Transformation of HepG2 nascent lipoproteins by LCAT: modulation by HepG2 d > 1.235 g/ml fraction

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Abstract We have previously shown that lecithin:cholesterol acyltransferase (LCAT) can transform ultracentrifugally isolated HepG2 lipoproteins (d < 1.235 g/ml) into particles that differ substantially from their nascent precursors. Transformed high density lipoprotein (HDL) subpopulations, as judged by nondenaturing gradient gel electrophoresis (GGE), resemble plasma HDL, i.e., HDL_{2a}- and HDL_{3a}-sized particles predominate. In HepG2 conditioned medium (CM), 60–70% of apoA-I is in the d > 1.235 g/ml fraction (lipid-poor apoA-I); hence we investigated whether inclusion of d > 1.235 g/ml fraction in LCAT incubations altered HDL subpopulations. After 18 h incubation of CM (containing lipoproteins and d > 1.235 g/ml fraction) with purified LCAT, the major transformation product on GGE was a large 9.7-nm particle (*HDL_{2b} pattern*); a minor component appeared at 7.4 nm (HDL_{3c}). Differences in particle size distribution between CM and isolated lipoprotein incubations were not the result of differences in LCAT activity; mass ratios of unesterified cholesterol:cholesteryl ester and phospholipid:cholesteryl ester were similar. Removal of apoA-I from the d > 1.235 g/ml fraction by immunoaffinity chromatography prior to incubation with the d < 1.235 g/ml fraction produced the same products (i.e., *HDL_{2b} pattern*) as incubations performed with the unaltered d > 1.235 g/ml fraction; therefore, lipid-poor apoA-I does not influence nascent HDL transformation. Cholesteryl ester was transferred from HepG2 HDL to LDL in CM incubations; however, cholesteryl ester transfer protein was not immunochemically identified. Removal of HepG2 LDL from CM prior to incubation with LCAT still resulted in the HDL_{2b} pattern. We conclude that HepG2 cells secrete a factor(s) that modifies nascent HDL transformation products into a predominantly HDL_{2b} subpopulation.—**McCall, M. R., A. V. Nichols, R. E. Morton, P. J. Blanche, V. G. Shore, S. Hara, and T. M. Forte.** Transformation of HepG2 nascent lipoproteins by LCAT: modulation by HepG2 d > 1.235 g/ml fraction. *J. Lipid Res.* 1993. **34:** 37–48.

Supplementary key words high density lipoprotein • HDL subclasses • gradient gel electrophoresis • cell culture

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 distinct from their nascent precursors in having been remodeled in the extracellular milieu. Recent work with the human hepatoblastoma-derived cell line, HepG2, suggests that these cells may be a useful source of 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 secretion of plasma proteins, lipoproteins, and lipid-poor apolipoproteins (2, 4) these cells express receptors for low density lipoproteins (LDL) (5–7) estrogen (8), insulin, and transferrin (9). It has also been reported that HepG2 cells produce hepatic triglyceride lipase (HTGL), (10) cholesteryl ester transfer protein (CETP) (11), and lecithin:cholesterol acyltransferase (LCAT) (12).

Previous studies with the HepG2 cell line in our laboratory have shown that HDL (d 1.063–1.235 g/ml) harvested from culture medium resemble HDL isolated from patients with familial LCAT deficiency (13). These HDL are enriched in surface lipids, i.e., phospholipid and unesterified cholesterol, relative to plasma HDL and appear as a mixed population of small, spherical (7.4 nm) and larger, discoidal particles when examined by electron microscopy. HepG2 LDL (d < 1.063 g/ml), on the other hand, are a relatively homogeneous population of spherical particles that are similar in size to plasma LDL but possess a triglyceride rather than cholesteryl ester-rich core.

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; PCR, polymerase chain reaction; TBS, Tris-buffered saline.

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We have recently demonstrated that LCAT can transform ultracentrifugally isolated HepG2 lipoproteins (d < 1.235 g/ml) into product particles that exhibit substantially different physical and chemical properties (14). HDL isolated from LCAT incubation mixtures were transformed into spherical particles possessing a particle size distribution similar to that of plasma HDL, i.e., HDL_{3a} was the predominant HDL species. Molar increases in HDL cholesteryl ester after LCAT incubations were paralleled by equimolar decreases in phospholipid and unesterified cholesterol, suggesting that LDL did not provide substrate or serve as an acceptor of cholesteryl ester formed in HDL. LDL isolated from incubation mixtures now possessed heterogeneity in particle size; the single prominent 25.6 nm size class observed in control incubations appeared to give rise to at least two additional smaller particle size populations. Changes in the LDL particle size distribution, however, were not accompanied by changes in particle morphology.

