Abnormalities in lipoproteins of d < 1.006 g/ml in familial lecithin:cholesterol acyltransferase deficiency

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Abstract Studies of different sized lipoproteins of d < 1.006 g/ml from patients with familial lecithin:cholesterol acyltransferase deficiency have yielded new evidence of abnormalities in this lipoprotein class. Lipoproteins of all sizes contain high amounts of unesterified cholesterol, low amounts of total protein, and particularly low amounts of apolipoproteins C-II and C-III. Lipoproteins 60 nm in diameter or larger include particles that show a notched appearance upon electron microscopy, and contain *a*) a high combined volume of phospholipid, unesterified cholesterol, and protein; *b*) high amounts of cholesteryl ester and apolipoproteins C-I and E, and *c*) two major tetramethylurea-insoluble proteins that can be separated by electrophoresis in the presence of sodium dodecyl-sulfate. In contrast, lipoproteins that are 40 nm in diameter or less appear to contain low amounts of cholesteryl ester, normal amounts of apolipoproteins C-I and E, and a single tetramethylurea-insoluble protein the size of that in control lipoproteins. Since these abnormalities occur in the lipoproteins of four different patients from four different families, they are probably effects of the enzyme deficiency. Most, however, appear to arise indirectly because in vitro experiments published earlier indicate that few are reversed by incubation in the presence of the enzyme and patient high density lipoproteins—**Glomset, J. A., K. Applegate, T. Forte, W. C. King, C. D. Mitchell, K. R. Norum, and E. Gjone.** Abnormalities in lipoproteins of d < 1.006 g/ml in familial lecithin:cholesterol acyltransferase deficiency. *J. Lipid Res.* 1980. **21:** 1116–1127.

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In recent experiments (1) with lipoproteins of d < 1.006 g/ml from patients with familial lecithin:cholesterol acyltransferase (LCAT) deficiency, we found that these lipoproteins change markedly when incubated in the presence of LCAT and patient high density lipoproteins (HDL). In one instance, for example, the content of cholesteryl ester (CE) in the lipoproteins of d < 1.006 g/ml increased nearly three-fold and that of apolipoprotein E (apoE) increased almost five-fold, while the contents of individual C apolipoproteins decreased by one-fourth to one-half. These observations raised the possibility that LCAT normally contributes to plasma lipoprotein metabolism not only by forming CE, but also by affecting the distribution of apolipoproteins between lipoproteins of d < 1.006 g/ml and HDL. One question that remained was whether the changes in apolipoprotein distribution caused by incubating patient plasma with LCAT corrected or exacerbated abnormalities in the native lipoproteins. Information was not available concerning the content of CE and apolipoproteins in individual subfractions of lipoproteins of d < 1.006 g/ml. The question seemed important, since a number of the abnormalities in familial LCAT deficiency appear to be only indirect effects of the enzyme lack (2).

As a step toward understanding the mechanisms by which LCAT might normally control the distribution of apolipoproteins in plasma, we decided to characterize the distribution of apoC and apoE in patient lipoproteins of d < 1.006 g/ml. We studied four patients from different families, who showed different degrees of hypertriglyceridemia and renal dysfunction. We isolated their plasma lipoproteins of d < 1.006 g/ml by preparative ultracentrifugation, subfractionated the lipoproteins by gel filtration on 2% agarose, analyzed the content of lipids and apo-

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoproteins; VLDL, very low density lipoproteins; apo, apolipoprotein; TMU, tetramethylurea; SDS, sodium dodecylsulfate; PL, phospholipid; UC, unesterified cholesterol; CE, cholesteryl ester; TG, triglyceride.

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lipoproteins in the subfractions, and related the results to lipoprotein size as determined by electron microscopy. As described below, we found evidence for a number of hitherto undetected abnormalities involving the distribution of both lipids and apolipoproteins.

METHODS

Patients

Four female patients from different Scandinavian families were studied: M.R., aged 31; A.A., aged 52; D.J., aged 64; and M.L., aged 57. Their clinical features, laboratory findings, and levels of plasma lipids and lipoproteins have been previously described (2–8). At the time of study, three were in relatively good health whereas the fourth, M.L., had begun to show signs of renal failure (serum creatinine 5.1 mg/dl). Patients A.A. and M.L. both had pronounced hyperlipemia (plasma triglycerides 950 mg/dl and 925 mg/dl, respectively) whereas patients M.R. and D.J. did not (plasma triglycerides 175 mg/dl and 89 mg/dl, respectively). Patients A.A., M.R., and M.L. had moderate proteinuria (0.5–1.5 mg/protein/ml) without hypoalbuminemia, whereas patient D.J. showed no clinical signs of renal dysfunction. All subjects gave written informed consent for the study.

For the experiments described below, the patients were admitted to the hospital and given ordinary food for 3–7 days before plasma was obtained by plasmapheresis. In the case of patient A.A., samples of plasma were obtained successively after she had *a*) ingested ordinary hospital food for 7 days and *b*) ingested the basic fat-free diet described previously (9) for 7 additional days. In all cases, plasma was obtained after the patients had fasted overnight, and acid citrate dextrose solution (U.S.P. formula A) was used as an anticoagulant. Plasma was also prepared from eight normal females (ages 24 to 56; plasma triglycerides 36 to 74 mg/dl) living in Seattle, as well as from six normal Norwegian females (ages 20 to 25, plasma triglycerides 35 to 71 mg/dl). Neither patients nor controls were taking medication or oral contraceptives when studied.

VLDL

Plasma from the Norwegian patients and controls was shipped to Seattle by air on ice. Lipoproteins of $d < 1.006$ g/ml, usually from one unit of plasma, were prepared by ultracentrifugation (10), starting within 48 hr of the time blood was drawn in Oslo. Plasma from the normal females living in Seattle was centrifuged immediately, after adding p-chloromercuriphenylsulfonate (final concentration 2 mM) to inhibit LCAT. After ultracentrifugation the lipoproteins from patient or control plasma were filtered through separate columns of 2% agarose (Bio-Gel A 50m, 100–200 mesh, Bio-Rad Labs, Richmond, CA) as described previously (11). The effluent from the columns was pooled to provide subfractions of approximately equal protein content, and the subfractions were concentrated by dialysis against Dextran T 500 (Pharmacia Fine Chemicals, Piscataway, NJ), preparatory to electron microscopy and lipid and apolipoprotein analysis.

