A study of the small spherical high density lipoproteins of patients afflicted with familial lecithin:cholesterol acyltransferase deficiency

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Abstract We studied the effects of the lecithin:cholesterol acyltransferase reaction on the size and composition of the small spherical high density lipoproteins of patients afflicted with familial lecithin:cholesterol acyltransferase deficiency. We isolated these lipoproteins by preparative ultracentrifugation and rate zonal ultracentrifugation, determined their diameter by gradient gel electrophoresis, and then calculated their composition by relating measurements of their lipid and apolipoprotein content to particle volume. Our results revealed lipoprotein particles 6.0–6.2 nm in diameter that contained approximately 2 molecules of apolipoprotein A-I, 37–38 molecules of phospholipid, 3–9 molecules of unesterified cholesterol, 1–2 molecules of cholesteryl ester, and 1–2 molecules of triacylglycerol. Upon being incubated with lecithin:cholesterol acyltransferase and a source of additional unesterified cholesterol, these lipoproteins increased in content of total cholesterol and in particle size to form discrete lipoprotein products 6.6–8.6 nm in diameter. The increase in size occurred despite a net decrease in product unesterified cholesterol and phospholipid and though the net change in total lipid volume was small. Moreover, specific product lipoproteins, isolated by rate zonal ultracentrifugation, contained an increased amount of apolipoprotein A-I. These results seem best explained by a process involving lecithin:cholesterol acyltransferase-induced particle rearrangement reactions. The possibility that a similar process normally occurs in vivo deserves to be explored.—**Chen, C., K. Applegate, W. C. King, J. A. Glomset, K. R. Norum, and E. Gjone.** A study of the small spherical high density lipoproteins of patients afflicted with familial lecithin:cholesterol acyltransferase deficiency. *J. Lipid Res.* 1984. **25:** 269–282.

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The high density lipoproteins (HDL) of patients who have familial lecithin:cholesterol acyltransferase (LCAT) deficiency (1) are most unusual. Some are disc-shaped and contain either apolipoproteins A-I and A-II (apoA-I, apoA-II) or apolipoprotein E (apoE) (2). Others are very small spheres (3–6) that contain mainly apo A-I (5–7). Whether disc-shaped or spherical, all of the HDL are rich in phosphatidylcholine (PC) and contain high relative amounts of unesterified cholesterol (UC) compared with cholesteryl ester (CE) (2, 8).

The disc-shaped HDL that contain apoE have been isolated recently and characterized in detail (2). Their dimensions and composition suggest a particle structure made up of a circular bilayer of lipid whose perimeter is limited by a three-tiered rim of apolipoprotein. In this respect the disc-shaped HDL closely resemble the "nascent" HDL that are released by perfused rat livers (9). Furthermore, like these "nascent" HDL (9), patient disc-shaped HDL react with LCAT in vitro to form spherical particles similar to normal HDL (10). It is possible, therefore, that the apoE-rich discs in the patients' plasma are nascent particles of hepatic origin.

It is also conceivable that the small spherical HDL are nascent particles, but less information is available regarding this possibility. These lipoproteins have been isolated and partially characterized (5–8), but their composition has not yet been determined completely, no molecular model of their structure has been proposed, and it is unclear why they are spherical while other HDL isolated from the same plasma are disc-shaped. Furthermore, the origin of the small spherical HDL also is unclear. The only clues available are the observations that the

Abbreviations: HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoE, apolipoprotein E; PL, phospholipid; PC, phosphatidylcholine; UC, unesterified cholesterol; TG, triacylglycerol; CE, cholesteryl ester; SDS, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)-tetraacetic acid, disodium salt; TRP, triacylglycerol-rich particles; UC-TRP, triacylglycerol-rich particles containing unesterified cholesterol; NEM, N-ethylmaleimide; SM, sphingomyelin; apoHDL, apolipoproteins of high density lipoproteins; lysoPC, lysophosphatidylcholine.

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concentration of small HDL diminishes when patients consume fat-free diets (11), that similar particles are present in rat mesenteric lymph (12), and that similar-sized HDL are released by mouse hepatocytes in culture (13). Finally, the effects of the LCAT reaction on the small spherical HDL have been defined only partially. The small HDL have been shown to react with LCAT in vitro and to remove cholesterol from erythrocyte ghosts (14), but how this affects the structure of the HDL is unknown. Thus, more information is needed that might relate these lipoproteins to normal HDL. In an attempt to obtain such information, we have examined the structure of the small HDL, particularly as it is influenced by the LCAT reaction. The results suggest that the small HDL are spherical because they contain a core of triacylglycerol (TG) and CE, and that action of LCAT on these HDL leads to the formation of product lipoproteins that approach the size range of normal HDL_3.

MATERIALS AND METHODS

Plasma

Two female patients from different Norwegian families were studied: M.R., aged 33, and D.J., aged 66. Their clinical features, laboratory findings, and plasma lipoproteins have been described previously (1, 15–18), and evidence has been obtained (19) that they both have low levels of a functionally defective LCAT enzyme. Both patients were in relatively good health at the time of the study and were receiving no special therapy. Plasma was prepared from patient blood as described previously (14).

Lipoproteins

Patient HDL (d 1.063–1.25 g/ml) were isolated by preparative ultracentrifugal flotation as described previously (14) except that NaN_3 was omitted from all solutions. The HDL were then subfractionated by rate zonal ultracentrifugation and, in some cases, also by gel filtration. Rate zonal ultracentrifugation was performed in a Beckman L2-65 ultracentrifuge equipped with a Z60 rotor (Beckman Instruments, Palo Alto, CA). The procedure used was that of Laggner, Stabinger, and Kostner (20), which employs a discontinuous NaBr gradient in the density range of 1.0–1.4 g/ml. Apparent flotation densities of HDL particles were determined by monitoring the conductivity of the NaBr gradient with a flow cell as the rotor was unloaded, and then employing a predetermined calibration curve for NaBr density vs. conductivity. Gel filtration was performed on K 26/100 columns of Sephacryl S200 or Sephacryl S300 (Pharmacia Fine Chemicals, Piscataway, NJ). The buffer solution (pH 7.4) for both types of column contained 0.15 M NaCl and 0.001 M EDTA. Lipoproteins obtained by ultracentrifugation or gel filtration were concentrated either in Spectrapor 3 dialysis tubing (Spectrum Medical Industries, Los Angeles, CA) against dry Dextran T500 (Pharmacia Fine Chemicals, Piscataway, NJ) or in a Diaflo concentrator provided with a PM10 membrane (Amicon Corporation, Lexington, MA).

LCAT

Unless otherwise stated, LCAT was prepared from fresh plasma of fasting male blood donors according to the method of Albers, Lin, and Roberts (21). The purity of the enzyme preparation was assessed by SDS-urea polyacrylamide gel electrophoresis (2). The enzyme activity was monitored by the method of Verdery and Gatt (22). Enzyme activities reported in the figure texts are in units of μmol UC esterified/ml LCAT per hr. The purified enzyme was kept at 4°C in a solution (pH 7.4) of 0.14 M NaCl and 0.01 M Tris, 0.001 M EDTA and used within 1–2 months after its final purification.

Erythrocyte membranes

Colorless erythrocyte membranes were prepared from fresh blood of healthy donors by the method of Hanahan and Ekholm (23). The membranes were kept at 4°C for no more than 20 hr prior to use.

Triacylglycerol-rich particles (TRP)

Artificially prepared TRP were isolated as described previously (24) from emulsions generously provided by AB Vitrum (Stockholm, Sweden) or from commercial preparations of Intralipid (AB Vitrum). The particles resembled chylomicrons in size and composition except for the absence of apolipoproteins. The ratio of phospholipid (PL) to free sterol to TG in the purified particles was about 9:1:74.