Our previous investigations on LCAT transformation of HepG2 lipoproteins excluded the HepG2 d > 1.235 g/ml fraction since this protein-rich fraction contains lipid-poor apolipoprotein (apo) A-I as well as other components which might influence the LCAT reaction. In the present study we investigated LCAT-induced transformation products from incubations containing the HepG2 d > 1.235 g/ml fraction. We found that inclusion of the protein-rich fraction produced particle size distributions that differed dramatically from those observed when only the isolated lipoprotein fraction was used.

MATERIALS AND METHODS

Cell culture and conditioned medium

HepG2 cells were grown and subcultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum as previously described (13, 14). 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 medium 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. Conditioned medium was concentrated by ultrafiltration (100- to 150-fold) and either dialyzed against buffer I (10 mM Tris, 150 mM NaCl, 0.27 mM EDTA, pH 7.4) or fractionated by ultracentrifugation (15) into one or more of the following density classes: d > 1.063 g/ml; d < 1.235 g/ml; d > 1.235 g/ml. Solution densities were adjusted with NaBr and ultracentrifugation was performed at 4°C using a Beckman 50.3 Ti rotor (40,000 rpm). Ultracentrifugation times were 36 h and 48 h for the 1.063 g/ml and 1.235 g/ml isolations, respectively. The lipoprotein fraction and/or infranatant were dialyzed against buffer I.

Preparation of lipoprotein-deficient plasma (LPDP) and apoA-I stripped HepG2 d > 1.235 g/ml conditioned medium

Lipoproteins were isolated from the plasma of a normolipidemic adult man by ultracentrifugation. Plasma was adjusted to d 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-apoA-I immunoaffinity column (16). In some experiments the HepG2 d > 1.235 g/ml fraction isolated from conditioned medium was passed through an anti-apoA-I immunoaffinity column to remove lipid-poor apoA-I. When this was done the apoA-I concentration in conditioned medium was checked to insure that the capacity of the column was not exceeded. Unbound column fractions were pooled, concentrated back to the volume of the original plasma sample or HepG2 d > 1.235 g/ml fraction, and then dialyzed against buffer I.

Preparation of purified lecithin:cholesterol acyltransferase

LCAT was isolated from human serum essentially as described Chen and Albers (17). Preparations of the purified enzyme have previously been characterized in our laboratory (14). The activity of purified LCAT prepared for this study (stored under nitrogen at 4°C in 10 mM Tris, 1 mM EDTA, pH 7.4) was checked prior to each experiment by the proteoliposome method (18).

Incubation mixtures and conditions

Concentrated HepG2 conditioned medium and ultracentrifugally isolated fractions were incubated at 37°C with LPDP or purified LCAT and 5 mM mercaptoethanol for the periods indicated in the figure legends. All incubation components (except mercaptoethanol) were sterile-filtered prior to use, and all incubations were performed in the dark under nitrogen. Incubations with purified LCAT contained 1.5% fatty acid-free human serum albumin. Incubations were terminated by the addition of the LCAT inhibitor, *p*-hydroxymercuriphenylsulfonic acid (PHMPS, final concentration 3.5 mM). HepG2 LDL (d < 1.063 g/ml) and HDL (d 1.063–1.235 g/ml)

were subsequently isolated from incubation mixtures by sequential ultracentrifugation. Incubations containing the d > 1.063 g/ml fraction were not subjected to the LDL isolation procedure after incubation with LCAT.

Lipoprotein analyses

Lipoprotein morphology and particle size distribution were assessed by electron microscopy (19) and nondenaturing gradient gel electrophoresis, respectively (20, 21). The gradient gel size intervals for the major plasma HDL subpopulations are those suggested by Nichols, Krauss, and Musliner (21). 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; $(HDL_{3aGGE}$, 8.8–8.2 nm; $(HDL_{3b})_{GGE}$, 8.2–7.8 nm; and $(HDL_{3c})_{GGE}$, 7.8–7.2 nm. Quantitative immunoassay of apoA-I was carried out as previously described (2). This assay can detect as little as 25 ng of apoA-I. Albumin was quantitated by single radial immunodiffusion (SRID) on plates purchased from Tago, Inc. (Burlingame, CA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (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 apoA-II after gradient gel electrophoresis of HepG2 HDL was determined by Western blotting (2). Lipoprotein chemical composition was determined by standard methods as previously described (2).

