Lecithin:cholesterol acyltransferase-induced transformation of HepG2 lipoproteins

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Abstract Previous studies with the human hepatoblastoma-derived HepG2 cell line in this laboratory have shown that these cells produce high density lipoproteins (HDL) that are similar to HDL isolated from patients with familial lecithin:cholesterol acyltransferase (LCAT) deficiency. Experiments were, therefore, performed to determine whether HepG2 HDL could be transformed into plasma-like particles by incubation with LCAT. Concentrated HepG2 lipoproteins ($d < 1.235$ g/ml) were incubated with purified LCAT or lipoprotein-deficient plasma (LPDP) for 4, 12, or 24 h at 37°C. HDL isolated from control samples possessed excess phospholipid and unesterified cholesterol relative to plasma HDL and appeared as a mixed population of small spherical (7.8 ± 1.3 nm) and larger discoidal particles (17.7 ± 4.9 nm long axis) by electron microscopy. Nondenaturing gradient gel analysis (GGE) of control HDL showed major peaks banding at 7.4, 10.0, 11.1, 12.2, and 14.7 nm. Following 4-h LCAT and 12-h LPDP incubations, HepG2 HDL were mostly spherical by electron microscopy and showed major peaks at 10.1 and 8.1 nm (LCAT) and 10.0 and 8.4 nm (LPDP) by GGE; the particle size distribution was similar to that of plasma HDL. In addition, the chemical composition of HepG2 HDL at these incubation times approximated that of plasma HDL. Molar increases in HDL cholesteryl ester were accompanied by equimolar decreases in phospholipid and unesterified cholesterol. HepG2 low density lipoproteins (LDL) isolated from control samples showed a prominent protein band at 25.6 nm with GGE. Active LPDP or LCAT incubations resulted in the appearance of additional protein bands at 24.6 and 24.1 nm. No morphological changes were observed with electron microscopy. Chemical analysis indicated that the LDL cholesteryl ester formed was insufficient to account for phospholipid lost, suggesting that LCAT phospholipase activity occurred without concomitant cholesterol esterification. LCAT alone appears sufficient to introduce heterogeneity into the HepG2 LDL particle size distribution and to transform HepG2 HDL into a plasma-like subclass distribution. —**McCall, M. R., A. V. Nichols, P. J. Blanche, V. G. Shore, and T. M. Forte.** Lecithin:cholesterol acyltransferase-induced transformation of HepG2 lipoproteins. *J. Lipid Res.* 1989. **30:** 1579–1589.

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The intravascular metabolism of high density lipoproteins (HDL) is a dynamic process involving a variety of enzymatic and exchange reactions. HDL isolated from plasma are most likely distinct from their cellular precursors (hepatic or intestinal), having been partially or completely remodeled in the extracellular milieu. Recent work with the human hepatoblastoma-derived cell line, HepG2, has indicated that these cells may be a useful model for studying HDL that have not been exposed to intravascular processing (1,2). Although some chromosomal abnormalities have been described (3), the HepG2 cell line has retained well-differentiated function, expressing many traits attributed to normal human hepatocytes. In addition to lipoproteins and lipid-poor apolipoprotein (2), these cells release into the culture media many of the major plasma proteins (4), and also express the receptors for low density lipoproteins (LDL) (5–7), estrogen (8), insulin, and transferrin (9).

Previous studies with the HepG2 cell line in this laboratory have shown that the "HDL" (d 1.063–1.235 g/ml) harvested from culture medium are remarkably similar to the HDL isolated from patients with familial lecithin:cholesterol acyltransferase (LCAT) deficiency (2,10). These "HDL" are enriched in surface lipids (phospholipid and unesterified cholesterol) relative to their plasma counterparts, and appear as a mixed population of small, spherical and larger, discoidal particles when examined with the electron microscope. The present study was undertaken to determine whether HepG2 "HDL" could be remodeled into mature plasma-like particles by incubation with a source of LCAT. In these studies, the $d < 1.235$ g/ml

Abbreviations: LDL, low density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; LPDP, lipoprotein-deficient plasma; HSA, human serum albumin; GGE, gradient gel electrophoresis; PHMPS, *p*-hydroxymercuriphenylsulfonic acid; SRID, single radial immunodiffusion.

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lipoprotein fraction isolated from HepG2 culture medium was used as substrate, and purified LCAT and lipoprotein-deficient plasma (LPDP) were used as sources of LCAT. It was anticipated that LPDP, presumably containing cholesteryl ester transfer protein (11) and conversion factor (12), might permit more complete remodeling of the lipoproteins. Our data indicate that both LPDP and LCAT can transform HepG2 "HDL" into a plasma-like HDL subclass distribution.

METHODS

Cell culture

HepG2 cells were grown and subcultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum as previously described (10). Cells were routinely grown in 175-cm^2 culture flasks and split 1:4 every 7–8 days. Prior to collection of media from which lipoproteins were to be isolated, confluent cell sheets were rinsed three times with Hank's Balanced Salt solution to remove residual fetal bovine serum. Cell sheets were then incubated for 24 h with 20 ml unsupplemented MEM. The serum-free media harvested after this incubation is referred to as "conditioned medium" and is the material from which HepG2 lipoproteins were isolated. Conditioned medium was centrifuged at 1000 *g* (30 min, 4°C) to remove detached cells and debris; gentamicin sulfate (0.1 mg/ml) and EDTA (1 mg/ml of the dipotassium salt) were added to prevent microbial and oxidative damage.

Lipoprotein isolation

The lipoproteins that accumulated in the 24-h conditioned medium were isolated by a single ultracentrifugation step. Conditioned medium was concentrated by ultrafiltration (approximately 150-fold), dialyzed, and adjusted to a density of 1.25 g/ml with NaBr. Following ultracentrifugation (40,000 rpm, 48 h, 4°C, using a Beckman 50.3 Ti rotor), the lipoprotein fraction ($d < 1.235$ g/ml) was pipetted from the top of the centrifuge tube and dialyzed against buffer I (10 mM Tris, 150 mM NaCl, 0.27 mM EDTA, pH 7.4).

Upon termination of the incubations, HepG2 "LDL" ($d < 1.063$ g/ml) and "HDL" (d 1.063–1.235 g/ml) were isolated by sequential ultracentrifugation (13). Ultracentrifugation (Beckman 50.3 ti rotor, 40,000 rpm, 4°C) times were 36 and 48 h for the "LDL" and "HDL" fractions, respectively.