Triacylglycerol-rich particles containing UC (UC-TRP)

UC-TRP were either isolated from specially prepared emulsions of UC, PL, and TG provided by AB Vitrum, or made from Intralipid-derived TRP by incubation with UC-containing liposomes. The latter were prepared by sonication of UC with PL isolated from Intralipid. In a typical preparation, 310 μmol of UC (Nu-Chek-Prep., Inc., Elysian, MN) dissolved in 5 ml of chloroform was mixed with 297 μmol of PL in 4.5 ml of chloroform and dried under argon. After addition of 100 ml of NaCl–EDTA solution, the mixture was sonicated at 40–50°C under argon for 1 hr with a probe sonicator (Branson Instruments, Inc., Danbury, CT) at a setting of 3–4. After sonication, the liposomes were centrifuged for 15 min at 1000 rpm to remove metal particles released by the probe. The molar ratio of UC to PL in the resulting liposomes was 0.89.

To prepare UC-TRP, the UC-containing liposomes (267.9 μmol of PL, 239 μmol of UC) were incubated with 10% TRP (25.36 μmol PL) at 37°C for 17.5 hr under argon. UC-TRP were isolated by centrifugation for 50 min at 25,000 rpm, 5°C, in a SW27 rotor. Then the UC-TRP were washed once with NaCl–EDTA by centrifugation as just described and once through a discontinuous sucrose density gradient as described previously (24). The molar ratio of cholesterol to PL in the resulting UC-TRP was 0.86.

Incubations

Patient small spherical HDL were incubated with or without LCAT in the presence of UC provided by patient very low density lipoproteins (VLDL), UC-TRP, or normal erythrocyte ghosts. In most cases 4% fatty acid-free human serum albumin (Sigma Chemical Co., St. Louis, MO) was included as well. The incubations were carried out in Falcon disposable culture tubes (Falcon, Oxnard, CA) at 37°C, usually under argon. At the end of the incubation the LCAT reaction was stopped by adding N-ethylmaleimide (NEM) to a final concentration of 0.01 M. Then the HDL were recovered by a combination of centrifugation (1.446×10^6 *g*-min to remove erythrocyte ghosts) and filtration through millipore filters (11). Alternatively, in experiments that employed TRP as a source of UC, the HDL were recovered by preparative and/or rate zonal ultracentrifugation.

Gradient gel electrophoresis

Freshly isolated or incubated HDL samples were separated by electrophoresis on PAA 4/30 polyacrylamide gradient gels run in a Model GE-4 apparatus (Pharmacia Fine Chemicals, Piscataway, NJ). The running buffer recommended by the manufacturer (0.08 M Tris-borate and 3 mM Na_2 EDTA, pH 8.35) was used, and gels were run at 125 V for 16 hr (2000 volt-hours) at 10°C. Subsequently, gels were stained with Coomassie Brilliant Blue R250 and then scanned at 570 nm using an Ortec Model 4310 densitometer (2).

The Pharmacia high molecular weight protein standards kit was used to run calibration tracks at the left and right sides of each gel. Nonhydrated molecular diameters for these proteins are: thyroglobulin, 11.62 nm; apo-ferritin, 10.18 nm; catalase, 8.16 nm; lactate dehydrogenase, 6.90 nm; bovine serum albumin, 5.38 nm (25). These values are 20–40% smaller than the presumably hydrated diameters reported by Blanche et al. (26), but require no assumptions about the degree of hydration.

Densitometric scans were digitized and resolved into component HDL distributions using programs available on the PROPHET computer system (27). Details are given in the Appendix to this paper.

Quantitative analysis of apolipoproteins and lipids

Quantitative apolipoprotein analysis was performed by densitometry of SDS-urea gels, as described previously (2). Analyses of neutral lipids and phospholipids also were performed by previously published methods (14). Regression lines fit to known standards had the following coefficients of variation: apoA-I, 4–7%; PC and other phospholipids, 2–4%; UC and CE, 3–4%; TG, 11%.

Calculations of particle composition

The molecular composition of HDL particles was calculated from the analytical data for lipids and apolipoproteins, using molar volumes reported by Sata, Havel, and Jones (28), and total particle volumes calculated for spheres having the mean diameter of the principal components resolved from the gradient gel scans. The following points should be kept in mind about such a procedure. *a*) Particles may actually be somewhat ellipsoidal. However, for an excentricity as high as 0.5, the ratio of surface volume to core volume is only 5% higher than that of a sphere. *b*) Apparent molar volumes for apoA-I associated with lipid, polar lipids on highly curved surfaces, and nonpolar lipids in a small core region may not be the same as the bulk phase values employed. *c*) The experimental compositions are actually volume weighted averages for the entire distribution of particle sizes in the sample. Since the mean particle volume will always be larger than the volume calculated on the basis of the mean particle diameter, the average number of apoA-I molecules per particle of mean diameter will be reduced by the ratio of these volumes. This should lead to apparent nonstoichiometric (and decreased) numbers of peptide molecules per particle, even if the actual number throughout the distribution is a constant integer. In addition, the unknown variation of lipid composition across the distribution will affect the values found for the hypothetical mean particle. If surface components vary as the square of the particle diameter, and core components as the cube, one might expect surface lipid to be underestimated and core lipid to be overestimated for the mean particle.

RESULTS

Characterization of freshly isolated small spherical HDL

A major goal of this investigation was to characterize the small spherical HDL of LCAT-deficient patients in sufficient detail to permit estimation of the number of lipid and apolipoprotein molecules contained in an average lipoprotein particle. To attain this goal we performed several experiments with the HDL of patients

M.R. and D.J. We isolated lipoproteins of d 1.063–1.25 g/ml by preparative ultracentrifugation, and by rate zonal ultracentrifugation obtained a subfraction that reproducibly contained small spherical HDL. Then we used gradient gel electrophoresis to determine the diameter of these HDL, measured their content of lipids and apolipoproteins, and calculated the contribution of each component to the total particle volume. The results of a typical rate zonal ultracentrifugation experiment are shown in **Fig. 1.** The shaded area in Fig. 1A corresponds to the subfraction containing small spherical HDL. This subfraction emerged in a density range of d 1.16–1.25 g/ml and behaved similarly upon recentrifugation (Fig. 1B). The results of a gradient gel electrophoresis experiment are shown in **Fig. 2.** We typically observed a major peak corresponding to particles about 6 nm in nonhydrated diameter. In the experiment shown in this figure we also observed minor peaks of both higher and lower mobility. Using the computer-assisted peak resolution procedure described in the Appendix, we determined that the major peak contributed 79.2% of the total stained area on the gradient gel. The particles within this peak