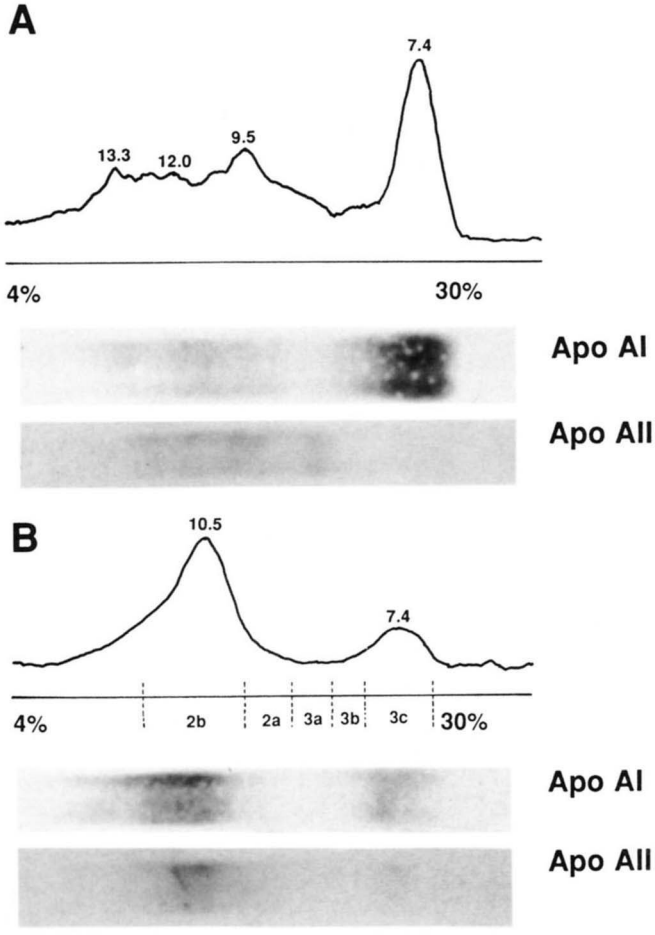

Fig. 1. Western blot particle size distribution of apoA-I and apoA-II in HepG2 HDL isolated from control (panel A) and 12-h LPDP (panel B) incubations. Incubations contained concentrated HepG2 conditioned medium, 5 mM mercaptoethanol, LPDP, and HSA. Samples were electrophoresed in 4–30% nondenaturing polyacrylamide gels, transferred to nitrocellulose, and immunoblotted for apoA-I and apoA-II. Densitometric scans of gels similarly electrophoresed and stained with Coomassie blue G250 are shown for comparison. Estimated Stokes' diameters (nm) are shown above appropriate peaks. Size estimates were based on gradient gel electrophoresis of standard globular proteins. Since some of the particles in the control incubation are discoidal (13) their peak positions can only be used as a relative size determinant.

Measurement of lipid transfer activity

Lipid transfer activity was assessed by methods that have been previously described (23–25). The standard assay system used [^{3}H]triglyceride and [^{14}C]cholesteryl ester, phosphatidylcholine liposomes as donor particles and isolated unlabeled plasma LDL as acceptor (23). In some experiments, however, radiolabeled LDL served as the donor and isolated unlabeled HDL served as the acceptor (24, 25). Incubations were performed at 37°C and terminated by the selective removal of LDL (23–25). Incubation times are provided in appropriate figure legends. Partially purified CETP was isolated (CM-cellulose fraction; (26)) and used as a positive control for incubations containing HepG2 conditioned medium.

CETP antibodies

Antisera to CETP were prepared and characterized as previously described by Morton (27). Monoclonal CETP antibody TP2 (28, 29) was generously provided by Dr. Yves Marcel. Both TP2 and the antisera have been previously shown to inhibit CETP activity.

RESULTS

Incubation of HepG2 conditioned medium with plasma d > 1.21 g/ml fraction as a source of LCAT

Concentrated HepG2 conditioned medium and LPDP depleted of lipid-poor apoA-I were incubated at 37°C for 12 h; control medium was maintained at 4°C in the presence of LPDP and the LCAT inhibitor PHMPS. Changes in nondenaturing gradient gel particle size distribution and apoA-I and apoA-II distribution are summarized in **Fig. 1.** Control HDL showed major peaks at 7.4, 9.5, 12.0, and 13.3 nm (Fig. 1), which are in good agreement with our previously published data on nonincubated HepG2 HDL. After incubation of conditioned medium with LPDP at 37°C, large shifts in the HDL particle size distribution were observed; the predominant

TABLE 1. Composition of major HepG2 lipoprotein fractions isolated from control (4°C) and active (37°C) LCAT incubation mixtures

Lipoprotein Fraction	Incubation Condition	Composition (% by weight)				
		Protein	CE	UC	PL	TG[a]
LDL[b]	4° control	34	12	13	42	
	37° LPDP	31	24	11	34	
HDL[c]	4° control	43 ± 3	5 ± 1	12 ± 1	40 ± 2	
	37° LPDP	44 ± 1	17 ± 1	3 ± 1	36 ± 1	
Plasma HDL[d]		45–55	15–20	3–5	26–32	2–7

Concentrated HepG2 conditioned medium and LPDP depleted of lipid-poor apoA-I were incubated at 37°C for 12 h; control medium was maintained at 4°C with LPDP and 3.5 mM PHMPS. Lipoproteins were subsequently isolated by sequential ultracentrifugation: LDL, d < 1.063 g/ml; HDL, d 1.063–1.235 g/ml; CE, cholesteryl ester; UC, unesterified cholesterol; PL, phospholipid; TG, triglyceride.