Preparation of lipoprotein-deficient plasma (LPDP)

Lipoproteins were removed from the plasma of a normolipidemic adult man by ultracentrifugation. Plasma was adjusted to a density of 1.215 g/ml with NaBr and ultracentrifuged at 40,000 rpm for 48 h at 4°C in a Beckman 50.3 Ti rotor. The lipoproteins were removed from the top of the tube by aspiration and the infranatant was collected and subsequently dialyzed against buffer II (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.05% NaN_3, pH 7.4). The dialyzed lipoprotein-deficient plasma was then passed through an anti-apolipoprotein (apo) A-I immunoaffinity column (14). The unbound column fractions were pooled, reconcentrated back to the volume of the original plasma sample, and then dialyzed against buffer I.

Preparation of purified lecithin:cholesterol acyltransferase (LCAT)

LCAT was isolated from human serum essentially as described by Chen and Albers (15). Preparations of the purified enzyme from our laboratory have been measured within the expected specific activity range (14,670 ± 860 nmol cholesteryl ester formed per h per mg) and the amino acid composition is similar to published information (15–17). SDS-PAGE analysis shows the purified enzyme as a single protein band with an estimated molecular weight of 63,000. Purified LCAT prepared for this study (stored under nitrogen at 4°C in 10 mM Tris, 1 mM EDTA, pH 7.4) was used within 2 weeks after preparation, during which time no loss of enzyme activity was observed.

Measurement of LCAT activity

The activities of LCAT in LPDP and the purified LCAT preparation were assayed using the proteoliposome method (18).

Incubation mixtures and conditions

Concentrated HepG2 ($d < 1.235$ g/ml) lipoproteins (0.27 μmol unesterified cholesterol and 0.56 mg protein per incubation) were incubated at 37°C with LPDP or purified LCAT and 5 mM mercaptoethanol (final volume 1.8 ml) for 4, 12, and 24 h. All incubation components (except mercaptoethanol) were sterile-filtered prior to use, and all incubations were performed in the dark under nitrogen. LCAT activities were estimated to be similar for the three sets of incubations (46 nmol cholesteryl ester formed per h per incubation). Incubations with purified LCAT contained 1.5% fatty acid-free human serum albumin (HSA). Incubations were terminated by the addition of the LCAT inhibitor *p*-hydroxymercuriphenylsulfonic acid (PHMPS, final concentration 3.5 mM).

Lipoproteins used for a given set of LPDP and LCAT incubations were isolated from the same batch of conditioned medium. Three different batches from three different cell passages were obtained; 1120 ml of conditioned media per batch (equivalent to 56 175-cm^2 culture flasks) were required for one set of LPDP and LCAT incubations.

On one occasion incubations were performed in which the isolated "LDL" ($d < 1.063$ g/ml) fraction was incubated with (37°C, 12 h) and without (4°C with added LCAT and

PHMPS) active LCAT (69 nmol cholesteryl ester formed per h per incubation). Incubations included 1.5% HSA and the active incubation was terminated with the addition of PHMPS (final concentration 3.5 mM). Each incubation contained 0.5 mg of "LDL" protein and 0.13 μmol unesterified cholesterol (isolated from approximately 500 ml conditioned media) in a 1.9-ml volume. All of the "LDL" for the experiment was obtained from the same batch of conditioned media and control incubations were maintained at 4°C with 3.5 mM PHMPS and either LPDP or purified LCAT and HSA.

Lipoprotein analyses

Lipoprotein morphology and particle size distribution were assessed by electron microscopy (19) and nondenaturing gradient gel electrophoresis, respectively (20,21). Quantitative immunoassay of apoA-I was carried out as previously described (2) and albumin was quantitated by single radial immunodiffusion (SRID) on plates purchased from Tago, Inc. (Burlingame, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (22) was used to estimate the relative amounts of apoA-I and apoE in HDL isolated from incubation mixtures. The distribution of apoA-I and apoE following gradient gel electrophoresis (GGE) of HepG2 "HDL" was determined by Western blotting (2). Lipoprotein chemical composition was determined by standard methods as previously described (2). The following estimated molecular weights were used to convert mass data to molar data: apoA-I, 28,100; cholesterol, 387; cholesteryl ester, 658; and phospholipid, 750.

Human HDL analysis

HDL used for reference gradient gel particle size distributions were obtained from two adult women, one normolipidemic and one with familial LCAT deficiency. The HDL (d 1.063–1.210 g/ml) was isolated from plasma by standard ultracentrifugation methods (13).

RESULTS

HepG2 d < 1.235 g/ml lipoproteins

The lipoproteins that accumulate in the 24-h conditioned medium of HepG2 cells have the characteristic particle size distribution seen in **Fig. 1** when isolated and electrophoresed on 4–30% nondenaturing gradient gels. The major HepG2 lipoprotein particle size classes observed with this electrophoretic method were previously isolated and characterized by our laboratory (2,10). HepG2 "LDL" isolated at d < 1.063 g/ml correspond to the first peak (far left) observed on the scan. We have demonstrated that HepG2 "LDL" appear as a relatively uniform population of spherical particles

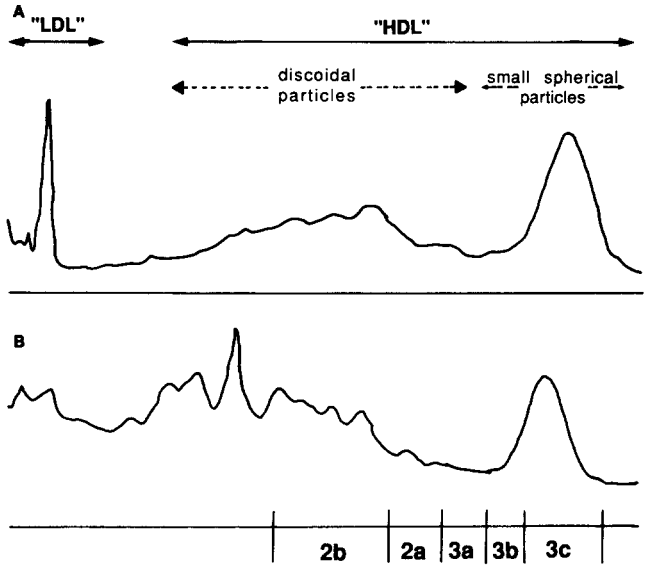

Fig. 1. Densitometric scans of Coomassie blue G250-stained 4–30% nondenaturing polyacrylamide gradient gels of HepG2 d < 1.235 g/ml lipoproteins (panel A) and HDL (panel B) isolated from the plasma of a patient with familial LCAT deficiency. The regions marked "discoidal particles" and "small, spherical particles" in panel A also refer to the same regions in panel B (26, 31). For comparative purposes the major human plasma HDL subpopulation size intervals assessed by GGE (20,21) have been indicated in panel B. The intervals correspond to standard globular proteins with the following Stokes' diameters: $(HDL_{2b})_{GGE}$, 12.9–9.7 nm; $(HDL_{2a})_{GGE}$, 9.7–8.8 nm; $(HDL_{3a})_{GGE}$, 8.8–8.2 nm; $(HDL_{3b})_{GGE}$, 8.2–7.8 nm; and $(HDL_{3c})_{GGE}$, 7.8–7.2 nm.