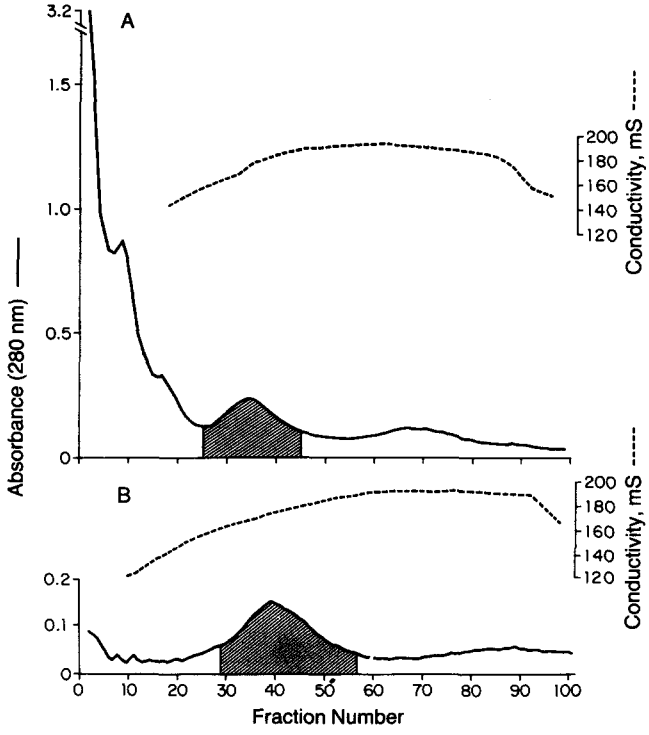

Fig. 1. Isolation of small spherical HDL from LCAT-deficient plasma by rate zonal ultracentrifugation. HDL (d 1.063–1.25 g/ml) were isolated from the plasma of patient M.R. by sequential ultracentrifugal flotation, then subfractionated by rate zonal ultracentrifugation using the method of Laggner et al. (20). A: Elution profile of HDL. Shaded area corresponds predominantly to small spherical HDL. B: Elution profile of recentrifuged material corresponding to the shaded area in A. Fractions were pooled, concentrated, and recentrifuged under conditions similar to those used in the experiment shown in A.

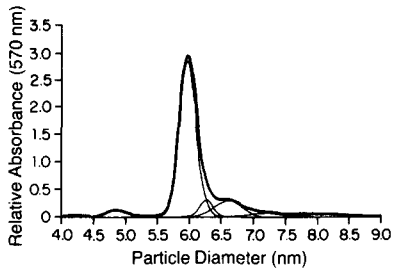

Fig. 2. Estimation of the diameter of the small spherical HDL of patient M.R. by gradient gel electrophoresis. Small spherical HDL were isolated from the plasma of patient M.R. as described in Fig. 1. Material corresponding to the shaded area in Fig. 1B was filtered through Sephacryl S 200, then concentrated by dialysis against Dextran T 500. An amount corresponding to 10 μg of protein was then applied to a precast polyacrylamide gradient gel (PAA 4/30), allowed to migrate for 16 hr at 125 v, then stained with Coomassie R250 and scanned at 570 nm. High molecular weight standards from Pharmacia were applied to the gel in duplicate. Peaks scanned at 570 nm were digitized, replotted (heavy line) on a linear scale, and resolved into component peaks using programs available on PROPHET. Note that the resolved component peaks were plotted using light lines, and that all values given are calculated for nonhydrated particles.

had a mean nonhydrated diameter of 5.98 nm, and the width parameter of the Gaussian fit to the peak indicated that the apparent diameters of 95% of the particles lay within ±0.45 nm (8%) of this value. Some of this variation can be attributed to diffusion because 95% of the particles of lactic dehydrogenase, i.e., the protein standard of nearest molecular weight, had apparent diameters that lay within 4% of the mean.

The remainder of the total stained area on the gradient gel was contributed by minor peaks, which corresponded to particles 4.84, 6.26, 6.61, and 7.2 nm in diameter. Attempts by repeated gel filtration on Sephacryl S 200 to obtain a more highly purified preparation of 5.98 nm diameter particles were unsuccessful. The particles seemed to be unstable and to decompose, yielding material of both smaller and larger molecular weight.

Four separate analyses of the major peak of small spherical HDL of patient M.R., performed over a period of several years, yielded an overall mean nonhydrated diameter of 6.07 ± 0.1 nm. Similar analyses of two separate preparations of the corresponding HDL of patient D.J. yielded a mean diameter of 6.16 nm. Thus, the two patients' small spherical HDL were similar in size and could be reproducibly isolated. These values complement previous estimates of the particle size range of the Norwegian patients' small spherical HDL (4.0–6.0 nm in diameter) made by electron microscopy (3), and seem to provide the most precise estimate of the size of these HDL that is currently available.

After determining the size of the small spherical HDL, we analyzed the apolipoprotein and lipid contents of the same preparations. In agreement with earlier information (6, 7), we found that apoA-I was the only apolipoprotein

present. In addition, we found that the lipid components not only included UC, PC, and sphingomyelin (SM) (molar ratio of PC:SM = 12–15:1) (8, 14), but also included both CE and TG. By expressing measurements of the contents of these components in terms of molecular volume (see Materials and Methods), we estimated the contribution each component could have made to a spherical HDL particle 6.0–6.1 nm in diameter (**Table 1**). The results suggested that an average particle of this size contains approximately 2 molecules of apoA-I, 37–38 molecules of PL, 3–9 molecules of UC, 1–2 molecules of CE, and 1–2 molecules of TG. Although information is not available regarding the distribution of these components within the particle, it is apparent that apoA-I and PL together make up more than 90% of the particle volume. Furthermore, enough apoA-I, PL, and UC is present to form a surface 2.0 nm thick, and enough CE and TG is present to form a core 2.0 nm in diameter. Thus, the composition of the particles seems consistent with the general pseudomicellar model of plasma lipoprotein structure proposed by Shen, Scanu, and Kézdy (29), Verdery and Nichols (30), and Jackson, Morrisett, and Gotto (31).

Action of LCAT on the small spherical HDL

Another goal of this investigation was to examine the effects of the LCAT reaction on the size and composition of the small spherical HDL. In three time-study experiments we incubated these HDL for 1–24 hr in the presence of both a purified preparation of LCAT and a source of additional UC, then used gradient gel electrophoresis to examine the distribution of HDL products formed. In other experiments, we incubated similar mixtures for 17–18 hr, then attempted to isolate specific HDL products and determine their size and composition.

The three time-study experiments yielded essentially similar results. Irrespective of whether patient VLDL, normal erythrocyte membranes, or UC-TRP were used as a source of additional UC, reaction of the small spherical HDL with LCAT led to the formation of a heterogeneous population of product HDL particles that ranged from 6.6 nm to 8 nm in diameter. Furthermore, product HDL 6.6–6.7 nm in diameter appeared at an early stage and then seemed to be replaced by larger particles. Results obtained in one of the time-study experiments are shown in **Fig. 3.** In this experiment the 6-nm diameter HDL of patient M.R. were incubated for 0, 3, 6, 10, or 24 hr with LCAT and UC-TRP. Then, both product HDL and materials of d < 1.006 g/ml and d 1.006–1.063 g/ml were isolated by preparative ultracentrifugation and compared with control material. Analysis of the HDL by gradient gel electrophoresis (Fig. 3) and subsequent curve resolution provided evidence for recurring particle diameters of 6.01 ± 0.05 nm (n = 6), 6.61 ± 0.03 nm (n = 6), 6.98 ± 0.05 nm (n = 6), 7.39 ± 0.10 nm (n = 4), and 7.84 ± 0.04 nm (n = 2). Plots of peak areas corresponding to the major products (**Fig. 4**) suggested that the concentration of product HDL 6.61 nm in diameter increased rapidly to reach a maximum at 6 hr and then declined, whereas the concentrations of product HDL 6.98 nm and 7.39 nm in diameter increased for the first 10 hr of the incubation and then remained essentially

TABLE 1. Estimated composition and properties of small spherical HDL

Patient	Nonhydrated Particle Diameter[a] (nm)	Particle Component	Component Mole Percent	Component Volume Percent[b]	Number Component Molecules per Particle[c]	Molecular Weight[d] (daltons)	Density[d] (g/ml)
M.R.	5.98	apoA-I	3.43	50.1	1.71	83,116	1.234
		PL	73.5	41.9	36.7		
		UC	18.5	5.1	9.25		
		CE	1.76	.88	.88		
		TG	2.74	1.95	1.37		
D.J.	6.08	apoA-I	3.88	51.6	1.83	86,881	1.225
		PL	80.6	41.8	38.13		
		UC	6.24	1.56	2.96		
		CE	4.75	2.17	2.25		
		TG	4.57	2.96	2.17		

Small spherical HDL of patients M.R. and D.J. were prepared by preparative and rate zonal ultracentrifugation (see Fig. 1). Composition of small spherical HDL was calculated by relating measurements of apolipoprotein and lipid content to particle volume.