Electron microscopy

The size distribution of particles in each subfraction of lipoproteins was determined by electron microscopy. An aliquot of each subfraction was dialyzed against a buffer solution (pH 7.4) containing 0.13 M ammonium acetate, 345 μM EDTA, and 124 μM merthiolate; then the lipoproteins were negatively stained with sodium phosphotungstate as described previously (12) and examined with a JEM 100 C (JEOL, Inc., Tokyo, Japan) electron microscope. Only preparations showing well spread particles were used; micrographs of random areas of the grid were taken at instrumental magnifications of 20,000 and 40,000×. Lipoprotein sizes were obtained by measuring the diameter of 100 to 200 free-standing particles by means of an ocular micrometer or a computerized program using a sonic digitizer (Science Accessories Corporation Graf-Pen., Southport, CT) which records X–Y coordinates and translates them into particle diameters. In the case of patient M.L., the lipoprotein samples had been previously frozen and thawed; these lipoproteins showed a high degree of aggregation but free-standing particles were also present. The latter had diameters consistent with fractions from the other three patients and are thus included in the results.

To correct for overestimation in particle size caused by flattening during negative staining, we compared subfractions of patient and control lipoproteins that had been stained as described above with those that had been fixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.4) and stained with sodium phosphotungstate (NaPTA), or fixed with OsO_4 and then shadowed according to the procedure of Sata, Havel, and Jones (13). In the latter case, polystyrene latex spheres were used as standards. Plots of apparent diameter of fixed particles versus that of nonfixed particles showed a similar linear relation for patients and controls ($D_{fixed} = 0.782 \times D_{nonfixed} + 6.80$ nm). This relation was used to adjust the measured diameters

of all negatively stained lipoproteins above 35 nm in apparent diameter.

Apolipoproteins

Total lipoprotein protein was measured by the method of Lowry et al. (14), after extraction of the samples and standards with ether (15). Bovine serum albumin was used as a standard. Total tetramethylurea-soluble protein was determined by the method of Kane (16). Quantitation of the individual apoproteins was performed by densitometry of polyacrylamide gels stained with Coomassie Brilliant Blue R250, after electrophoresis of replicate aliquots of each fraction in one of three different gel systems.

For measurement of apoC-II, apoC-III_1 and apoC-III_2, lipoproteins were delipididated with TMU (16) and separated by electrophoresis in a basic gel system (17) modified to include 8 M urea. For measurement of apoC-I and apoE, lipoproteins were extracted twice with ether, boiled for 5 min in 2% SDS, and analyzed by electrophoresis on 3-mm thick slabs of 15% polyacrylamide gels containing both SDS and urea (18). This system was employed because analysis of patient lipoproteins by two-dimensional electrophoresis (not shown) in a system successively utilizing the two systems described above revealed that the basic gel system containing 8 M urea did not adequately separate apoC-I and apoE from contaminating proteins of larger molecular weight.

Analysis of apoproteins larger than apoE was accomplished by extracting aliquots of the whole lipoprotein fractions with ether, boiling them in 0.1% SDS for 5 min, then separating them by electrophoresis in 0.1% SDS on gels of 10% or 4% polyacrylamide (19). TMU-insoluble apoprotein was shown to give 4% gel patterns identical to those of total apoprotein except that the content of smaller apoproteins, travelling with the bromphenol blue dye front, was greatly reduced.

After electrophoresis, all gels except the 15% polyacrylamide slabs were fixed in 12.5% trichloroacetic acid for 30 min at room temperature. Gels containing SDS were then extracted overnight with 25% isopropanol–10% acetic acid to remove SDS. All gels were stained 16–20 hr with 0.25% Coomassie Brilliant Blue R 250 in 45% methanol–9% acetic acid (20) and then destained as described previously (18), first in 25% isopropanol–10% acetic acid, then in 10% methanol–7% acetic acid.

Quantitation of the apolipoproteins was done by densitometry of stained bands using either a Quickscan (Helena Instruments, Beaumont, TX) or an Ortec gel scanner (Ortec, Inc., Oak Ridge, TN) fitted with the green filter supplied with the instrument and a slit size of 0.5 × 5.0 mm. Because of variability in staining and destaining, secondary lipoprotein standards, at three to five protein levels in duplicate or triplicate, were analyzed in parallel with the unknowns, either on the same slab gel or within the same cassette of cylindrical gels. Calibration curves were prepared from plots of the integrated peak areas of the standard apoproteins versus the apoprotein concentration, for each individual slab gel or set of 18 cylindrical gels. Under the conditions of gel electrophoresis, staining, and destaining described above, the linear staining range was 1–8 μg for the C apolipoproteins, and 1–10 μg for apoE. Any samples that fell above this range were re-analyzed at lower concentrations. Use of a neutral density filter established that the chromogenicities of the stained proteins ranged from approximately 3.0×10^{-2} OD-cm/μg (apoC-III_2) to 3.8 OD-cm $\times 10^{-2}/\mu$g (apoC-II), though variation between gels was as much as 10%.

The secondary lipoprotein standards used on each gel were calibrated in the same gel systems against primary apolipoprotein standards prepared from normal very low density lipoproteins (VLDL). Preparation of the apoC-II and apoC-III standards followed published procedures (21–23). Final purification of apoC-I was obtained by chromatography on a column of SP-Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) using a linear gradient of 0.005 to 0.10 M ammonium acetate, pH 4.6, in 6 M urea. Final purification of apoE was performed by preparative SDS gel electrophoresis (18). Amino acid analysis of duplicate or triplicate aliquots was used to establish the concentration of each primary standard solution based on amino acyl residue mass; corrections were made for ½ cystine or tryptophan where necessary from published amino acid compositions. In every case, the amino acyl composition of the standards matched published values.

For estimation of the molecular weight of the apoprotein bands separated in the 4% polyacrylamide gel system, we used standards obtained from Dr. E. H. Fischer on parallel gels: myosin, mol wt 200,000; debranching enzyme, mol wt 160,000; and phosphorylase B, mol wt 96,000. Collagenase, mol wt 109,000 (Worthington Biochemical Corporation, Freehold, NJ) was also used. Apparent molecular weights for the major peaks on the 4% gels were extrapolated from the plot of log mol wt versus relative mobility of these standards; they must be considered only as estimates, owing to the nonlinear behavior of large proteins in SDS-polyacrylamide gel electrophoresis (24).

Lipid analysis

UC, CE, and phospholipid (PL) were measured as described previously (10). Triglyceride (TG) also was measured as described previously (10) except that TG was separated from PL by the method of Carlson and Wadstrom (25).