when negatively stained and examined with the electron microscope. The particles collectively termed "HDL" in Fig. 1 are composed of small, spherical particles (7.5 nm in diameter as assessed by electron microscopy) and of larger, discoidal particles ranging in size (long axis) from approximately 10 nm to 21 nm (by electron microscopy) (2). "LDL" and "HDL" are rich in phospholipid and unesterified cholesterol and poor in cholesteryl ester when compared with their plasma counterparts. Typically, HepG2 "LDL" comprise less than 35% of the total HepG2 lipoprotein mass.

A scan of a gel electrophoresed with HDL isolated from a patient with familial LCAT deficiency has been included in Fig. 1 (panel B) for comparative purposes. The general features of the two scans are similar; both HDL fractions contain spherical $(HDL_{3c})_{GGE}$-sized particles and a variety of larger-sized discoidal lipoprotein complexes. The major difference lies in the fact that familial LCAT-deficient HDL possess larger discoidal complexes than HepG2 HDL. Our data suggest that HepG2 lipoproteins are potentially excellent substrates for LCAT.

Particle size distribution of HepG2 "HDL" following incubation

Densitometric scans of Coomassie blue G250-stained 4–30% gradient gels of HepG2 "HDL" (d 1.063–1.235

g/ml) isolated from control, 4-, 12-, and 24-h incubation mixtures are shown in **Fig. 2**, along with a scan of human plasma HDL from a normal lipidemic subject. The particle size distribution obtained for control incubations (LCAT, left; LPDP, right) agrees well with the distribution shown for unincubated "HDL" in Fig. 1. Progressive changes in the particle size distribution are observed with time of incubation with LPDP and LCAT. Although qualitatively similar, the mass distribution differs depending upon the LCAT source. At 4 h, major shifts have already occurred in the purified LCAT incubation; a prominent peak is observed at the $(HDL_{3a})_{GGE}$ and $(HDL_{3b})_{GGE}$ boundary (8.1 nm), a lesser peak at 10.1 nm (HDL_{2b}), and a slight shoulder at 7.6 nm (HDL_{3c}) are also apparent. The most striking change in the 4-h LPDP incubation is a shift of the small spherical particles (7.4 nm) to the larger pore regions of the gel ($(HDL_{3b})_{GGE}$ and larger). It is not until the 12-h incubation with LPDP that the "HDL" particle size distribution begins to differ dramatically from that observed for the control incubation. The 12-h LPDP distribution is characterized by a major peak in the $(HDL_{3a})_{GGE}$ region and a shoulder in the $(HDL_{2b})_{GGE}$ region. Changes in the HDL particle size distribution subsequent to the 4-h LCAT incubation and the 12-h LPDP incubation were relatively small. It is of interest to note that particle size distributions for HDL isolated from the 4-h LCAT incubation and the 12-h LPDP incubation approximate the particle size distribution for plasma HDL (bottom panel). In both instances, a major peak is observed around the $(HDL_{3a})_{GGE}$ region and a smaller peak or shoulder is observed in the $(HDL_{2b})_{GGE}$ region.

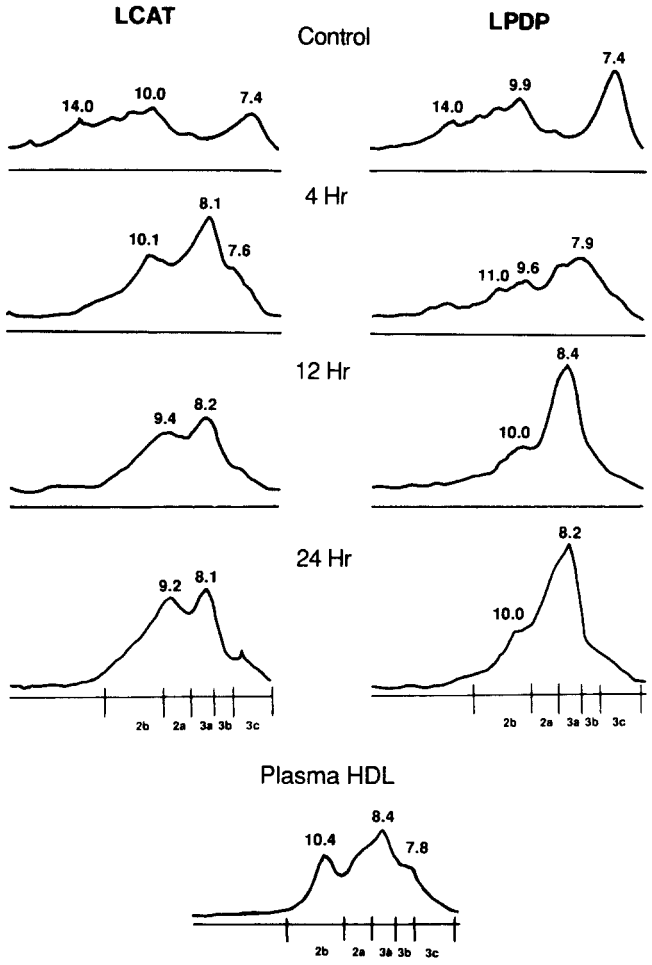

Fig. 2. Densitometric scans of Coomassie blue G250-stained 4–30% nondenaturing polyacrylamide gradient gels of HepG2 "HDL" (d 1.063–1.235 g/ml) isolated from incubation mixtures. Incubations contained HepG2 d < 1.235 g/ml lipoproteins isolated from the same batch of conditioned medium, 5 mM mercaptoethanol, and either LPDP or purified LCAT plus HSA. Incubations were carried out for 4, 12, and 24 h at 37°C and were terminated by the addition of PHMPS. Control incubations were maintained at 4°C with PHMPS and an LCAT source. The initial LCAT activity for the LPDP and LCAT incubations was similar. Panels on the left-hand side show the LCAT incubation series while the LPDP incubation series is on the right. Estimated Stoke's diameters (nm) are shown above appropriate peaks. The major human plasma HDL subpopulation size intervals assessed by GGE have also been included in the figure. The profile on the bottom is a selected example of normal human HDL and is similar to normal profiles reported by Blanche et al. (20).