[a] Obtained from gradient gel calibration for major particle shown in Fig. 2 for patient M.R., and similarly from a single experiment (not shown) for patient D.J.

[b] Calculations do not take into account possible alteration of apparent molar volumes because of condensing effects involving PL and UC or PL and apoA-I.

[c] Calculated from volume percentages for a particle of the diameter indicated. Estimated coefficient of variation and absolute standard deviations for the derived numbers of molecules per particle are: apoA-I, 6–7% (±0.1 molecule per particle); PL, 6–8% (±2.6); UC, 9–10% (±0.9); CE 6–10% (±0.1); TG, 16% (±0.3).

[d] Calculated from compositional data.

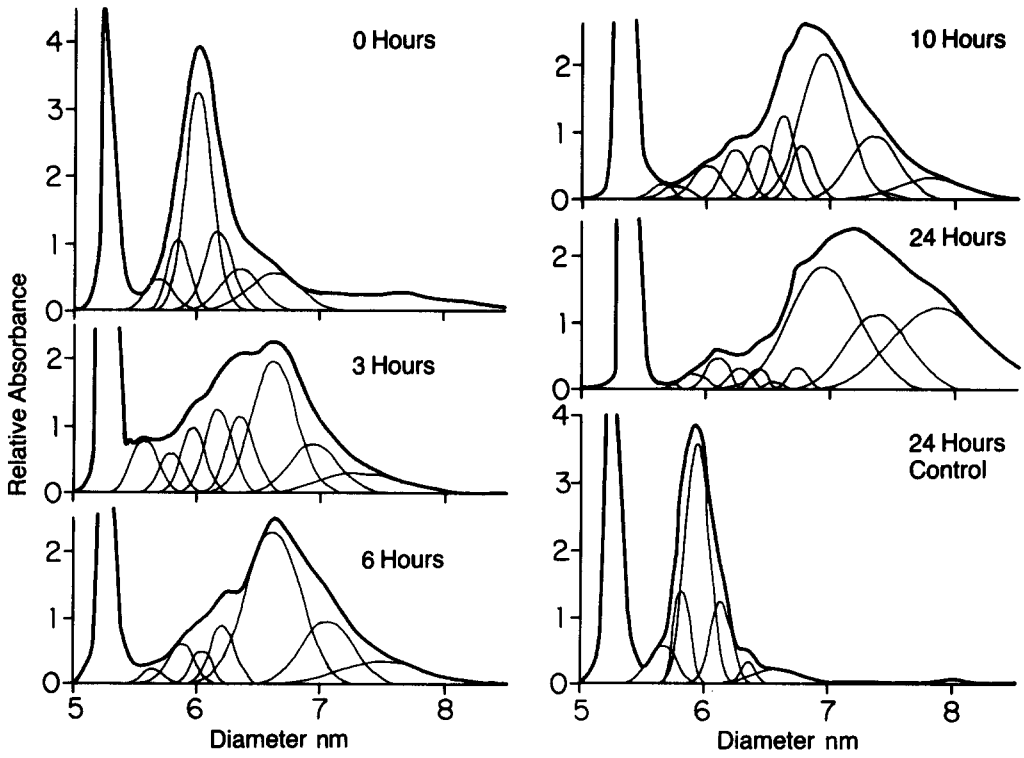

Fig. 3. Gradient gel electrophoretic analysis of the HDL products formed by action of LCAT on the small spherical HDL. The small spherical HDL of patient M.R., prepared by methods similar to those shown in Fig. 1, were incubated at 37°C with LCAT (158 units/ml) in the presence of UC-TRP and fatty acid-free albumin. After 3, 6, 10, or 24 hr the HDL products were isolated by preparative ultracentrifugation in a SW 60 rotor and examined by gradient gel electrophoresis. The gel scans were digitized and plotted as in Fig. 2, and the curve envelopes resolved by the computer-assisted peak resolution procedure (see Appendix) were plotted separately using light lines. Note that the particles 5.3 nm in diameter correspond to residual human serum albumin.

constant. On the other hand, the concentration of product HDL 7.84 nm in diameter began to increase after 6 hr and then rose in a linear fashion. Thus, incubation with LCAT led to an increase of 50% to 120% in particle volume of the HDL. Though the basis for this remains to be completely clarified, the formation of the various discrete product HDL clearly depended on the LCAT reaction because small spherical HDL that were incubated for 24 hr in the absence of LCAT did not increase in diameter (Fig. 3).

Analysis of the lipid and apolipoprotein content of the HDL recovered at the various time points in this experiment (**Fig. 5, Table 2**) demonstrated that the LCAT-dependent increase in particle volume was accompanied by an almost 40-fold increase in the content of CE, and also by a 6- to 7-fold increase in the content of total cholesterol. The increase in content of total HDL cholesterol evidently occurred because much of the UC required for the LCAT reaction was provided by the UC-TRP that had been included in the incubation mixture, whereas very little HDL CE transferred to the UC-TRP in return (Table 2). Nevertheless, the UC-TRP did not provide enough UC or PL to the HDL to make up for the deficits in these lipids caused by the LCAT reaction. Meanwhile, the LCAT reaction had little effect on the contents of HDL apoA-I and TG (Fig. 5).

Thus, incubation of the small, relatively homogeneous substrate HDL with LCAT led to the formation of a highly heterogeneous population of larger product HDL, and the increase in HDL size was accompanied by a large *relative* increase in the content of CE. Nevertheless, the *absolute* increment in HDL CE was far too small to account for the 50 to 120% increase in particle volume revealed by gradient gel electrophoresis. This raises an important question about the mechanism of the LCAT-induced increase in HDL particle size.

Two experiments in which specific product HDL were isolated and characterized provided further information about the effects of the LCAT reaction on the 6-nm diameter substrate HDL. In one of these experiments we incubated the small spherical HDL of patient M.R. for 17.5 hr with LCAT and UC-TRP, but in the absence of other proteins. Then we isolated product HDL by rate zonal ultracentrifugation and examined them by gradient

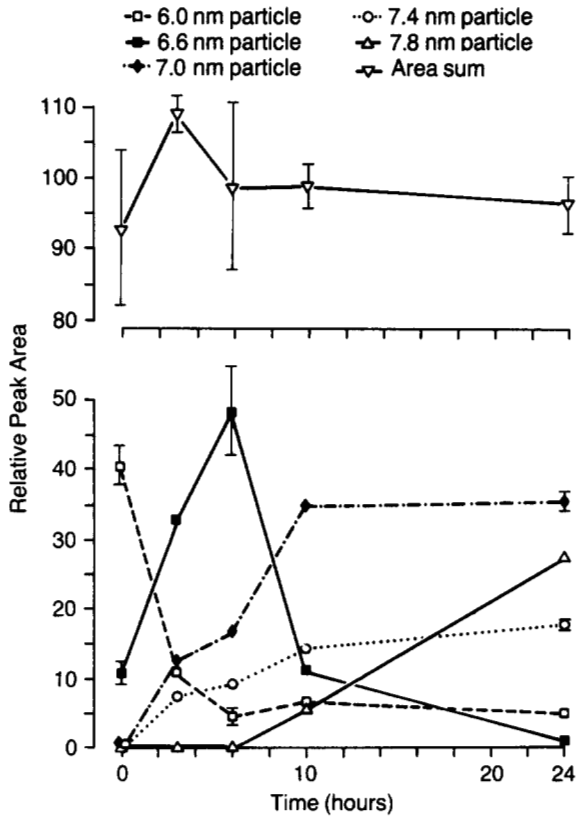

Fig. 4. Effect of the LCAT reaction on HDL peak area. The areas of HDL peaks identified in the experiment shown in Fig. 3 were determined by integration and plotted ±SD as a function of time. Particle sizes are average values found at the five sampling times and have ranges (95% confidence interval) of less than ±0.05 nm.