Calculations and data treatment

The following steps were taken to determine the mean composition of the lipoproteins in a given subfraction obtained by gel filtration. First, the mean volume of the lipoproteins in the subfraction was calculated from the volume-weighted mean diameter (D_{vol}) of the particles observed in the electron microscope. For the patients, actual particle frequency counts from electron micrographs were used to compute $D_{vol} = (\sum f_i D_i^3 / \sum f_i)^{1/3}$ where f_i is the number of particles of diameter D_i in the narrow size range i. For the controls, test counts showed close adherence to Gaussian frequency distributions. Therefore, weighted diameters were computed as $D_{vol} = D(1 + 3CV^2)^{1/3}$ where D is the unweighted mean size and CV is the coefficient of variation of the size distribution. Second, the lipid and protein contents of the corresponding subfraction were expressed in terms of volume, using the same mass to volume conversion factors employed by Sata et al. (13). Third, the relative contribution of each component to the total volume was calculated and used to determine the contribution of that component to the mean lipoprotein particle volume. Finally, the number of molecules per lipoprotein particle of each component, or the mass per particle of the component in the case of protein, was calculated from the volume to mass conversion factors and Avogadro's number.

In calculating linear regressions for putative surface components on lipoprotein surface area, we used the area weighted diameter (D_{area}) determined as $D_{area} = (\sum f_i d_i^2 / \sum f_i)^{1/2}$. In most cases the mean diameter determined by electron microscopy differed from D_{vol} or D_{area} by no more than a few nm. However, for the patient's larger lipoproteins, size distributions were broad and skewed. D_{vol} and D_{area} therefore had to be calculated. The distribution of control lipoproteins was essentially normal in all instances, however, so the following approximation could be used: $D_{area} = D(1 + CV^2)^{1/2}$.

RESULTS

Since plasma lipoproteins of $d < 1.006$ g/ml are known to be heterogeneous in size and composition (see, for example, reference 13), an important early step in this investigation was to subfractionate control and patient lipoproteins of this density so that similar sized lipoproteins could be compared. To accomplish this we filtered the lipoproteins through separate columns of 2% agarose gel, and monitored the subfractionation by measuring the protein in the effluent. The lipoproteins of the controls and of patient D.J. yielded a single peak that emerged in a position corresponding to that of normal VLDL (13). The lipoproteins of patients A.A., M.L., and M.R. yielded a comparable peak, but also an additional peak that emerged with the void volume and comprised 10% to 32% of the total protein applied. On the basis of previously reported diet experiments (9), we assume that the void volume peak contained chylomicrons and/or chylomicron remnants.

Assuming that the "VLDL" peak of control and patient lipoproteins corresponded to a population of particles, partially separated on the basis of size (13), we subdivided it into 5–6 successive subfractions of approximately equal protein content, and examined the lipoproteins in each subfraction by electron microscopy and chemical analysis.

Abnormalities detected by electron microscopy

Electron microscopy revealed that the subfractions of control lipoproteins contained round particles that decreased from about 74 nm to 33 nm in mean diameter with increasing subfraction number. Each of the subfractions was heterogeneous and showed a coefficient of variation from the mean diameter of about 15%. The lipoprotein subfractions of patient D.J. resembled those of the controls in the shape and distribution of their contained particles. However, those of patients A.A., M.L., and M.R. differed from those of the controls in two important respects. First, there were large lipoproteins (85 to 200 nm in diameter) in the void volume peak. Second, both these lipoproteins and some of the larger "VLDL" frequently appeared flattened in negatively stained preparations (**Fig. 1B**).

Though fixation with OsO_4 prevented flattening, and yielded particles that appeared round, from 18% to 21% of the particles that were 60 nm in diameter or larger had notches or nicks in their periphery (Fig. 1C). Occasionally, the notched regions seemed to be cavities covered with a membrane-like material (Fig. 1C), and there was frequently more than one notch per particle.

In other respects the distribution of patient "VLDL" resembled that of the control lipoproteins. The mean diameters of the particles in successive subfractions

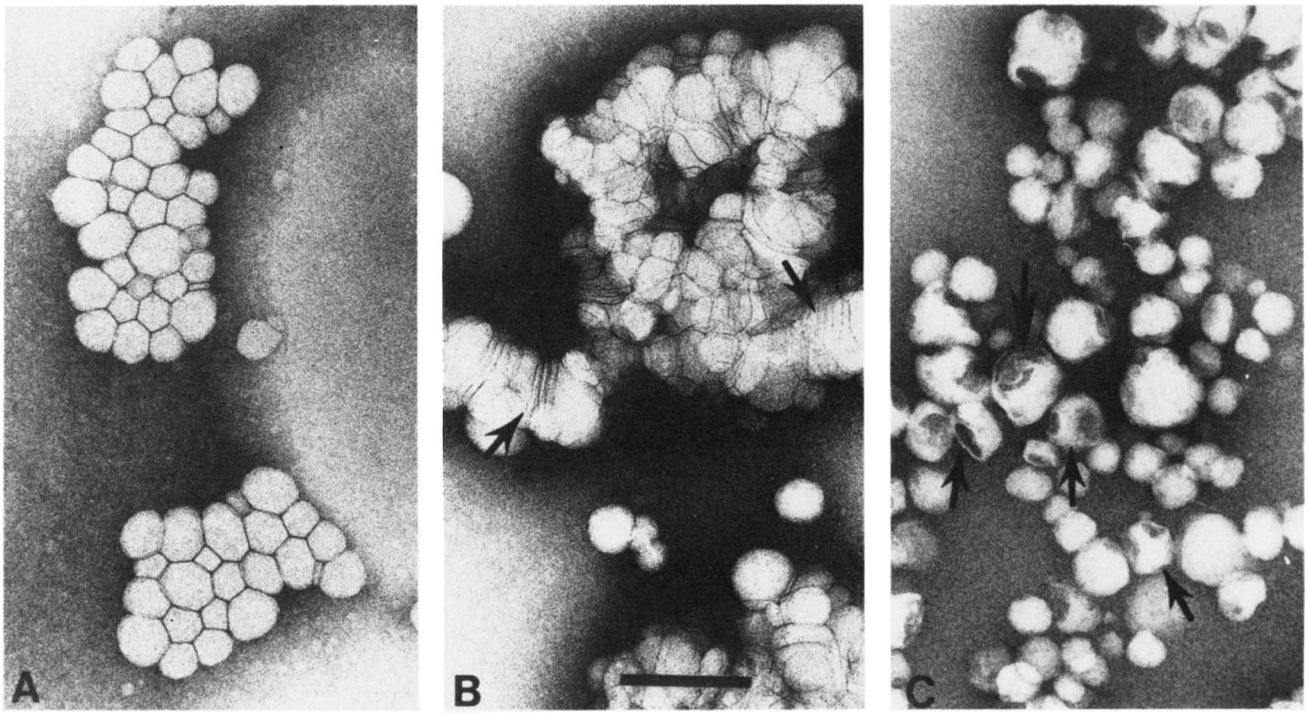

Fig. 1. Electron micrographs of normal and patient large "VLDL." a. Normal VLDL, 65 nm in diameter, negatively stained with sodium phosphotungstic acid (NaPTA). The particles are generally round when free standing, but become polygonal when aggregated as demonstrated here. b."VLDL," 75 nm in diameter, from patient M.R. Negatively stained with NaPTA. Numerous large flattened structures (arrows) can be seen when particles are aggregated. c. Same "VLDL" as in b., but fixed with OsO_4, then stained with NaPTA. Fixation appears to prevent flattening, but many particles appear to contain notches or cavities that trap NaPTA (small arrows). Occasionally a membranous film is seen over a notched region (large arrow). The bar marker represents 100 nm and applies to all micrographs.

ranged from about 73 nm to 29 nm, with coefficients of variation of about 22%.