Morphology of HepG2 "HDL" isolated from incubation mixtures

Electron micrographs of negatively stained HepG2 "HDL" isolated from control and 12-h LCAT incubations are shown in **Fig. 3**. Similar results were obtained for the corresponding LPDP incubations. Control "HDL" appear as a mixed population of small spherical (7.8 ± 1.3 nm in diameter) and larger discoidal particles (17.7 ± 4.9 nm long axis). These data agree quite well with our previously reported data on unincubated HepG2 "HDL" (2). Following 12 h of incubation, essentially all the discoidal particles have been transformed; a relatively uniform population of spherical particles that are 8.8 ± 1.6 nm in diameter can be seen (Fig. 3B). Although not shown, a small number of discoidal particles are still evident at 4 h. The majority of particles, however, are spherical.

Chemical composition of HepG2 "HDL" isolated from incubation mixtures

The composition data presented in **Table 1** represent the mean ± SD of three incubations, each incubation representing lipoproteins isolated from the conditioned medium of a different cell passage. Triglyceride determinations were not performed due to the relatively small amounts of sample available and the low triglyceride concentrations found in HepG2 "HDL". The composition of plasma HDL is shown for comparative purposes. The composition of "HDL" isolated from the control incubations agrees quite well with data previously reported (2) for similarly isolated unincubated HepG2 "HDL". Increases in cholesteryl ester and decreases in phospholipid and unesterified cholesterol were associated with the ac-

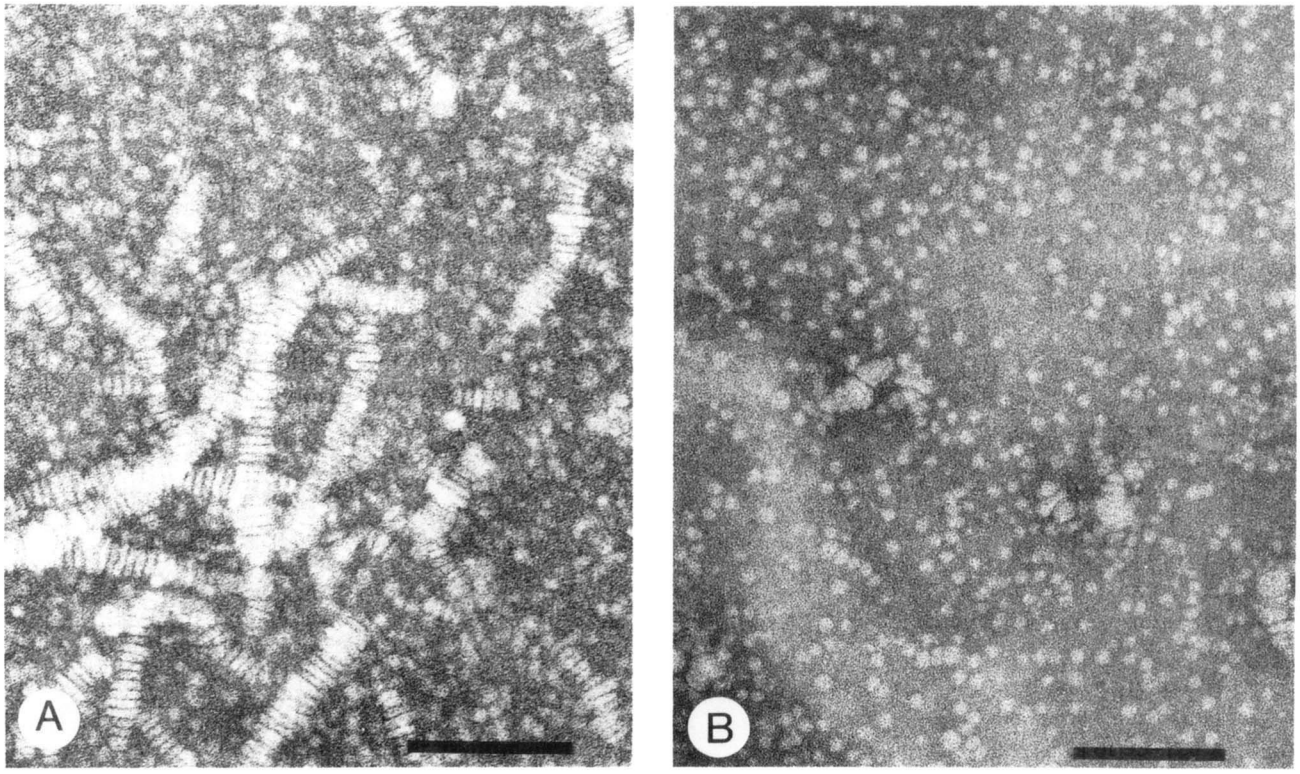

Fig. 3. Electron micrographs of negatively stained HepG2 "HDL" (d 1.063–1.235 g/ml): panel A, LCAT control; panel B, LCAT 12-h incubation. The bar marker in the lower right-hand corner of each micrograph indicates 100 nm. By 12 h there was a complete absence of the discoidal particles and only round profiles were present. Similar results were obtained for LPDP (data not shown).

tive LCAT preparations. These compositional changes, reflecting alterations in the ratio of core to surface lipid, are consistent with the disc-to-sphere transformations observed with electron microscopy. Although the trends observed for the LPDP and purified LCAT incubations were similar, there were quantitative differences. The molar ratio of phospholipid to cholesteryl ester was consistently higher in the LPDP incubations (Table 1), suggesting lower rates of cholesterol esterification.

In both the LCAT and LPDP incubations the percent "HDL" protein tended to increase with time of incubation. This trend most likely reflects loss of LCAT-gener-

TABLE 1. Chemical composition of HepG2 "HDL" isolated from incubation mixtures[a] containing LPDP or purified LCAT

LCAT Source	Incubation	Composition (% by weight)				Molar Ratio PL/CE
		Protein[b]	UC[c]	CE	PL	
	h					
LPDP	Control	42.3 ± (3.4)	12.2 ± (3.5)	5.5 ± (1.6)	40.0 ± (1.6)	6.3
LPDP	4	46.1 ± (2.1)	8.1 ± (0.6)	11.4 ± (1.3)	34.4 ± (1.1)	2.7
LPDP	12	46.7 ± (1.3)	6.2 ± (0.6)	16.2 ± (0.8)	30.6 ± (0.5)	1.7
LPDP	24	48.2 ± (2.1)	5.1 ± (0.3)	18.1 ± (1.1)	28.6 ± (0.8)	1.4
LCAT	Control	41.5 ± (1.1)	12.6 ± (0.8)	5.0 ± (1.8)	40.9 ± (1.5)	6.8
LCAT	4	49.1 ± (1.9)	4.2 ± (0.6)	20.3 ± (0.7)	26.4 ± (1.1)	1.2
LCAT	12	52.7 ± (1.4)	2.1 ± (0.5)	24.5 ± (0.4)	20.7 ± (0.9)	0.7
LCAT	24	51.4 ± (2.0)	1.6 ± (0.4)	26.9 ± (0.8)	20.1 ± (1.7)	0.6
Plasma HDL (32)		45–55	3–5	15–20	26–32	

Each value represents the average of three experiments ± standard deviation. Each experiment (set of incubations with LPDP and LCAT) used lipoproteins from a different cell passage. Triglyceride was not determined.