TABLE 2. Effect of LCAT on the distribution of lipids among lipoprotein fractions isolated from a mixture that originally contained patient small spherical HDL, UC-TRP, and albumin

Lipoprotein Fraction	UC	CE	PL
	nmol lipid/incubation mixture		
d < 1.006 g/ml			
−LCAT	334	5	327
+LCAT	148	17	292
	−186	+12	−35
d 1.006–1.063 g/ml			
−LCAT	32	1	48
+LCAT	10	2	20
	−22	+1	−28
d 1.063–1.25 g/ml			
−LCAT	31	6	271
+LCAT	8	234	132
	−23	+228	−139

Data from the experiment shown in Figs. 3–5. Lipoprotein fractions isolated after a 24-hr incubation by preparative ultracentrifugation. Results shown are mean values of duplicate analyses.

gel electrophoresis. Rate zonal ultracentrifugation yielded a relatively sharp HDL peak in addition to rapidly floating material and non-floating material (**Fig. 6**). The apparent flotation density of material in this peak ranged from d 1.20 to d 1.26 g/ml with a median value of d 1.23 g/ml. When the material corresponding to the shaded area of the HDL peak was analyzed by gradient gel electro-

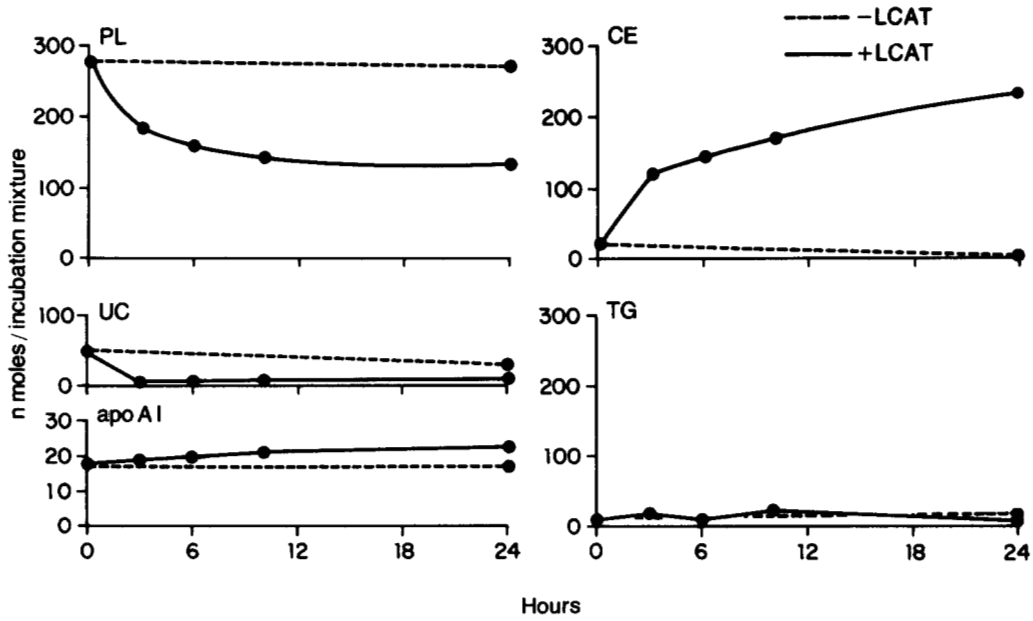

Fig. 5. Composition of product HDL formed by the LCAT reaction. The aggregate composition of HDL products, isolated by preparative ultracentrifugation in the experiment shown in Figs. 3 and 4, was analyzed by methods described in Materials and Methods and plotted as a function of time.

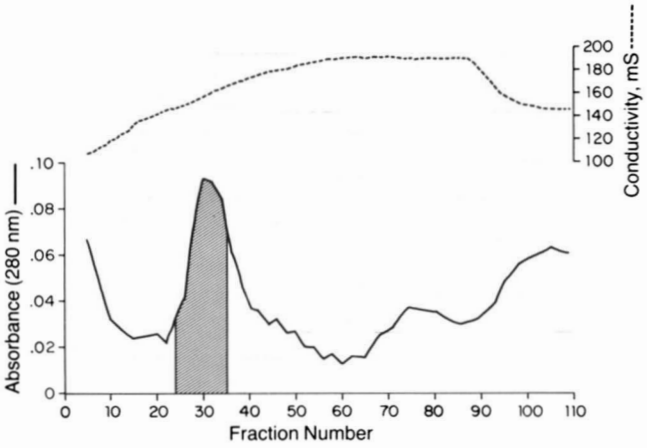

Fig. 6. Rate zonal ultracentrifugation of the lipoprotein product formed upon incubation of small spherical HDL, LCAT, and UC-TRP. Small spherical HDL isolated from 246 ml plasma of patient M.R. were incubated for 17.5 hr at 37°C with LCAT (69 units/ml) and UC-TRP (UC:PL = 0.86) in 10 mM Tris buffer (pH 7.4) and 10 mM mercaptoethanol. Reaction was terminated by addition of NEM to a final concentration of 10 mM. Reaction product was isolated by rate zonal ultracentrifugation as described in Fig. 1 after removal of UC-TRP by ultracentrifugal flotation at 10^6 g-min.

phoresis (**Fig. 7A**), a major component, corresponding to particles 6.85 nm in diameter, and several minor components were seen. The major component contributed 65.8% of the total stained area on the gradient gel, whereas the most prevalent minor component, which corresponded to particles of 7.1 nm in diameter, contributed most of the remainder (25.9% of the total stained area). Analysis of the lipid and apolipoprotein content of the product HDL (**Table 3**) suggested that the HDL 6.85 nm in diameter might contain approximately 3 molecules of apoA-I per particle, 35 molecules of PL, 4 molecules of UC, 14 molecules of CE, and 2 molecules of TG. Among the molecules of PL were 9 molecules of lysoPC, which had remained on the HDL presumably because human fatty acid-poor albumin had not been included in the incubation mixture.

The particle volume of these product HDL was 51% greater than that of the substrate HDL. Moreover, apoA-I accounted for 64% of the volume, as compared to 50–51% in the original substrate. If the substrate particle had increased in size merely to accommodate excess CE, the volume percentage of apoA-I should have decreased. Thus, it seems likely that the LCAT reaction led to some form of particle rearrangement which may have included particle fusion.

The ratio of the combined volume of presumptive surface components (apoA-I + PL + UC) to that of presumptive core components (CE + TG) was 8.04 for the 6.85-nm particle, whereas the pseudomicellar model of Shen et al. (29) would predict a ratio of 8.54 for a 2.0-nm surface monolayer. A 1.8-nm monolayer thickness may be calculated for our particle using the same model. Thus, the 6.85-nm particles appear to be slightly deficient in surface lipid.