Abnormalities in "VLDL" composition

Since electron microscopy of the control lipoproteins had revealed round particles, we assumed the lipoproteins to be spherical, and calculated the mean volume of the lipoproteins as described under Methods. We then expressed measurements of the lipid and protein components in individual subfractions in terms of volume, and based on the relative contribution of each component to the sum of the component volumes, calculated the amount present per particle. Finally, we computed regressions for the entire population of particles 74 to 33 nm in diameter as a means of estimating the composition of its individual lipoprotein components. To identify abnormalities in patient lipoproteins, we treated data for them in the same way. However, we included only patient VLDL-sized particles in the regression analyses. Moreover, since we had demonstrated by electron microscopy that patient lipoproteins include a significant proportion of unusually shaped particles greater than 60 nm in diameter, we calculated regressions for particles 29 nm to 60 nm in diameter as well as for particles 29 nm to 73 nm in diameter.[2] Upon comparing the results obtained for control and patient lipoproteins, we discovered abnormalities in the distribution of both lipids and apolipoproteins.

The lipid abnormalities appeared to involve constituents of the lipoprotein surface and core. Thus, the UC and PL of control and patient lipoproteins must have been largely associated with the surface since the amounts of these components were linear functions of lipoprotein surface area (**Fig. 2**). However, patient lipoproteins of all sizes contained abnormally high amounts of UC, and those that were 60 nm in diameter or larger contained abnormally high amounts of PL (Fig. 2, **Table 1**).

When we calculated the relative contribution of CE to the particle volume of patient "VLDL" (Fig. 2), we found an additional abnormality. The content of CE was a linear function of particle volume in patient lipoproteins, but not in control lipoproteins, which showed a very high degree of scatter (Fig. 2). Furthermore, no simple relation appeared when the lipoproteins of individual control subjects were considered

[2] Both types of calculation revealed the same fundamental abnormalities.

separately (not shown). In six of eight subjects, however, the content of CE was high in large VLDL, decreased to a minimum value in VLDL of about 50 nm diameter, and then either increased (five subjects) or remained constant with decreasing particle size. In the remaining two subjects the content of CE was lowest in large "VLDL" and increased with decreasing particle size. The net effect of this was that patient large "VLDL" contained more CE than did corresponding control VLDL, whereas the opposite was true for patient and control small VLDL (Fig. 2, Table 1).

A second type of abnormality involving constituents of the lipoprotein core was seen only in patient "VLDL" 60 nm in diameter or larger. Calculated contents of "core" lipid (i.e., CE + TG) per particle were lower than those of corresponding control lipoproteins (Table 1). Both this and the relative excess of UC and PL calculated for the same large lipoproteins probably reflected the presence of notched particles in the distribution of patient lipoproteins.

Abnormalities in the distribution of apolipoproteins in patient "VLDL" were of several types. First, all sizes of "VLDL" examined contained less total protein, less TMU-soluble protein, and less apoC-II, apoC-III$_1$, and apoC-III$_2$ than did corresponding control lipoproteins (**Figs. 3** and **4,** Table 1). Second, the sum of apoC-I + apoC-II + apoC-III$_1$ + apoC-III$_2$ + apoE calculated for all sizes of patient "VLDL" amounted to only 74 ± 7.5% of the measured total TMU-soluble protein, whereas the corresponding sum for control lipoproteins amounted to 95.5 ± 23.5% of the measured total TMU-soluble protein ($P < 0.001$). Though we cannot yet fully account for this discrepancy, analysis of patient lipoproteins by disc gel electrophoresis in 0.1% SDS showed that several proteins entering gels at 10% polyacrylamide had apparent molecular weights in the range of 40,000 to 120,000 daltons (not shown). These proteins were not otherwise characterized, but presumably contributed to the total TMU-soluble protein. Fourth, additional abnormalities involved the apolipoproteins of patient "VLDL" 60 nm in diameter or greater. Contents of apoC-I and apoE in these lipoproteins were high (Figs. 3 and 4, Table 1), and two major TMU-insoluble apolipoproteins (and several minor ones) could be separated by electrophoresis in SDS on gels of 4% polyacrylamide. One of these TMU-insoluble apolipoproteins (apparent molecular weight 220,000 daltons) predominated in the largest lipoproteins, as judged by densitometry (**Fig. 5**). However, the relative contribution of this peak decreased with decreasing lipoprotein diameter, as the contribution of the second major peak increased.[3] The "220,000" dalton