[a]Incubation mixtures contained HepG2 d <1.235 g/ml lipoproteins, a source of LCAT (purified LCAT incubations contained 1.5% HSA), and 5 mM β-mercaptoethanol.

[b]Protein values represent total protein (measured by a modification of the Lowry method (23)) minus albumin (measured by SRID).

[c]UC, unesterified cholesterol; CE, cholesteryl ester; PL, phospholipid.

ated lysolecithin from the HDL fraction. The concentration of the major apolipoprotein associated with HepG2 "HDL", apoA-I, was not significantly altered following incubation with active LCAT or LPDP. The control LPDP incubations contained 0.25 ± 0.10 mg/ml apoA-I while the 4-, 12-, and 24-h incubations contained 0.26 ± 0.08 mg/ml, 0.33 ± 0.06 mg/ml, and 0.30 ± 0.10 mg/ml apoA-I, respectively. Essentially identical results were obtained for the control and active LCAT incubations. Densitometric scans of 4–20% SDS-PAGE gels electrophoresed with similar amounts (5–6 μg) of "HDL" protein [HDL protein (measured by a modification of the Lowry procedure (23)) minus albumin (measured by SRID)] were in agreement with the quantitative apoA-I data; i.e., the staining intensity of the control apoA-I band was not altered following LPDP or LCAT incubations (data not shown).

Molar amounts of lipid found in control and active LCAT incubations are shown in **Table 2**. The numbers in bold print are the differences between control values and the 4-, 12-, and 24-h incubation values. In both the LCAT and LPDP incubation series, molar increases in cholesteryl ester were paralleled by equimolar decreases in phospholipid and unesterified cholesterol. Total "HDL" cholesterol remained constant. It appears, therefore, that all the "HDL" substrate used by the LCAT reaction can be accounted for by "HDL" cholesteryl ester formed. These data suggest that the LCAT-induced changes in "HDL" occurred without any lipid transfer from "LDL".

ApoA-I and ApoE distribution in HepG2 "HDL" isolated from incubation mixtures

Incubation of HepG2 lipoproteins with LCAT resulted in a redistribution of apoA-I and apoE-containing "HDL" particles. ApoA-I is the major protein associated with HepG2 "HDL" and, as shown in **Fig. 4** (panel A), appears with most, if not all, of the major "HDL" subpopulations. The LCAT-induced changes in the apoA-I distribution correspond with the observed changes for total protein as deter-

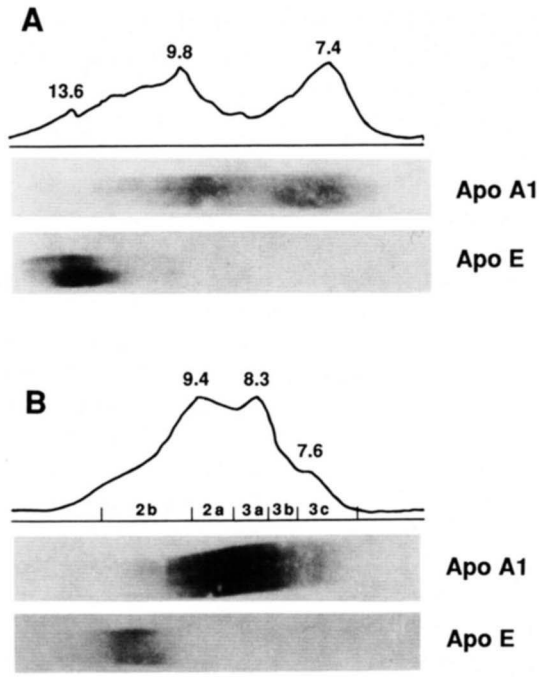

Fig. 4. Western blot particle size distribution of apoA-I and apoE in HepG2 "HDL" isolated from control (panel A) and 12-h LCAT (panel B) incubations. Samples were electrophoresed in 4–30% nondenaturing polyacrylamide gels, transferred to nitrocellulose, and immunoblotted for apoA-I and apoE. Densitometric scans of similarly electrophoresed gels stained with Coomassie blue G250 are shown for comparison. Estimated Stokes' diameters are shown above appropriate peaks. The major human plasma HDL subpopulation size intervals are also indicated in the figure.

TABLE 2. Mole amounts[a] of lipid in HepG2 "HDL" (d 1.063–1.235 g/ml) isolated from incubation mixtures

LCAT Source	Incubation	TC[b]	UC	CE	PL
	h		*μmol*		
LPDP	Control	0.27 ± (0.01)	0.21 ± (0.01)	0.06 ± (0.01)	0.38 ± (0.12)
LPDP	4	0.27 ± (0.01)	0.15 ± (0.01)	0.12 ± (0.01)	0.32 ± (0.02)
			− 0.06	**+ 0.06**	**− 0.06**
LPDP	12	0.28 ± (0.04)	0.11 ± (0.02)	0.17 ± (0.02)	0.28 ± (0.02)
			− 0.10	**+ 0.11**	**− 0.10**
LPDP	24	0.29 ± (0.03)	0.09 ± (0.01)	0.20 ± (0.02)	0.27 ± (0.03)
			− 0.12	**+ 0.14**	**− 0.11**
LCAT	Control	0.25 ± (0.04)	0.20 ± (0.02)	0.05 ± (0.02)	0.34 ± (0.02)
LCAT	4	0.27 ± (0.03)	0.07 ± (0.02)	0.20 ± (0.02)	0.23 ± (0.02)
			− 0.14	**+ 0.15**	**− 0.11**
LCAT	12	0.26 ± (0.01)	0.03 ± (0.01)	0.23 ± (0.01)	0.17 ± (0.01)
			− 0.17	**+ 0.18**	**− 0.17**
LCAT	24	0.28 ± (0.02)	0.03 ± (0.01)	0.25 ± (0.02)	0.16 ± (0.02)
			− 0.17	**+ 0.20**	**− 0.18**

Each value represents the average of three experiments ± (standard deviation). Each experiment used lipoproteins from a different cell passage. Differences between 0 hr (Control) and each later time point are indicated in bold print.