The design of the second incubation experiment was similar to that of the experiment just described except that human serum albumin poor in fatty acid was included in the incubation mixture. The incubation led to a heterogeneous distribution of product HDL (not shown) that was generally similar to that observed at the 24-hr time period in Fig. 3. When the product HDL were subfractionated by rate zonal ultracentrifugation and subsequently analyzed by gradient gel electrophoresis, only the material in the leading edge of the HDL peak appeared sufficiently homogeneous to merit further examination. This material covered a range of apparent flotation den-

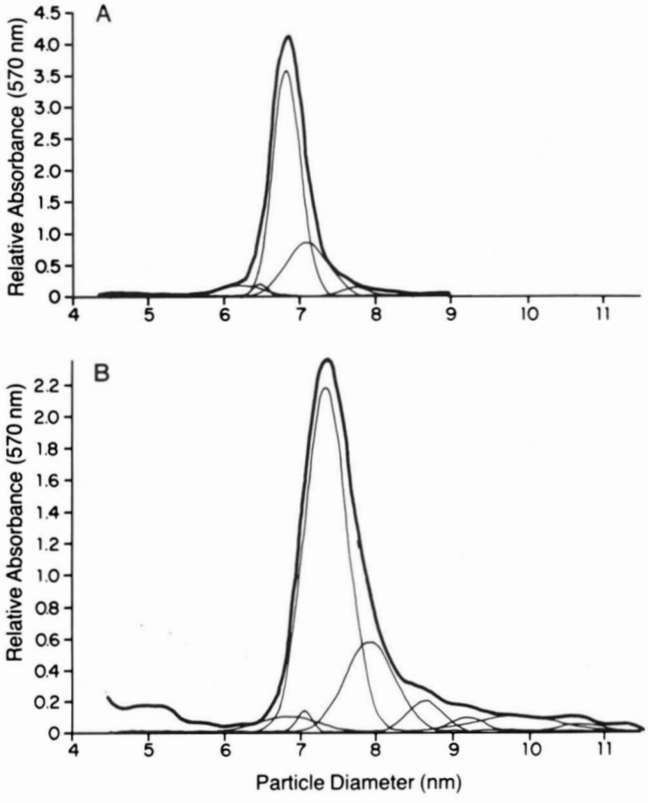

Fig. 7. Gradient gel electrophoresis of product of LCAT reaction with small spherical HDL. A: Reaction product corresponding to the shaded area under the peak in Fig. 6 was concentrated, dialyzed against NaCl-EDTA, analyzed by gradient gel electrophoresis, and plotted as described in Fig. 2. The curve envelope of product HDL was resolved into components and plotted as in Fig. 3. B: Small spherical HDL of patient M.R. were incubated for 18 hr with LCAT (171 units/ml), UC-TRP, and human serum albumin, then isolated by preparative and rate zonal ultracentrifugation. Material corresponding to the leading edge of the HDL peak obtained in the zonal ultracentrifuge (d 1.17–1.22 g/ml) was pooled, concentrated, dialyzed against 0.15 M NaCl–1 mM EDTA (pH 7.4), analyzed by gradient gel electrophoresis, and plotted as in Fig. 2. The curve envelope product HDL was resolved into components and plotted as in Fig. 3.

TABLE 3. Estimated composition and properties of product lipoproteins 6.85 nm in diameter formed by reaction of LCAT with small spherical HDL

Nonhydrated Particle Diameter of Major Component[a] (nm)	Particle Component	Component Mole Percent	Component Volume Percent[b]	Number Component Molecules per Particle[c]	Molecular Weight[d] (daltons)	Nonhydrated Density[d] (g/ml)
6.85	apoA-I	5.05	64.0	3.25	137,200	1.230
	PC	31.6	15.4	20.1		
	lysoPC	14.6	3.6	9.4		
	SM	8.6	4.0	5.2		
	PE	.93	0.7	0.6		
	UC	6.2	1.5	4.0		
	CE	21.8	9.4	14.0		
	TG	2.8	1.7	1.8		

Small spherical HDL of patient M.R. were incubated for 17.5 hr with LCAT and UC-containing TRP, then reisolated by rate zonal ultracentrifugation as shown in Fig. 6.

[a] Obtained from gradient gel calibration for experiment shown in Fig. 7.

[b] See footnote b, Table 1.

[c] Calculated from volume percentages for a 6.85 nm particle. Estimated coefficient of variation and absolute standard deviations for the derived numbers of molecules per particle: apoA-I, 7% (±0.25); PC, LPC, SM, PE, 16% (±3.0, ±1.5, ±0.8, ±0.1); UC, 16% (±0.6); CE 15% (±2.1); TG 22% (±0.4).

[d] Calculated from compositional data.

sities from d 1.17 to d 1.22 g/ml with a median value of 1.19 g/ml. Upon being analyzed by gradient gel electrophoresis (Fig. 7B), it could be resolved into one major and several minor components. The major component corresponded to particles about 7.35 nm in diameter, and accounted for 57.9% of the total stained area on the gradient gel, whereas particles 7.93 nm in diameter contributed the bulk of the remainder (21.0% of the total stained area). Analysis of the lipid and apolipoprotein content of these HDL products (**Table 4**) suggested that the average lipoprotein particle 7.35 nm in diameter might contain approximately 3 molecules of apoA-I, 35 molecules of PL, 7 molecules of UC, 53 molecules of CE, and 6 molecules of TG.

The presence of 3 molecules of apoA-I in each 7.35-nm diameter lipoprotein particle is consistent with the possibility that both these particles and the product lipoprotein particles 6.85 nm in diameter isolated in the previous experiment were formed by similar mechanisms. However, the 7.35-nm diameter HDL clearly contained much more CE than did the 6.85-nm HDL, and seemed to be much more deficient in surface lipid. The ratio of the combined volume of apoA-I + PL + UC to that of CE + TG was 1.99, whereas a ratio of 4.68 would have been predicted by the model of Shen et al. (29). Our experimental ratio corresponds to a surface monolayer 1.25 nm thick.

The factors that led to the formation of these different-sized HDL products that contained very different amounts of lipid largely remain to be identified. However, one of these factors was probably albumin because we found, in an additional experiment (not shown), that smaller HDL

TABLE 4. Estimated composition and properties of product lipoproteins 7.35 nm in diameter formed by reaction of LCAT with small spherical HDL

Nonhydrated Particle Diameter of Major Component[a] (nm)	Particle Component	Component Mole Percent	Component Volume Percent[b]	Number Component Molecules per Particle[c]	Molecular Weight[d] (daltons)	Nonhydrated Density[d] (g/ml)
7.35	apoA-I	2.60	42.86	2.69	146,587	1.171
	PL[e]	33.82	21.66	34.96		
	UC	6.69	2.06	6.91		
	CE	50.82	28.58	52.5		
	TG	6.06	4.84	6.26		

Small spherical HDL of patient M.R. incubated for 18 hr with LCAT, UC-TRP, and human serum albumin poor in fatty acid, then reisolated by rate zonal ultracentrifugation.

[a] Obtained from gradient gel calibration for experiment shown in Fig. 8.

[b] See footnote b, Table 1.

[c] Calculated from volume percentages for a 7.35 nm particle. Estimated coefficient of variation and absolute standard deviations for the derived numbers of molecules per particle: apoA-I, 7% (±0.2); PL, 8% (±2.9); UC, 10% (±0.7); CE, 8% (±4.1); TG, 16% (±1.0).

[d] Calculated from compositional data.

[e] LysoPC accounts for 3.9 mole % of total phospholipid recovered.

products were formed in the absence of albumin than were formed in its presence.