[a] Triglyceride concentrations were not determined.

[b] Values represent a single experiment in which three different incubations were pooled.

[c] Values represent three separate incubations from a single pool of HepG2 conditioned medium.

[d] Plasma values are shown for comparison (ref. 30).

HDL species appeared in the HDL_{2b} region while a minor component was observed in the HDL_{3c} region (Fig. 1). This distribution is referred to as the *HDL_{2b} pattern.* Changes in the particle size distribution of Coomassie blue-stained gels were accompanied by a redistribution of apolipoproteins A-I and A-II (Fig. 1). Nascent HepG2 HDL possessing apoA-I were transformed into two populations of particles that corresponded in size with the two populations observed by Coomassie blue staining, i.e., HDL_{2b} and HDL_{3c} sized particles. HDL possessing apoA-II were transformed into predominantly HDL_{2b} sized particles.

The 37°C incubation with LPDP resulted in increases in HDL cholesteryl ester and decreases in unesterified cholesterol and phospholipid (**Table 1**). These compositional changes, reflecting alterations in the ratio of core to surface lipids, are consistent with the disc-to-sphere transformations observed with electron microscopy (**Fig. 2**). Discoidal particles 16.4 ± 4.0 nm in diameter were converted to spherical particles 10.6 ± 1.6 nm in diameter.

Increases in cholesteryl ester and decreases in unesterified cholesterol and phospholipid were observed in the isolated LDL fraction (Table 1). LDL compositional changes, however, were not associated with alterations in particle morphology (data not shown) and particle size was unchanged or slightly increased (**Fig. 3**, panel B). Summing the major lipid components for LDL and HDL revealed that decreases in the mole amount of unesterified cholesterol (−0.13 μmol) and phospholipid (−0.12 μmol) corresponded to increases in the mole amount of

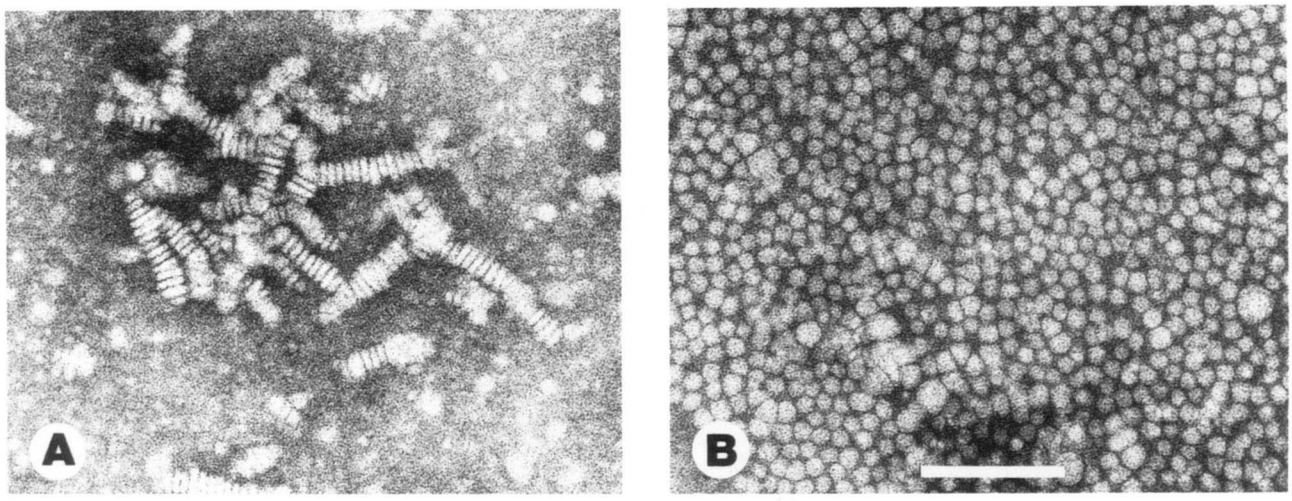

Fig. 2. Electron micrographs of negatively stained HepG2 HDL (d 1.063–1.235 g/ml): panel A, LPDP control; panel B, LPDP 12-h incubation. Both micrographs were taken at the same magnification. The bar marker in panel B indicates 100 nm.