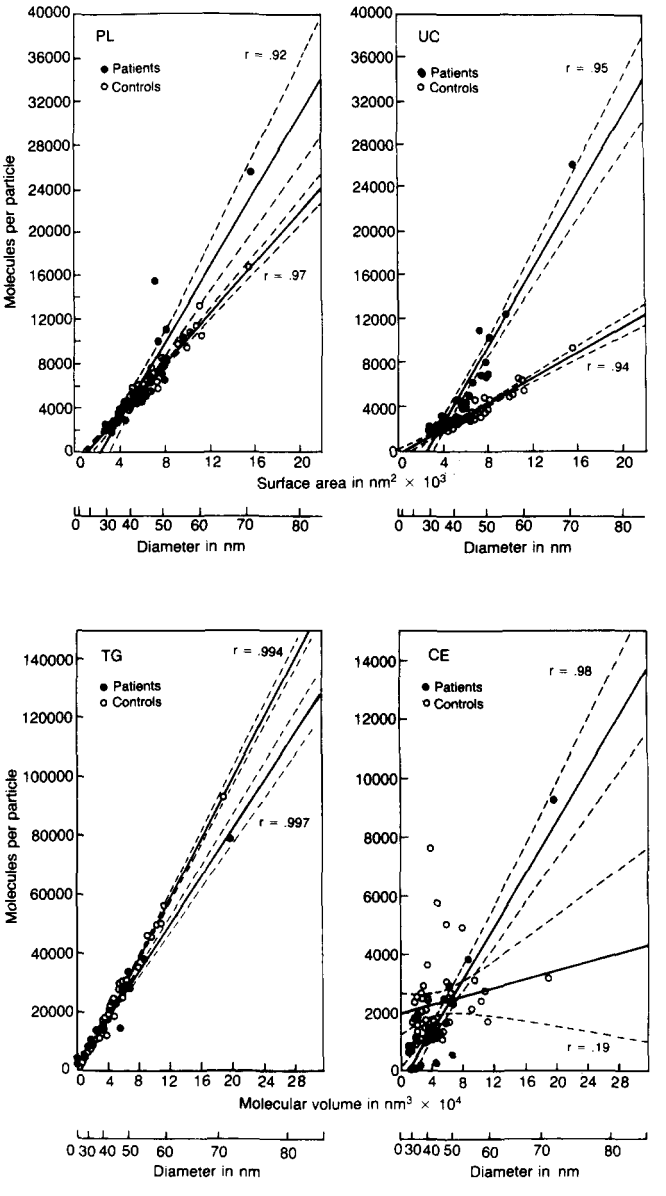

Fig. 2. VLDL lipids in familial LCAT deficiency. Lipoproteins of d < 1.006 g/ml from four female patients and eight controls were separately filtered through 2% agarose gel, particle dimensions in successive subfractions were determined by electron microscopy, and the content of lipids and apolipoproteins in the subfractions was determined by chemical analysis. The mean number of molecules of each component contained in subfraction lipoproteins was then calculated based on the contribution of the component to the mean particle volume. The figures show regressions and 95% confidence limits calculated for particles 30 to 90 nm in diameter. Significant differences between regression coefficients for patient and control lipoproteins were observed for each component measured (PL, $P < 0.005$; UC, $P < 0.001$; TG, $P < 0.001$; CE, $P < 0.001$).

[3] The apparent molecular weight of the second major peak, "347,000" daltons, was similar to that of the single "apolipoprotein B" peak observed in normal VLDL. Further attempts to define the molecular size of this protein were not made, however, and its assigned molecular weight should be regarded as preliminary.

TABLE 1. Composition of VLDL of 40 nm and 60 nm diameter; values predicted from regression lines shown in Figs. 2–4

Component	Units	40 nm			60 nm		
		Patients	Controls	P^b	Patients	Controls	P
Total protein	g × 10^{-21}/particle	2950 ± 320[a]	5230 ± 240	<0.001	5500 ± 660	9290 ± 430	<0.001
TMU soluble protein		1430 ± 130	2300 ± 90	<0.001	3540 ± 270	5800 ± 170	<0.001
apo E	molecules/particle	6.03 ± 1.01	7.36 ± 0.69	n.s.	25.5 ± 2.0	9.40 ± 1.50	<0.001
apo CI		9.50 ± 1.53	6.41 ± 0.49	n.s.	21.7 ± 3.1	13.1 ± 1.0	<0.05
apo CII		6.09 ± 0.79	12.20 ± 0.7	<0.001	13.3 ± 1.59	37.4 ± 1.5	<0.001
apo $CIII_1$		12.4 ± 1.9	49.4 ± 2.6	<0.001	25.1 ± 3.8	124.0 ± 6.0	<0.001
apo $CIII_2$		14.9 ± 2.6	42.5 ± 2.4	<0.001	33.4 ± 5.2	110.0 ± 5.0	<0.001
PL	molecules/particle	4920 ± 490	4730 ± 130	n.s.	15800 ± 1000	12000 ± 200	<0.001
UC		4430 ± 350	2700 ± 90	<0.001	15500 ± 700	6340 ± 170	<0.001
CE		937 ± 171	2260 ± 250	<0.001	4610 ± 310	2840 ± 410	<0.01
TG		13800 ± 600	12200 ± 300	<0.05	47100 ± 1100	53000 ± 600	<0.001

[a] Standard error of value predicted from regression line, calculated as standard error = (standard error of estimate) times $\sqrt{1/n + (x - \bar{x})^2/(var_x)(n - 1)}$ where x, $\bar{x}$ refer respectively to surface area or particle volume for 40 or 60 nm and the mean for all areas or volumes in the regression, var_x = variance of the areas or volumes, and n = number of points used in the regression computation (patients, n = 23; controls, n = 44).

[b] Two-tailed test with Student's t calculated as $(x_1 - x_2)/\sqrt{(s.e._1)^2 + (s.e._2)^2}$ with approximate degrees of freedom given as d.f. = $\{[(s.e._1)^2 + (s.e._2)^2]^2/[(s.e._1)^4/(n_1 - 1) + (s.e._2)^4/(n_2 - 1)]\} - 2$, in which the removal of 2 additional degrees of freedom for each regression line has been taken into account.

TMU-insoluble protein was observed in the lipoproteins of all patients, including patient D.J., and was present in the VLDL of patient A.A. after she had consumed a fat-free diet for 1 week. Since this peak was barely (if at all) detectable in normal VLDL, its presence in similar-sized patient lipoproteins can be considered abnormal.

A final abnormality in patient large "VLDL" was observed when the contribution of surface components (i.e., UC + PL + total protein) to total particle volume was calculated. In patient lipoproteins of 40 nm diameter, the high content of UC and low content of protein compensated for one another so that the combined volume of UC + protein + PL in these lipoproteins was similar to that in control VLDL (33.3% ± 2.8% (S.E.M.) and 34.2% ± 1.0% of the total particle volume, respectively). In contrast, corresponding volumes for patient and control "VLDL" of 60 nm diameter differed significantly (30.0% ± 1.7% versus 22.9% ± 0.5% of the respective total particle volumes; $P < 0.001$). Similarly, values projected for lipoproteins of 80 nm diameter were respectively 24.5% ± 1.7% and 17.2% ± 0.6% of the total particle volume ($P < 0.001$). The higher combined volume of surface constituents calculated for patient large lipoproteins compared with control lipoproteins clearly reflected the high contents of UC and PL and low contents of CE and TG, and thus the presence of the notched particles observed by electron microscopy (Fig. 1C). A notch would clearly have increased the surface area at the expense of core volume.

Patient lipoproteins > 85 nm in diameter

Since no control lipoproteins > 85 nm in diameter were detected, comparison of patient and control lipoproteins of this size was not possible. However, it was possible to compare lipoproteins of this size from patients A.A., M.L. and M.R. with smaller lipoproteins from the same patients. We did this by predicting the composition of the large lipoproteins based on regression equations calculated for lipoproteins < 85 nm in diameter, and then comparing the predicted values with those observed for the large lipoproteins alone. Good agreement was found for TG and for total and TMU-soluble protein. However, the observed contents of PL, UC, and apoC-III_1 + apoC-III_2 were 20% to 30% higher than predicted, and that of apoC-II was about 40% higher than predicted, consistent with an increased contribution of surface components, per particle. On the other hand, for reasons that remain to be explained, the ratio of observed to predicted decreased progressively with increasing particle size for CE, apoC-I and apoE.

