportional to the ability of that fraction to compete with ^{125}I-labeled LDL for binding sites, the latter possibility seems unlikely.

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