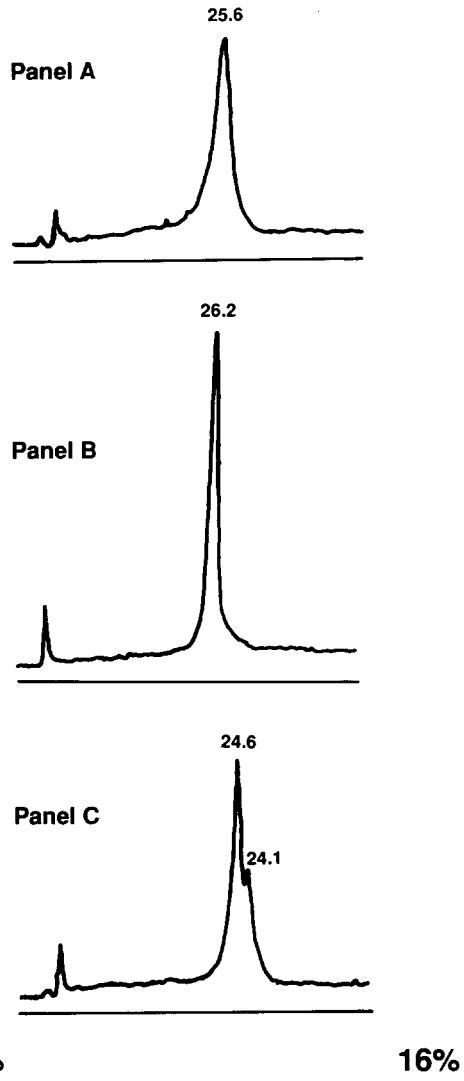

Fig. 3. Densitometric scans of Coomassie blue G250-stained 2–16% nondenaturing polyacrylamide gradient gels of HepG2 LDL (d < 1.063 g/ml) isolated from 12-h incubation mixtures containing LPDS as a source of LCAT. Panel A, 4°C conditioned medium control; panel B, 37°C conditioned medium with active LCAT; panel C, 37°C d < 1.235 g/ml fraction with active LCAT. Estimated Stokes' diameters (nm) are shown above appropriate peaks.

cholesteryl ester (+0.13 μmol), suggesting that all substrates and products of the LCAT reaction can be accounted for. Interestingly, it was observed in the HDL fraction that more unesterified cholesterol (0.13 μmol) and phospholipid (0.12 μmol) were consumed during the 12-h incubation than cholesteryl ester formed (0.08 μmol; **Table 2**), suggesting that some HDL cholesteryl ester was transferred to LDL.

The preceding data differ in several important aspects from our previously published findings (14) where incubation mixtures consisted of ultracentrifugally isolated HepG2 lipoproteins (d < 1.235 g/ml fraction from conditioned medium) and LPDP. These earlier studies demonstrated that ultracentrifugally isolated lipoproteins transformed by LPDP possessed HDL that closely resembled that of plasma HDL where HDL_{2a} and HDL_{3a} sized particles predominated (**Fig. 4,** panel D). This particle size distribution is hereafter referred to as the *HDL_{2a-3a} pattern*. In addition, our earlier work demonstrated that the particle size distribution of LDL was considerably altered after incubation of the isolated lipoprotein fraction with LPDP; LDL possessed additional particles of smaller size which appear to have been derived from the 25.6 nm LDL peak (Fig. 3, panel C). Quantitation of lipids in this earlier work also provided little evidence for the transfer of HDL cholesteryl ester to lower density lipoproteins (i.e., LDL) in incubations performed with LPDP and ultracentrifugally isolated HepG2 lipoproteins. The differences in particle size distribution between the conditioned media (*HDL_{2b} pattern*) and isolated lipoprotein (*HDL_{2a-3a} pattern*) incubations cannot be attributed to differences in LCAT activity as these very different particle size distributions can be obtained from incubation products possessing similar UC/CE and PL/CE mole ratios (14).

Incubations with purified LCAT

To better understand the formation of the *HDL_{2b} pattern* observed in incubations with conditioned medium as opposed to the *HDL_{2a-3a} pattern* observed in incubations with isolated lipoproteins, the following experiment was performed. HepG2 concentrated conditioned medium was divided into two pools; one pool was stored at 4°C and the other was fractionated by ultracentrifugation into d > 1.063, d < 1.235, and d > 1.235 g/ml fractions. Subsequently, the conditioned medium and ultracentrifugally isolated fractions were dialyzed into buffer I and a series of incubations were carried out at 4°C and 37°C (Fig. 4). Purified LCAT was used in these studies to eliminate LPDP as a potential source of cholesteryl ester transfer activity. All incubations contained equivalent LCAT activity (40 nmol cholesteryl ester formed/h of incubation) and cholesterol (0.15 mg cholesterol/1.75 ml incubation volume). Incubations were for 18 h and were performed as described in Methods.