Effect of diet on lipoproteins of patient A.A.

The change from a fat-containing diet to a fat-free diet was accompanied by several changes in the lipoproteins of d < 1.006 g/ml of patient A.A. The concentration of total protein in this density class increased from 348 μg/ml plasma to 444 μg/ml plasma; the proportion of this protein contained in particles greater than 85 nm diameter decreased from 29% to

12%; the maximum size of lipoproteins greater than 85 nm diameter decreased from 211 nm to 93 nm; and the composition of lipoproteins less than 85 nm in diameter changed as indicated in **Table 2.** The most significant changes appeared to involve CE, apoC-III, and apoE. The content of CE increased three- to four-fold, consistent with previous dietary experiments (9)

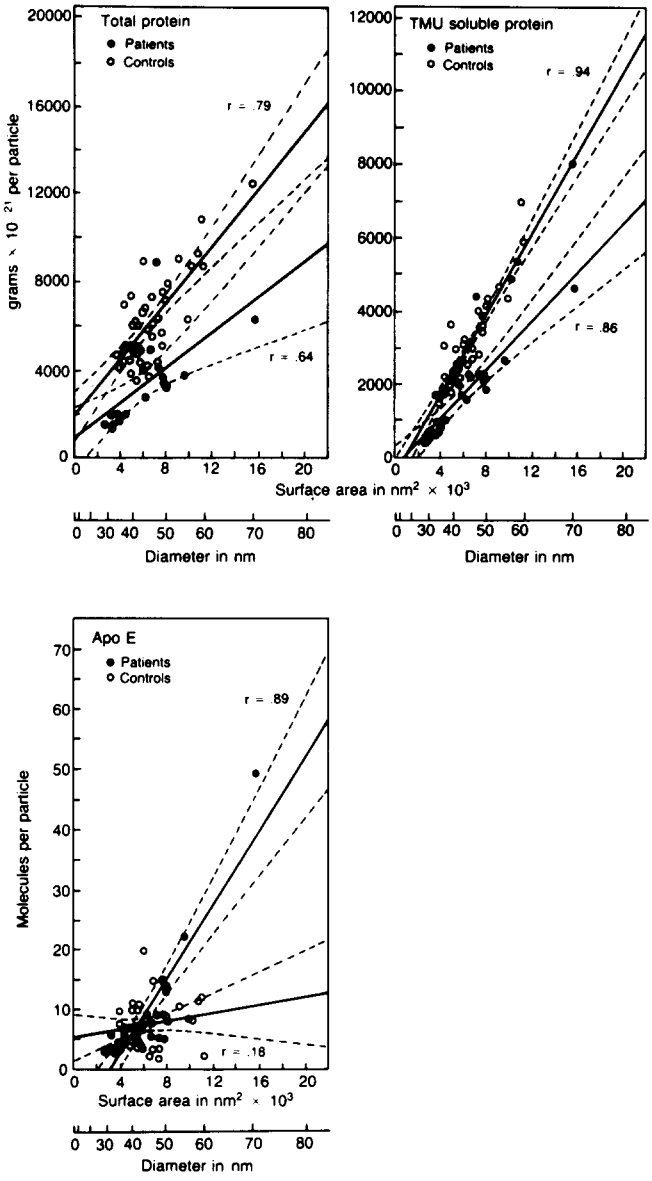

Fig. 3. VLDL total protein, TMU-soluble protein, and apoE in familial LCAT deficiency. Lipoproteins of d < 1.006 g/ml from four female patients and eight controls were analyzed as described for Fig. 2. The figures show regressions and 95% confidence limits calculated for the above mentioned proteins. Significant differences between regression coefficients for patient and control lipoproteins were observed for TMU-soluble protein ($P < 0.001$) and apoE ($P < 0.001$), whereas the difference in total protein coefficients was not significant.

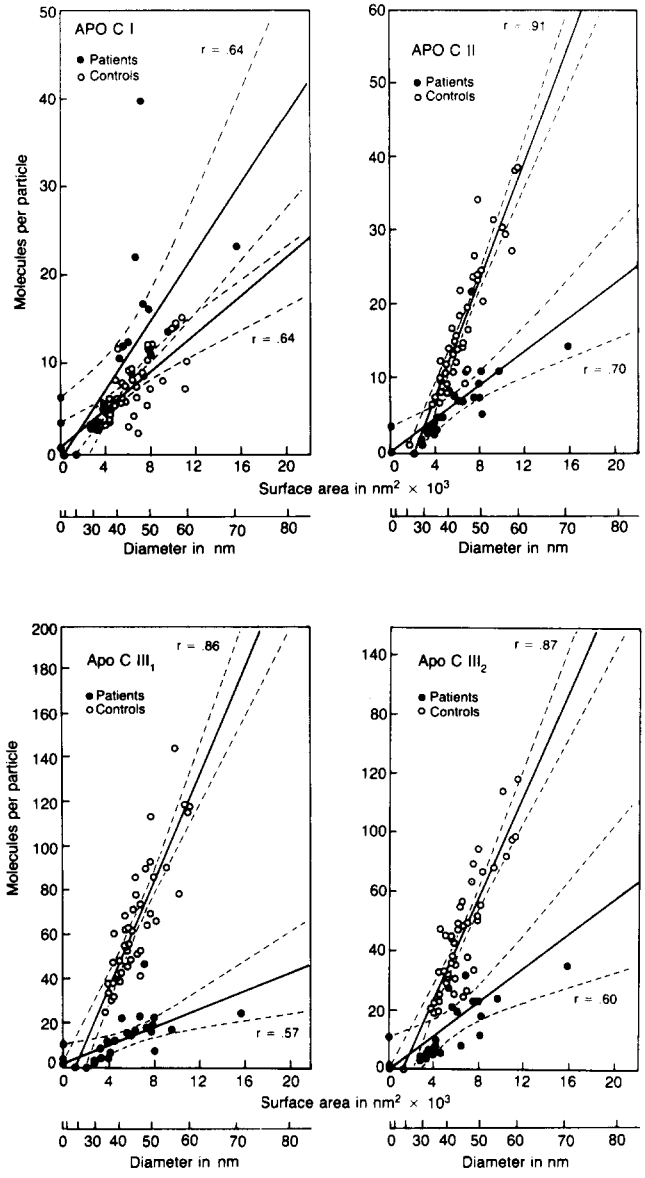

Fig. 4. VLDL apoC in familial LCAT deficiency. Lipoproteins of d < 1.006 g/ml from four female patients and eight controls were analyzed as described for Fig. 2. The figures show regressions and 95% confidence limits calculated for individual apoC. Significant differences between regression coefficients for patient and control lipoproteins were observed for components apoCII ($P < 0.001$); apoCIII$_1$ ($P < 0.001$); apoCIII$_2$ ($P < 0.001$), but not for apoCI ($P < 0.1$).

showing that content of CE in patient lipoproteins of d < 1.006 g/ml is inversely related to content of TG in the diet. The proportion of apoC-III$_2$ compared with apoC-III$_1$ doubled; and the content of apoE in lipoproteins of 60 nm diameter increased by about 78%. However, none of the changes corrected the abnormalities shown in Table 1 to be associated with familial LCAT deficiency.