DISCUSSION

After unusually small HDL were discovered in the plasma of Norwegian LCAT-deficient patients in 1970 (4, 14), various aspects of these particles, such as shape, size, chemical composition, and reactivity toward LCAT were studied. Negative staining electron microscopy revealed (3, 4) that the small HDL are spherical particles, 3.5–6 nm in diameter. Protein analyses indicated that they contain mainly apoA-I (7). Lipid analyses demonstrated a high relative content of lecithin compared with sphingomyelin (molar ratio = 12–15:1) and a low relative content of unesterified cholesterol compared with lecithin (molar ratio 1:3–4) (8, 14). Finally, the HDL were shown to react readily with LCAT and to remove cholesterol from erythrocyte ghosts (14).

Later, small HDL also were observed in the plasma of LCAT-deficient patients from other parts of the world. Thus, Utermann and his colleagues (5) isolated a fraction containing similar lipoproteins from the plasma of patients of Sardinian origin, found that these lipoproteins ranged from 6 to 15 nm in diameter, and showed by qualitative methods that the main apolipoprotein component was apoA-I. Similarly, Soutar, Knight, and Myant (6) isolated small spherical HDL from the plasma of three Irish sisters, found that apoA-I was the sole apolipoprotein present and measured the relative content of protein, total cholesterol, and phospholipid.

In none of these studies, however, was enough information obtained about a given preparation of the small spherical HDL to allow construction of a molecular model. This would have required not only a reasonably homogeneous preparation of small spherical HDL, characterized in terms of mean particle size, but also complete, quantitative data regarding the content of apolipoproteins, polar lipids, and nonpolar lipids in the preparation.

In the present investigation we set out to obtain a complete set of data of this type for Norwegian patient small spherical HDL. We isolated the HDL by zonal ultracentrifugation, measured the mean particle diameter by gradient gel electrophoresis, and not only measured the contents of apoA-I, phospholipid, and unesterified cholesterol, but also measured the contents of cholesteryl ester and triacylglycerol.

Our measurements yielded very similar results for the small spherical HDL of the two patients studied. In several different experiments the mean nonhydrated diameter of these lipoproteins seemed to be almost precisely 6 nm. In addition, an average particle seemed to contain 2 molecules of apoA-I, 37–38 molecules of PL, 3–9 molecules of UC, 1–2 molecules of CE, and 1–2 molecules of TG. This distribution of components is notable in several respects. Thus, the relative content of protein, PL, UC, and TG observed is in good agreement with that observed in a previous study of another Norwegian patient's small spherical HDL (8). Furthermore, the content of apolipoprotein relative to phospholipid is in good agreement with the results obtained by Soutar et al. (6). The relative content of cholesterol appears to differ from that observed by Soutar et al. (6), but their tabulated data show a much higher content of total cholesterol relative to phospholipid (molar ratio = 1:1) than indicated by their direct analyses of HDL subfractions obtained by gel filtration (see Fig. 6, reference 6). These analyses show a molar ratio of about 1:2, which is clearly in better agreement with our data.

The low relative content of UC consistently observed in studies carried out in this laboratory (14, 8 and the present study) is in sharp contrast to the relatively high content of this lipid found (2) in the patients' disc-shaped HDL. However, the number of molecules of UC that we have found in patient small spherical HDL is close to that predicted from the equation of Shen et al. (29) for normal circulating HDL of comparable size. As these authors point out, small lipoproteins isolated from an equilibrium mixture will be deficient in UC relative to larger lipoproteins. This is a direct consequence of the higher surface tension and thus free energy for UC on a highly curved surface.

There is clearly very little room for UC in the particle core because the core volume calculated from the equation of Shen et al. (29) is only large enough to accommodate the CE and TG that are present. The core of CE and TG presumably acts as a determinant of particle shape. This would explain why the 6-nm diameter HDL are spherical, while other HDL recovered from the same plasma are disc-shaped.

Another goal of the present investigation was to study the effect of the LCAT reaction on the structure of the small HDL. In particular, we wanted to examine the possibility that incubation of the small spherical HDL with LCAT and a source of UC might lead to the formation of larger HDL particles. The results of our experiments clearly support this conclusion. (See, for example, Fig. 4). In addition, it is evident that the increase in particle size is related to an increase in content of total cholesterol. Thus, substantial amounts of CE accumulated under the conditions of our experiments, where LCAT and a source of UC were present, but CE transfer protein was absent.

The ability of the small spherical HDL to accumulate large amounts of CE contrasts sharply with the much more limited ability of artificially prepared vesicles containing apoA-I, PL, and UC to accumulate CE under similar circumstances (32, 33). The basis for this difference

between patient small spherical HDL and artificially prepared vesicles remains to be fully explained, but may well depend on the ability of the small spherical HDL to form a core, as in the case of native (5, 9, 34–36) and artificially prepared (33) discoidal HDL.

Our incubation experiments also support the conclusion that action of LCAT on the small spherical HDL can lead to the formation of product HDL of discrete sizes, as opposed to a population of product HDL particles of gradually increasing size. This conclusion depends heavily on the computer-assisted peak resolution procedure used to analyze the gradient gels, and is therefore subject to the limitations of this procedure (see Appendix). Nevertheless, the fact that similar-sized particles were detected at different time points within the same experiment (see Fig. 3) and also in different experiments can be taken as a strong indication of the procedure's general validity.

It is of interest that Nichols et al. (37) observed the formation of discoidal protein-lipid complexes of discrete sizes upon subjecting apoA-I and egg yolk PC to detergent dialysis. Both their results and ours suggest that apoA-I can in some way act as a determinant of HDL particle size.

It is of interest also that the discrete HDL products formed in our experiments were 50% to 120% larger in volume than the substrate HDL, though the contribution of CE to this increment in volume was relatively small in some experiments. In addition, both HDL products isolated by rate zonal ultracentrifugation in our experiments appeared to contain three molecules of apoA-I per particle, as opposed to the two molecules of apoA-I found in the substrate HDL. Because the only apoA-I present in our experiments was that provided by the substrate HDL, some type of particle rearrangement reaction must have been involved. One possibility is that action of LCAT on the substrate HDL led to lipoprotein particle fusion. However, more than a simple fusion reaction must have occurred. Such a reaction would have caused the formation of product HDL about 7.5 nm in diameter, which contained not three, but four molecules of apoA-I.

The idea that HDL can participate in fusion events is not new. Similar mechanisms have been invoked by other investigators (38, 39) to account for results obtained in in vitro experiments with HDL. However, the possibility that LCAT can *promote* fusion events has not been considered until now. It might promote fusion by generating lysolecithin. This lipid has been shown to induce fusion of erythrocytes (40), though its potential significance as a fusigen for HDL is questionable in view of more recent studies done with lecithin-containing vesicles (41, 42). In any event, much more work will be required to establish LCAT-induced HDL fusion as a potentially important mechanism. Fortunately, 6-nm diameter HDL that resemble those isolated from patient plasma can now be prepared artificially, and it has already been shown that incubation of these HDL in the presence of LCAT and erythrocyte membranes leads to the formation of discrete product HDL 7.2 nm in diameter (43). Further experiments with these readily accessible artificial HDL should help clarify the LCAT-dependent mechanisms that led to the increase in HDL size observed in the present experiments.

A final conclusion supported by our experiments is that the LCAT reaction can lead to formation of product HDL that are deficient in surface lipid. This is particularly so in the case of product HDL 7.35 nm in diameter. It is noteworthy that the calculated surface thickness of the 7.35-nm product is only 1.25 nm. How these lipoproteins are able to remain as discrete particles in an aqueous environment is a question of interest. The product HDL contain no albumin and only a very small amount of lysoPC (See Table 4). Therefore, the possibility that apoA-I may have unfolded to cover much of the particle surface must be considered. The thickness attributable to an amphipathic helix would be expected to be about 1.2 nm and the thickness attributable to an extended chain would be much lower.