TABLE 2. Mole amounts of specific lipids in HepG2 HDL isolated from control (4°C) and active (37°C) LPDP incubation mixtures

Incubation	CE	UC	PL
		umol	
4° Control	0.040 ± 0.007	0.164 ± 0.003	0.287 ± 0.020
37° LPDP	0.124 ± 0.007	0.037 ± 0.003	0.167 ± 0.007
Δ from control	+0.084	−0.127	−0.120

Values represent the mean ± standard deviation of three separate incubations from a single pool of HepG2 conditioned medium. Triglyceride was not determined.

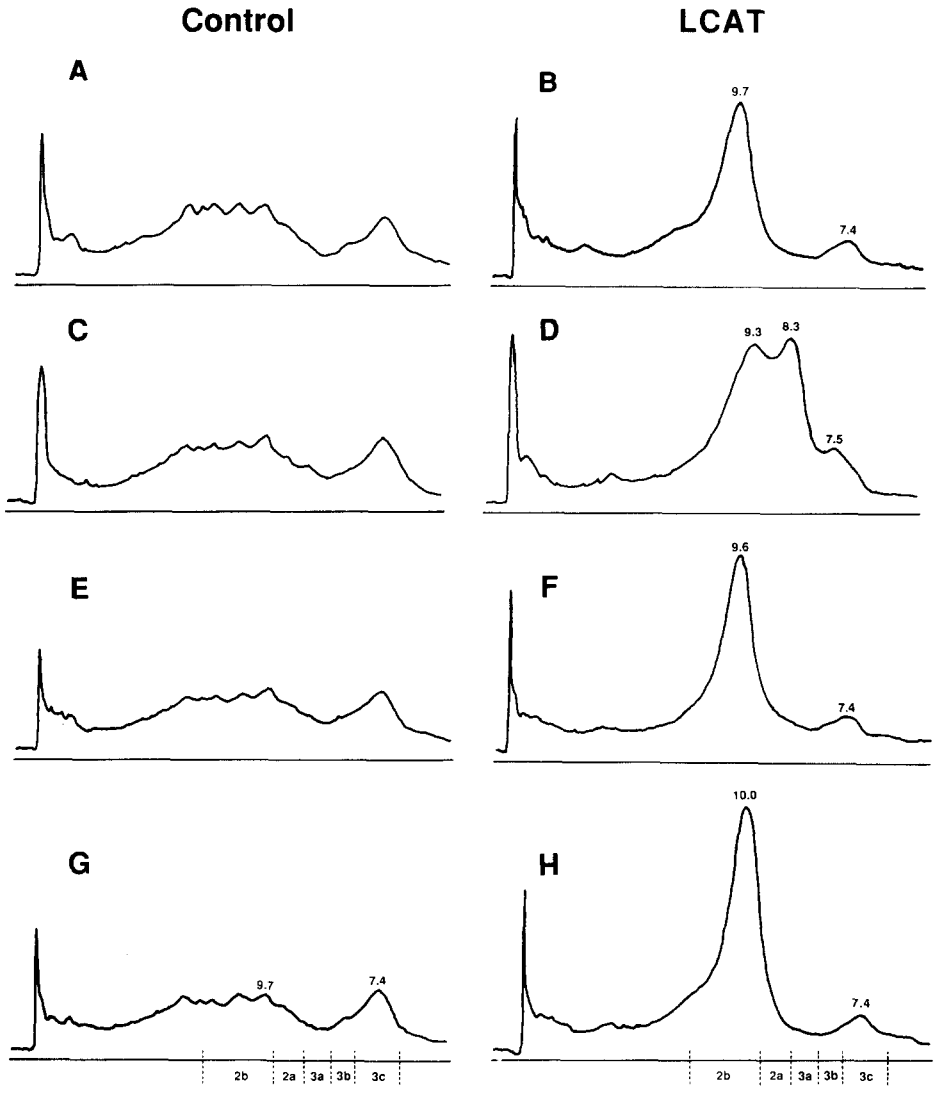

Fig. 4. Densitometric scans of Coomassie blue G250-stained 4–30% nondenaturing polyacrylamide gradient gels of HepG2 HDL isolated from 18-h incubation mixtures. Incubations contained LCAT, 5 mM mercaptoethanol, HSA, and one of the following: unfractionated conditioned medium (panels A and B); d < 1.235 g/ml fraction isolated from conditioned medium (panels C and D); reconstituted conditioned medium, i.e., d < 1.235 g/ml fraction + d > 1.235 g/ml fraction (panels E and F); conditioned medium depleted of LDL, i.e., d > 1.063 g/ml fraction (panels G and H). Control incubations are shown in panels A, C, E, and G. Estimated Stokes' diameters (nm) are shown above appropriate peaks. The major human plasma HDL subpopulation size intervals have been included (21) in panels G and H.