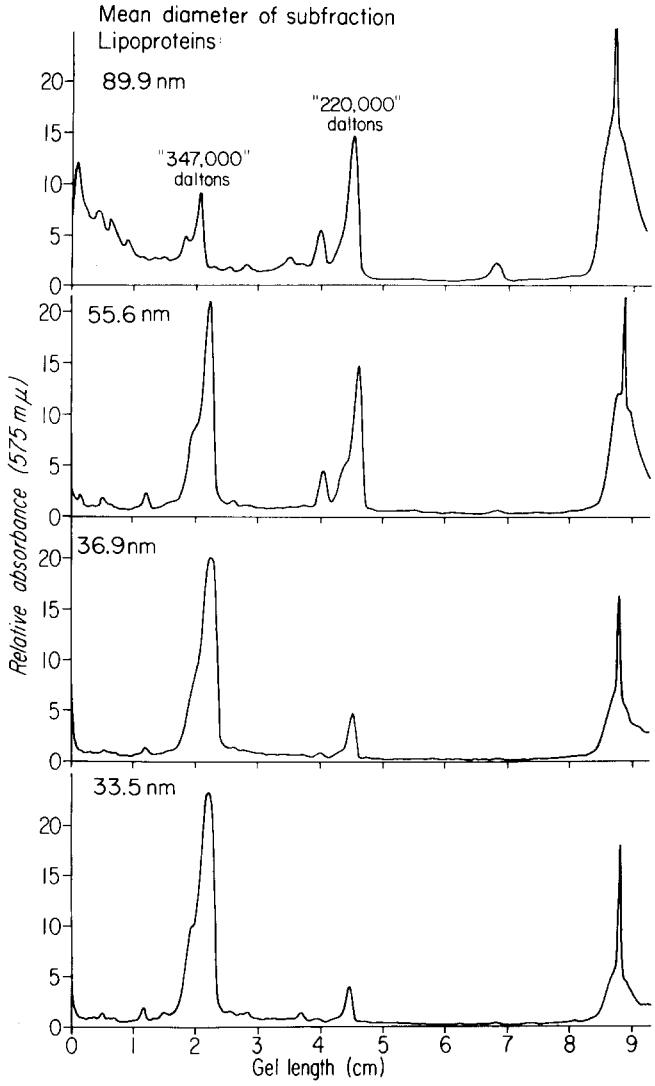

Fig. 5. Resolution of larger VLDL apolipoproteins of patient M.R. by gel electrophoresis in SDS. Subfractions of the lipoproteins of d < 1.006 g/ml of patient M.R. were extracted with ether, boiled in SDS, and then electrophoretically separated on gels of 4% polyacrylamide. Apparent molecular weights for the major peaks were extrapolated from plots of the mobility of standards (see METHODS). They must be considered tentative, owing to the nonlinear behavior of large proteins in SDS-polyacrylamide gel electrophoresis and the possibility that apolipoprotein B aggregates in the presence of SDS (32, 33).

DISCUSSION

Any attempt to characterize lipoproteins of d < 1.006 g/ml must take their heterogeneity into account. Lipoproteins of both intestinal and hepatic origins contribute to this density class; chylomicrons and chylomicron remnants as well as VLDL can be present; and a great deal of variation in size and composition can be expected. This heterogeneity is not easy to deal with since current techniques do not effectively separate intestinal lipoproteins from hepatic lipoproteins or subfractionate VLDL into discrete molecular species. As a minimum, however, the lipoproteins in the class of d < 1.006 g/ml can be partially separated on the basis of size. In the present investigation we employed gel filtration to accomplish this separation, and then compared lipoproteins of LCAT-deficient patients with those of controls. Even so, complications arose. The lipoproteins of three of the patients included large particles that probably were chylomicrons, and, as will be argued below, the lipoproteins of all four patients may have included chylomicron remnants. In contrast to this, it seems likely that the control lipoproteins predominantly included hepatic VLDL. There were other limitations to our investigation as well. We attempted to estimate the content of lipids and apolipoproteins in individual lipoprotein particles, using a regression analysis approach. We did not, however, measure minor constituents of the lipoproteins nor quantitatively analyze individual "apolipoprotein B" components; and, in calculating the composition of the lipoproteins based on constituent and lipoprotein particle volumes, we disregarded potential packing effects such as those that have been described for UC and PC (26).

Despite these limitations, our calculations of the composition of normal VLDL seem to be in relatively good agreement with those reported by others (13, 27); and our calculations of the composition of patient lipoproteins clearly reveal a number of hitherto undetected abnormalities. Previous experiments had shown that patient lipoproteins of d < 1.006 g/ml migrate with an abnormally slow electrophoretic mobility (4), often include chylomicrons (9), and contain a high proportion of UC relative to CE (11). The present experiments show that lipoproteins of all sizes and from all four patients including patient D.J. contain high amounts of UC per particle, and low amounts of apoC-II, apoC-III_1, and apoC-III_2. Since these abnormalities seem unrelated to the presence or absence of hyperlipemia or renal dysfunction, they are very likely characteristics of LCAT deficiency. Our experiments show in addition that patient lipoproteins of 60 nm or more in diameter are unusual in several respects. They contain increased amounts of PL, UC, CE, apoC-I, and apoE; they have a major TMU-insoluble protein that seems to be smaller than that found in normal VLDL; and some of the lipoproteins have a notched appearance upon electron microscopy. Finally, our measurements suggest that patient lipoproteins of 40 nm or less in diameter contain less CE than do their normal counterparts.