One might expect product HDL, deficient in surface lipid, to be highly reactive. They might fuse with one another, as suggested above, or under other circumstances interact with amphipathic plasma proteins. Alternatively, they might interact with other lipoproteins or with cells. It would be of special interest to know how they interact with lipid transfer proteins because net transfer reactions involving either CE or PL might tend to normalize the structure of the product HDL particles produced by the action of LCAT alone. In an unpublished experiment related to this question we incubated the small spherical HDL of patient M.R. with LCAT, UC-TRP, and patient plasma proteins of $d > 1.25$ g/ml.[2] CE transfer activity was clearly present in this experiment because about two-thirds of the CE formed by the LCAT reaction transferred to the UC-TRP. Nonetheless, product HDL of discrete sizes were observed on gradient gels. Product HDL about 7.5 nm in diameter were prominent after 1 hr of incubation, whereas major HDL products 7.8 nm in diameter and 8.6 nm in diameter were observed subsequently. Furthermore, in another experiment (10) we incubated the plasma proteins of $d > 1.063$ g/ml of patient D.J. with LCAT and UC-TRP and again observed that the LCAT reaction led to the formation of discrete HDL 7.5 nm in diameter. These results suggest that events similar to those observed in the present experiments can also occur in more complicated systems.

[2] Applegate, K. R., C. D. Mitchell, W. C. King, and J. A. Glomset. Unpublished results.

The relation between the substrate and product HDL studied in our experiments and normal plasma HDL is still unclear. It seems reasonable to postulate that the small spherical HDL closely resemble or are identical to a form of nascent HDL that circulates transiently in the blood of normal individuals. Furthermore, there are several reasons for believing that they might be of intestinal origin. Thus, the present studies show that patient small spherical HDL contain both CE and TG, which would be compatible with this possibility. A previous study (11) showed that patient small spherical HDL decrease in concentration when the patients consume fat-free diets. Forester et al. (12) have isolated similar particles from rat mesenteric lymph. The possibility must nonetheless be considered that both the substrate and product HDL studied in the present experiments may have been artificially influenced by the prolonged ultracentrifugation procedures employed in their isolation. This possibility cannot be totally discounted even though *a*) the properties of the substrate HDL seem consistent with those of patient small spherical HDL isolated earlier (14) by gel filtration without prior ultracentrifugation at d 1.25 g/ml; *b*) we reproducibly obtained preparations of both substrate and product HDL; and *c*) the product HDL were similar in size to product HDL analyzed by gradient gel electrophoresis without prior ultracentrifugation.[3]

If patient small spherical HDL actually are normal nascent HDL, one must still question the relation between the product HDL formed in the present experiments and the HDL "products" that are ordinarily found in plasma. As mentioned above, our incubation mixtures did not include lipid transfer proteins and other potentially important plasma components that might have influenced the composition and properties of the final lipoprotein particles produced. Furthermore, the product HDL isolated in the present experiments contain only apoA-I, whereas, most normal HDL contain both apoA-I and apoA-II. Nevertheless, normal HDL have been reported (44) to include particles that contain apoA-I but no apoA-II, and evidence has recently been obtained[4] that this type of particle is present in both normal HDL_2 and HDL_3. Thus, it is conceivable that small spherical HDL similar to those described in this investigation appear transiently in normal plasma, react with LCAT, fuse, and become converted to CE-containing, HDL_3-sized particles. This possibility clearly deserves to be investigated further in view of the paucity of available information concerning the mechanisms that form HDL_3-sized particles in normal humans.

[3] Applegate, K., and W. C. King. Unpublished results.
[4] Cheung, M. C., and J. J. Albers. Personal communication.

APPENDIX

Analysis of gradient gel scans

All processing and deconvolution of gradient gel data was done using the PROPHET computer system (27) and various programs available on the system or written by one of us (K. A.).

Public procedure DIGITIZE (45) was used to enter gradient gel data for samples and standards into tables of R_f values versus optical density.

Calibration curves

The two calibration tracks of any given gel were found to be nearly identical. However, R_f values showed a greater variation from gel to gel. In addition, plots of log (diameter) versus R_f for the calibration proteins were slightly sigmoidal, rather than linear. We therefore created a separate calibration curve for each gel, averaging the two sets of protein R_f values. Rather than attempting to fit some analytical function to these small data sets by least squares, we elected to run a cubic spline interpolating curve (46) through the experimental points using public procedure INTERPOLATE (47). Because of the smooth nature and moderate curvature of the data, such a procedure should yield a valid empirical calibration curve.

Curve deconvolution

In general, the gradient gel scans showed multiple maxima and inflection points, and the positions of these features seemed to be invariant from sample to sample, although the relative heights varied. This implied that the samples might contain several physically distinct subpopulations of HDL particles, and that deconvolution of the gel scans could yield useful data on mean particle sizes, size ranges, and relative proportions of each HDL subpopulation.

Although a theory of gradient gel electrophoresis has been developed (48), its application to Pharmacia gradient gels is limited, since detailed information on the gradient and pore size distribution is not provided by the manufacturer. Furthermore, even for simple linear gradients, the predicted shape of a peak is a complex function of particle heterogeneity, diffusion coefficients, and pore size gradient. Therefore, we used an empirical approach in which we transformed the data from the original R_f scale to a linear scale in particle diameter, using the experimental calibration curves, and then resolved each curve into a set of Gaussian components.

Several preliminary tests indicated that this was a reasonable approach. First, least squares fitting of a set of Gaussians to several gel scans in the optical density versus diameter representation gave much lower sums of squares than comparable fits of the original data. Second, curve resolution of transformed data using skewed peaks of the type described by Gladney, Powden, and Swalen (49), Grushka (50), and Anderson, Gibb, and Littlewood (51), produced poorer fits than a set of Gaussians, and parameters with less statistical significance. Third, particle size distributions in our samples, whether expressed in terms of diameter D, D^2, or D^3, were sufficiently narrow to be statistically indistinguishable from Gaussian distributions. Thus, as a matter of convenience for calculation and presentation of results, we elected to use the diameter as our particle size parameter, even though formulations employing D^2 or D^3 might more accurately reflect the interaction of a particle with the pores in a gradient gel.

Curve fitting was carried out using a modelling program, MKMODEL (52, 53), which in turn involves a non-linear least squares program called MLAB. The modelling function was a sum of Gaussians defined by height, standard deviation (width), and position parameters. The number of components was usually chosen to agree with the number of peaks and shoulders visible on the curve, as this generally gave the best fit. If extra components were added, the program tended to superimpose them on the original peaks, whereas if a component was omitted, the program would converge slowly to a poor fit, or not converge at all.

The program generally returned estimates of standard error in the fitted height and width parameters of 2–5% for major components and up to 10% for minor components. Standard error estimates for

peak positions were <1% and frequently 0.2–0.3%. Deviations of experimental points on the gel scan from the fitted curve envelope were on the order of 0.05% of the maximum curve height, which is approximately the noise level in the original scans.

Peak areas

It was not possible to express the amount of each resolved component in terms of particle concentration in the absence of detailed knowledge concerning the dye binding characteristics of each component particle. We therefore expressed the amounts of the HDL subspecies in terms of relative areas proportional to stain intensity on the original gels. This was accomplished by numerical integration of the quotient of each Gaussian peak function G(D) and the slope of the calibration curve over the appropriate range of diameters D: thus, peak area in units of optical density $\times R_f = \int [G(D)/(dD/dR_f)]dD$.

Error estimates for peak areas were computed using the standard errors of the individual Gaussian parameters combined to produce maximum deviations. The resultant standard errors were on the order of 5% for major components, but ranged up to 20% for some minor components.

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