[a]Triglyceride was not determined.

[b]TC, total cholesterol.

mined by Coomassie G250. This relationship is illustrated in Fig. 4, panel B. Note the correspondence between the densitometric scan of the protein-stained gel and the apoA-I blot. In general, the densitometric scans shown in Fig. 2 for both LCAT and LPDP incubations closely reflect the changes observed (blots not shown) in the apoA-I distribution. The apoE-containing HDL in control incubations were found in the larger pore regions of the gel. These particles appear to band at a position that corresponds to a standard globular protein 13.6 nm in diameter (Fig. 4, panel A). Following incubation with LCAT or LPDP, apoE-containing particles are smaller. The transformed apoE particles in Fig. 4 (panel B) are in the particle size range corresponding to plasma $(HDL_{2b})_{GGE}$. Additional incubation time with LCAT did not result in further shifts in the apoE distribution. Shifts in the apoE distribution resulting from incubation with LPDP were slightly less pronounced than those observed with purified LCAT. Densitometric scans of 4–20% SDS-PAGE gels electrophoresed with similar amounts of "HDL" protein revealed that the ratio of apoE to apoA-I decreased from control values following incubation with active LCAT or LPDP. The apoE to apoA-I ratios for control, 4-, 12-, and 24-h incubations with LCAT are 0.54 ± 0.04, 0.30 ± 0.06, 0.32 ± 0.04, and 0.34 ± 0.04, respectively. Similar results were obtained with the LPDP incubations except that at 4 h the apoE to apoA-I ratio was only 0.45 ± 0.06. Since the concentration of apoA-I was the same for control and active incubations, these data indicate that the concentration of apoE in the HDL fraction decreased by as much as 56% in the active LCAT incubations.

Particle size distribution HepG2 "LDL" isolated from incubation mixtures

Densitometric scans of the Coomassie blue G250-stained 2–16% gradient gels of LDL isolated from the purified LCAT incubation mixtures are shown in **Fig. 5**. The estimated particle diameter for LDL isolated from control incubations (25.6 nm) agrees well with our previously reported data for HepG2 "LDL" (2). The particle size distribution of "LDL" after incubation differed appreciably from the control. The data suggest that upon incubation with purified LCAT the predominant 25.6-nm particle observed for the control may give rise to smaller 24.6- and 24.1-nm particles. LPDP induced similar changes in the particle size distribution; however, the changes were less pronounced than those observed with purified LCAT (data not shown).

Morphology of HepG2 "LDL" isolated from incubation mixtures

"LDL" from control and 12-h LCAT incubations are seen in **Fig. 6**. It is apparent that active LCAT does not affect the morphology of particles even though GGE indicates that some modification of particle size has occurred. Similar micrographs were obtained at other incubation times with both LPDP and purified LCAT. Particle sizes estimated from micrographs for the control and 12-h LCAT incubation are 25.8 ± 2.2 nm and 25.9 ± 2.5 nm, respectively.

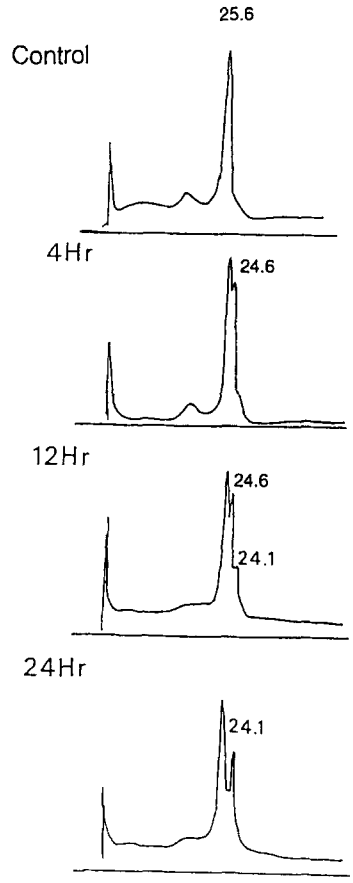

Fig. 5. Densitometric scans of Coomassie blue G250-stained 2–16% nondenaturing polyacrylamide gradient gels of HepG2 "LDL" (d < 1.063 g/ml) isolated from incubation mixtures. Details of incubations are provided in the Methods section and in Fig. 2. The control, 4-h, 12-h, and 24-h purified LCAT incubations are shown. Similar results were obtained for the LPDP incubations. Estimated Stokes' diameters in nm are shown above appropriate peaks. The small peak observed to the left of the 25.6 nm peak is in the same size region (31 nm) as the LDL dimer reported by Thrift et al. (2).

Chemical composition of HepG2 "LDL" isolated from incubation mixtures

Table 3 summarizes changes in "LDL" composition following incubation of HepG2 d < 1.235 g/ml lipoproteins with purified LCAT. The data were obtained from pooled samples representing lipoproteins collected from the three different cell passages. Incubations containing purified LCAT exhibited a trend toward increasing cholesteryl ester and decreasing phospholipid with time of incubation. Similar but smaller changes in the percentages of choles-