Several of these abnormalities are of special interest. For example, the low content of relatively acidic apo-

TABLE 2. Effect of fat-free diet on "VLDL" of patient A.A.; values predicted from regression lines (not shown)

Component	Units	40 nm Particles				60 nm Particles			
		Fat-free Diet	Normal Diet	Fat-free vs Normal (P)	Fat-free vs Controls (P)	Fat-free Diet	Normal Diet	Fat-free vs Normal (P)	Fat-free vs Controls (P)[b]
Total protein	g × 10^{-21}/particle	2490 ± 240[a]	2370 ± 70	n.s.	<0.001	5570 ± 290	4050 ± 250	<0.01	<0.001
TMU soluble protein		1320 ± 160	1270 ± 30	n.s.	<0.001	3960 ± 190	3100 ± 100	<0.01	<0.001
Apo E	molecules/particle	9.83 ± 1.02	7.40 ± 0.60	n.s.	n.s.	32.50 ± 1.21	18.3 ± 2.2	<0.01	<0.001
Apo CI		5.63 ± 0.16	6.73 ± 0.26	<0.05	n.s.	13.37 ± 0.18	18.8 ± 0.9	<0.01	n.s.
Apo CII		4.54 ± 0.24	5.89 ± 0.28	<0.01	<0.001	11.93 ± 0.28	16.1 ± 1.0	<0.05	<0.001
Apo $CIII_1$		10.10 ± 0.7	14.1 ± 0.3	<0.01	<0.001	24.81 ± 0.84	31.2 ± 1.2	<0.001	<0.001
Apo $CIII_2$		11.13 ± 0.8	7.30 ± 0.20	<0.01	<0.001	27.48 ± 0.94	16.7 ± 0.7	<0.001	<0.001
PL	molecules/particle	4370 ± 270	3750 ± 110	n.s.	n.s.	13700 ± 300	9850 ± 390	<0.001	<0.001
UC		4010 ± 250	3690 ± 110	n.s.	<0.01	13800 ± 300	10600 ± 400	<0.001	<0.001
CE		948 ± 106	205 ± 25	<0.001	<0.001	3520 ± 110	990 ± 90	<0.001	n.s.
TG		14400 ± 500	15600 ± 100	<0.05	<0.01	51000 ± 600	55200 ± 500	<0.001	<0.05

[a] Statistics calculated as in Table 1.
[b] Calculated from control data shown in Table 1.

lipoproteins (apoC-II, apoC-III_1, and apoC-III_2) in patient lipoproteins of d < 1.006 g/ml may account for the slow electrophoretic mobility of these lipoproteins noted previously (4). More importantly, the low content of these apolipoproteins provides critical new evidence that LCAT normally influences their distribution in plasma. Since our results strongly suggest that the content of apoC-II and apoC-III in patient lipoproteins of d < 1.006 g/ml is low in vivo,[4] whereas a previous study (1) showed that the content of these apolipoproteins in the same density class is *lowered* when patient plasma is incubated with LCAT in vitro, the normal effect of the LCAT reaction on apoC metabolism must be complex. Clearly, more experiments are needed to establish the basis for this effect. Meanwhile, our current working hypothesis is that the reservoir capacity of HDL for apoC is diminished in familial LCAT deficiency, partly because the concentration of HDL is low (28) and partly because the HDL has not reacted with LCAT (1). If this hypothesis is correct, less apoC would be expected to be available for in vivo transfer from HDL to newly secreted, triglyceride-rich lipoproteins and the turnover of apoC in patient plasma might be increased. To test this hypothesis it will be important to measure both the total content of apoC-II and apoC-III in patient plasma and the turnover of these apolipoproteins. Similar measurements might be made in Tangier disease, a condition characterized by low levels of HDL in plasma and a low content of apoC in VLDL (29).

[4] We have no evidence that loss of apoC occurred during ultracentrifugation or gel filtration.

Another abnormality of potential interest is related to the content of apoC-I in patient lipoproteins. That the content of this apolipoprotein in patient "VLDL" is normal or high, whereas contents of apoC-II and apoC-III are low, may reflect differences in the binding properties previously noted (30) for these apolipoproteins. Whether these differences between apoC-I and the other apoCs have any metabolic significance remains to be determined.

A third interesting abnormality demonstrated in the present investigation involves apoE. Our measurements indicate that the content of this apolipoprotein is normal in patient "VLDL" of 40 nm diameter, but is increased in the larger VLDL. Previous studies showed, however, that the content of apoE in patient "VLDL" *increases* when patient plasma is incubated with LCAT in vitro (1). This indicates that the LCAT reaction is only one of several mechanisms that lead to an increased content of apoE in triglyceride-rich lipoproteins, and is compatible with the possibility that absence of LCAT from the plasma leads to a removal defect involving apoE or a lipoprotein containing apoE.

That defective removal of apoE-rich lipoproteins indeed occurs in familial LCAT deficiency is suggested by the following. Several abnormalities involving the patient large "VLDL" are consistent with a chylomicron remnant removal defect. First, the plasma of hyperlipemic patients afflicted with familial LCAT deficiency contains large lipoproteins of d < 1.006 g/ml that decrease in concentration when the patients consume fat-free diets (9). Second, the notched particles observed in "VLDL"-sized particles in the present

investigation may be chylomicron remnants, since similar structures have been described by Blanchette-Mackie and Scow (31) in preparations of lipoprotein lipase-treated chylomicrons. Third, our finding that patient "VLDL" greater than 60 nm in diameter contains appreciable amounts of a TMU-insoluble protein that has an apparent molecular weight of 220,000 daltons is compatible with evidence obtained by Kane and colleagues[5] that triglyceride-rich lipoproteins of intestinal origin contain a type of TMU-insoluble protein smaller than the apolipoprotein B normally found in VLDL. Finally, the increased content of CE in patient large VLDL probably reflects the presence of lipoproteins of intestinal origin, since patient plasma CE appears to be formed by the intestinal mucosa and may be cleared only slowly from the plasma (2).

Another abnormality of potential interest concerns CE. The content of this lipid in patient lipoproteins, <85 nm in diameter, was a simple function of core volume, whereas the content of CE in control lipoproteins clearly was not. It seems likely that the content of CE in patient lipoproteins depends mainly on the rate of CE synthesis by the intestinal mucosa and the rate of CE removal from the plasma, whereas the content of CE in control lipoproteins is affected in addition by transfer of LCAT-derived CE from HDL (1) and possibly also by transfer from other plasma lipoproteins.

In conclusion, the present investigation has revealed a number of abnormalities associated with the lipoproteins of $d < 1.006$ g/ml of LCAT-deficient patients, but other characteristics of these lipoproteins remain to be investigated. For example, it will be important to measure the contents of the major TMU-insoluble proteins in patient lipoproteins of this density. Why there should be a chylomicron remnant removal defect in familial LCAT deficiency remains an open question. These and other abnormalities associated with this condition will have to be explained if the normal role of LCAT in plasma lipoprotein metabolism is ultimately to be understood.

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