Densitometric scans of Coomassie blue-stained 4–30% gradient gels showing the particle size distribution of HDL from control and active LCAT incubations are shown in Fig. 4. Active LCAT incubations with conditioned medium (panel B), reconstituted conditioned medium (panel F), and conditioned medium minus LDL (panel H) resulted in very similar particle size distributions. In each case the *HDL_{2b} pattern* is conspicuous; moreover these profiles resemble Fig. 1, panel B depicting HDL isolated from incubations containing conditioned medium and LPDP. Incubations with the d < 1.235 g/ml fraction, which excludes the protein-rich d > 1.235 g/ml fraction, resulted in the *$HDL_{2a\text{-}3a}$* pattern (Fig. 4; panel D). This particle size distribution possessed major components in the HDL_{2a} and HDL_{3a} regions of the gel; a minor component was also observed in the HDL_{3c} region. Clearly, subsequent to incubation with LCAT there were distinct differences in HDL particle size distribution dependent on the presence or absence of the protein-rich d > 1.235 g/ml fraction from conditioned medium in the incubation mixture.

Table 3 shows the percentage composition data for HDL and the mole ratios for PL/CE and UC/CE of HDL from the incubations described above. These data are

TABLE 3. Composition of HepG2 HDL isolated from control (4°C) and active (37°C) LCAT incubation mixtures

Incubation		Composition (% by weight)					Mole Ratios	
CM or CM Fractions	Temp °C	Protein[a]	CE	UC	PL	TG	PL/CE	UC/CE
CM	4	41.6	5.9	12.7	36.7	3.1	5.38	3.62
CM	37	47.5	22.8	3.7	22.1	3.9	0.84	0.28
d < 1.235 g/ml	4	41.3	5.4	12.6	37.7	3.0	6.08	3.92
d < 1.235 g/ml	37	48.4	22.6	3.7	22.2	3.1	0.85	0.28
d < and d > 1.235 g/ml	4	43.1	5.0	12.1	36.8	3.0	6.41	4.10
d < and d > 1.235 g/ml	37	48.4	21.6	3.3	20.8	2.8	0.84	0.26
d > 1.063 g/ml	4	40.9	5.9	12.5	37.8	2.8	5.53	3.55
d > 1.063 g/ml	37	48.6	21.7	3.7	23.4	2.7	0.93	0.28

LCAT incubations were performed as described in Methods with concentrated HepG2 conditioned medium (CM) or with density fractions ultracentrifugally isolated from CM. HDL was subsequently isolated from the incubation mixtures and its chemical composition was determined. Each value represents the average of duplicate 18-h incubations except for the d > 1.063 g/ml incubations which were done in triplicate. Standard deviations for the percent protein, CE, UC, PL, and TG measurements in the d > 1.063 incubation were 0.9, 0.4, 0.2, 0.3, 0.6, and 0.7, 0.7, 1.5, 0.5, and 0.5 for the 4°C and 37°C incubations, respectively.

[a] Protein values represent total protein (measured by a modification of the Lowry method [31]) minus albumin (measured by SRID).

remarkably similar among experimental incubations suggesting that observed differences in particle size distribution result from redistribution of protein and/or lipid within the HDL density class.

It can be inferred from the data presented in Fig. 4 and Table 3 that procedural differences between our earlier experiments with isolated HepG2 lipoproteins and the present studies with conditioned medium cannot account for the HDL particle size differences observed. It can also be inferred from the reconstituted conditioned medium incubation that ultracentrifugal isolation of HepG2 lipoproteins prior to incubation with LCAT does not significantly influence subsequent transformation of the particles (compare panels B and F; Fig. 4). In addition, it appears that HepG2 LDL is not a required participant in the transformation of HepG2 HDL into the *HDL_{2b} pattern* (compare panels B and H; Fig. 4).

Incubations with purified LCAT and conditioned medium or reconstituted conditioned medium resulted in LDL particle size distributions that were indistinguishable from one another (data not shown). After incubation, the LDL band in each case became narrower as judged by nondenaturing gradient gel electrophoresis, suggesting a more homogeneous population of particles. In addition, particle sizes were unaltered or slightly larger (data not shown). These data resembled those observed when conditioned medium was incubated with LPDP, Fig. 3, panel B. In contrast, incubations with isolated HepG2 lipoproteins resulted in increased LDL size heterogeneity (data not shown) similar to that observed in Fig. 3, panel C for incubations containing LPDP and isolated lipoproteins.