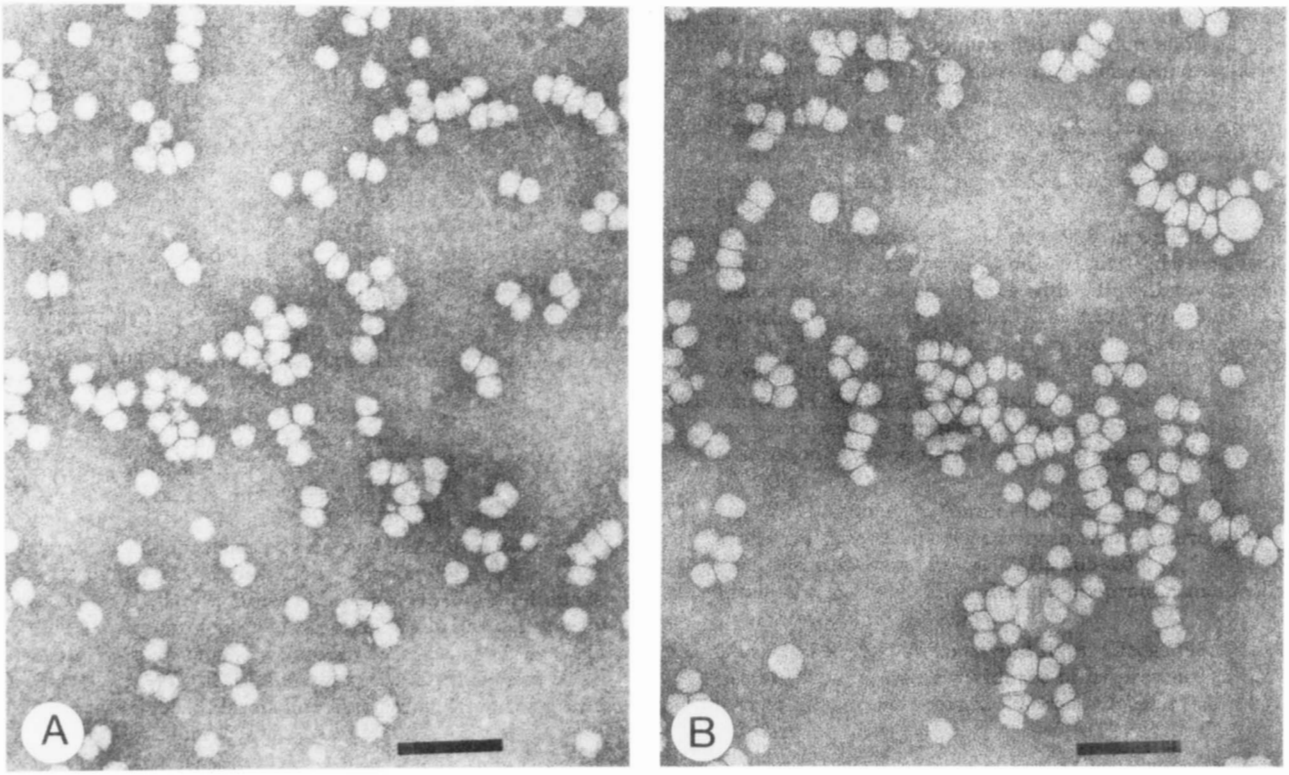

Fig. 6. Electron micrographs of negatively stained HepG2 "LDL" (d < 1.063 g/ml): panel A, LCAT control; panel B, purified LCAT 12-h incubation. The bar marker in the lower right-hand corner of each micrograph indicates 100 nm.

teryl ester, phospholipid, and unesterified cholesterol were observed for the LPDP incubations (data not shown). The amount of triglyceride recovered from incubation mixtures was at or below the detection limits of our assay; thus, the percentage composition data presented in Table 3 contain triglyceride values that are based on our previously reported HepG2 "LDL" composition (2). (See Table 3 legend for details.)

TABLE 3. Chemical composition of HepG2 "LDL" isolated from incubation mixtures containing purified LCAT[a,b,c]

	Composition (% by weight)				
Incubation	Protein	UC	CE	PL	TG[a]
h					
Control	29.4	9.0	11.6	25.0	(25.0)
4	29.8	7.8	17.4	21.8	(23.2)
12	29.7	10.1	16.5	21.4	(22.3)
24	32.4	8.5	17.6	20.3	(21.2)

[a]The amount of triglyceride (TG) recovered from incubation mixtures was insufficient for analysis. The triglyceride mass used to calculate the percentage composition data presented in this table assumes that 25% of the control "LDL" mass is triglyceride; this assumption is based on our previously reported HepG2 "LDL" composition (2). In addition, we assume that "LDL" triglyceride mass does not change with time of incubation. Data presented in Table 2 indicate that cholesteryl ester-triglyceride exchange between "HDL" and "LDL" probably did not occur in these incubations.

[b]LDL from three experiments were pooled in order to obtain sufficient lipoprotein mass on which to carry out protein, phospholipid, and cholesterol quantitation.

[c]Incubation mixtures contained HepG2 d < 1.235 g/ml lipoproteins, LCAT, and 5 mM β-mercaptoethanol.

Since our data provided evidence that the transformations induced by purified LCAT and LPDP in the "HDL" fraction were independent of the d < 1.063 g/ml lipoproteins it was hypothesized that LCAT may act directly on HepG2 "LDL" to produce the observed changes in particle size and composition. To test this hypothesis, a single experiment was performed in which HepG2 d < 1.063 g/ml lipoproteins were incubated with purified LCAT and HSA for 12 h at 37°C. The control was kept on ice with 3.4 mM PHMPS. Following incubation, the LDL was reisolated by ultracentrifugation. The gradient gel results for the active incubation (data not shown) were almost identical to those observed for the 12-h LCAT incubation with d < 1.235 g/ml lipoproteins (Fig. 5, 12 h), i.e., a smaller-sized LDL was generated. Analysis of chemical composition indicated that there was no change in triglyceride and only a 0.05 mM increase in cholesteryl ester. The increase in cholesteryl ester, however, was accompanied by a 0.16 mM decrease in phospholipid. These data suggest that LCAT phospholipase activity occurred without concomitant cholesterol esterification.

DISCUSSION

The small spherical and larger discoidal "HDL" that accumulate in the 24-h conditioned medium of HepG2 cells can be remodeled by incubation with purified LCAT or LPDP into core-containing spherical particles. The populations of particles generated by 4 h of incubation with LCAT or 12 h of incubation with LPDP are similar in their physical and chemical properties to plasma HDL. The predominant subpopulations correspond to plasma $(HDL_{3a})_{GGE}$ and $(HDL_{2b})_{GGE}$. These data indicate that LCAT alone is the major factor responsible for the transformation of HepG2 "HDL" into mature plasma-like particles.

The HDL particle size distributions obtained for the LCAT and LPDP incubations differed in relative amounts of the various HDL_{GGE} subclasses. LPDP incubations were characterized by a more skewed particle size distribution with relatively more $(HDL_{3a})_{GGE}$- and $(HDL_{3b})_{GGE}$-sized particles. A factor presumably related to these differences in particle size distribution was the lower rate of cholesteryl ester formation in the LPDP incubation series. It is not clear why lower rates of cholesterol esterification were observed as both incubation series were estimated to contain similar LCAT activity levels at the start of the experiments. It is likely that the proteoliposome assay employed was not suitable for estimating LCAT activity in the two LCAT sources. Conceivably, the 30-min proteoliposome incubation underestimated the effects of degradative enzymes and other negative effectors present in LPDP and not in the purified LCAT preparation.

Conversion factor (12) and cholesteryl ester transfer protein (CETP) (11) are two other factors presumably present in LPDP that may have influenced the particle subclass distribution. It is unclear at present how much, if any, of the observed differences between LPDP and LCAT transformed particles is related to the activity of these factors.

Gradient gel analysis of the apoA-I-containing HepG2 "HDL" following incubation with either purified LCAT or LPDP indicates that the small spherical particles tend to become larger while the largest discoidal particles tend to decrease in size. Generation of a cholesteryl ester core in an apoA-I-containing discoidal particle might be expected to produce a more compact, spherical lipoprotein. Indeed, it has been demonstrated that recombinant discoidal structures (19 nm × 4.6 nm by electron microscopy) made from apoA-I (four apoA-Is per particle) and phosphatidylcholine (dioleoylphosphatidylcholine) are transformed by LCAT and a source of additional substrate cholesterol into spherical particles 12.5 nm in diameter (by electron microscopy; also containing four apoA-Is per particle) (24). Similarly, smaller recombinant discoidal structures containing three apoA-Is per particle, egg yolk phosphatidylcholine, and unesterified cholesterol (13.5 nm × 4.4 nm by electron microscopy) are transformed by LCAT into spherical three apoA-I-containing particles 8.6 nm in diameter (25). Whether core formation in the discoidal HepG2 particles alone can account for observed changes in the gradient gel pattern is unknown. It is possible, however, that other mechanisms are also involved, e.g., apolipoprotein transfer or particle fusion.

The increase in particle diameter observed for the apoA-I-containing small, spherical particle is likely the result of a complex transformation involving both particle fusion and apolipoprotein transfer. The small, spherical HDL from the plasma of a patient with familial LCAT deficiency has been isolated by Chen and coworkers (26) and characterized. The molar composition of this LCAT-deficient plasma-derived particle (26) is almost identical to that of the 7.4-nm particle isolated from the conditioned medium of HepG2 cells by density gradient ultracentrifugation (**Table 4**). It has been demonstrated that the small particle isolated from LCAT-deficient plasma can be transformed by LCAT and a source of unesterified cholesterol and phospholipid into particles the size of $(HDL_{3b})_{GGE}$. This transformation appears to involve particle fusion and the product has been calculated to contain three apoA-I molecules per particle (26). LCAT transformation studies by Nichols et al. (25) using analogue particles containing two apoA-I molecules have directly demonstrated, by chemical crosslinking with dimethylsuberimidate, that the 8.2-nm fusion product contains three apoA-I molecules. Our data suggest that the HepG2 7.4-nm particle participates in the LCAT-induced fusion pathway just discussed.

Unmodified HepG2 apoE-containing particles are discoidal and band on 4–30% nondenaturing gradient gels in the size interval of spherical particles 12–17 nm in diameter (densitometric peak corresponds to 13.6 nm). A similar size distribution and particle morphology has been described for apoE-containing HDL isolated from the plasma of LCAT-deficient patients (27) and from African green monkey liver perfusates (28,29). In the latter cases, the addition of LCAT to the plasma (30) or perfusate (29) results in the formation of spherical, core-containing HDL particles which, when isolated, are almost

TABLE 4. Estimated composition of small, spherical "HDL" isolated from HepG2 conditioned media[a] and the plasma of a patient with familial LCAT deficiency (reference 26, subject DJ)

	Number of Component Molecules per Particle[b]				
	ApoA-I	PL	UC	CE	TG
HepG2	2	38	2	7	n.d.
Familial LCAT deficiency	2	38	3	2	2

[a]Calculated from previously published data (2, 10).
[b]Values have been rounded off to the nearest whole number; n.d., not determined.

devoid of apoE. Even though we found similar disc-to-sphere transformations by LCAT of the HepG2 apoE-containing "HDL", we observed a more modest decrease (based on SDS-PAGE) in apoE. The difference between our results and those reported for LCAT-deficient plasma and monkey liver perfusates may relate to the larger amounts of $d < 1.063$ g/ml lipoproteins found in the latter studies. It is possible that the VLDL and LDL present in these studies allowed for more complete transformation or particle remodeling than that observed in the HepG2 incubation mixture.

The incubations in our experiments were performed with the $d < 1.235$ g/ml lipoprotein fraction isolated from conditioned medium. This experimental design allowed the HepG2 apoB-containing lipoproteins that possess elevated phospholipid and unesterified cholesterol to serve as possible additional sources of substrate for the LCAT reaction. Our data provide suggestive evidence that in both incubation series (LCAT or LPDP) the LCAT reaction in the "HDL" fraction proceeded without significant interaction with the $d < 1.063$ g/ml lipoproteins. In the LPDP and LCAT incubations the molar amount of total cholesterol in the "HDL" fraction did not change. Moreover, the molar increase in cholesteryl ester for a given incubation time corresponded exactly with the molar decrease in unesterified cholesterol and phospholipid. These results for HepG2 lipoproteins are not consistent with previously published data in which African green monkey liver perfusate lipoproteins (29) or plasma lipoproteins from LCAT-deficient patients (30) were incubated with LCAT. In both instances, evidence was provided for the transfer of surface lipids from the $d < 1.063$ g/ml lipoproteins to HDL. HepG2 $d < 1.063$ g/ml lipoproteins possess LDL-like particles but no detectable very low density lipoproteins (VLDL). The "LDL" are analogous to the LDL isolated from African green monkey liver perfusates and LCAT-deficient plasma in that they are rich in triglyceride, phospholipid, and unesterified cholesterol, but poor in cholesteryl ester. It is not clear why they appear to be such poor surface-lipid donors to HDL although it may in part be due to the relatively low concentration of "LDL" in the $d < 1.235$ g/ml fraction.

Gradient gel analysis showed that both LPDP and LCAT produced changes in the particle size distribution of the $d < 1.063$ g/ml HepG2 lipoproteins. In both cases the apparent generation of particles with smaller diameter could reflect alterations in core and/or surface composition. Changes in chemical composition, however, were difficult to quantify, due to the small amounts of material available for analysis. In the single experiment in which sufficiently large amounts of "LDL" were used (incubations contained HepG2 "LDL", purified LCAT and HSA) to quantitate lipid compositional changes, it was found that LCAT can directly affect the composition of HepG2 "LDL", resulting in the production of particles of smaller diameter. Although not conclusive, the results of this experiment indicate that when HepG2 "LDL" are used as substrate in the LCAT reaction, considerably more phospholipid is lost than cholesteryl ester formed and that lipid transfer or exchange with "HDL" is not requisite for the observed decrease in particle size.

The data from these experiments provide additional support for the suitability of using HepG2 "HDL" as a model for the conversion of "nascent" HDL to mature plasma forms. The fact that the apoA-I to apoA-II weight ratio is similar in HepG2 lipoproteins (2) and LCAT-deficient plasma (30) makes such studies even more physiologically relevant. Our data suggest that HepG2 "HDL" may be extremely useful in examining the apoliprotein exchanges and/or particle fusions required for the conversion of precursor HDL to mature plasma-like forms.

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