Possible role of lipid-poor apoA-I in the transformation process

It is known that 60–70% of apoA-I present in conditioned medium is in the d > 1.235 g/ml fraction (2). To examine the potential role of lipid-poor apoA-I in modulating LCAT-induced transformation, incubations were carried out with the d > 1.235 g/ml fraction stripped of apoA-I by immunoaffinity chromatography. The concentration of apoA-I in the d > 1.235 g/ml fraction decreased from 0.20 mg/ml to levels that could not be detected by ELISA after immunoaffinity chromatography. Incubation conditions were essentially as described for the preceding experiment except that 12- and 24-h incubations were examined. The particle size distribution and the mole ratios of PL/CE and UC/CE for this experiment are indicated in **Fig. 5.** The data are consistent with results obtained from incubations with unaltered conditioned medium, suggesting that lipid-poor apoA-I does not influence the LCAT-induced transformation of HepG2 HDL. SDS-PAGE provided suggestive evidence that the apoA-I concentrations in HDL were similar in incubations performed with conditioned medium and conditioned medium stripped of lipid-poor apoA-I (**Fig. 6**). Subsequent quantitation of apoA-I confirmed this impression. HDL isolated from 24-h incubations containing conditioned medium and conditioned medium stripped of lipid-poor apoA-I possessed 0.13–0.14 mg/ml and 0.13–0.14 mg/ml apoA-I, respectively. The values are for duplicate incubations. It can also be inferred from SDS-PAGE (Fig. 6) that HDL apoE decreased after transfor-

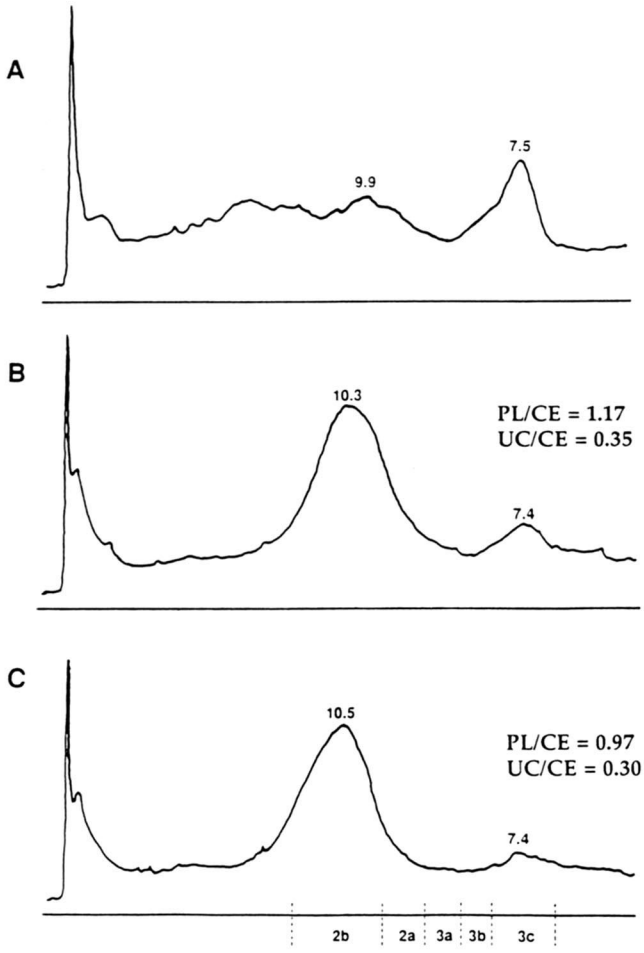

Fig. 5. Densitometric scans of Coomassie blue G250-stained 4–30% nondenaturing polyacrylamide gradient gels of HepG2 HDL isolated from control (panel A), 12-h (panel B), and 24-h (panel C) incubation mixtures. Incubations contained LCAT, 5 mM mercaptoethanol, HSA, and both of the following fractions isolated from HepG2 conditioned medium: the d < 1.235 g/ml fraction and the d > 1.235 g/ml fraction from which apoA-I had been removed by an immunoaffinity column. Estimated Stokes' diameters (nm) are shown above appropriate peaks. The major human plasma HDL_{GGE} subpopulation size intervals have been included in panel C.

mation by LCAT. This phenomenon was previously observed in our studies on isolated HepG2 lipoproteins transformed by LCAT (14).

Lipid transfer activity

To assess whether CETP could account for the unusual HDL particle size distribution observed in incubations containing LCAT and conditioned medium, triglyceride and cholesteryl ester transfer activity in conditioned medium was examined. The dependence of lipid transfer activity on the time and the amount of conditioned medium added to the incubation system is shown in **Fig. 7,** A and B, respectively. The relationships for both triglyceride and cholesteryl ester transfer are linear. The percentage of triglyceride transferred at any given time or for any given conditioned medium addition was consistently twofold greater than the percentage of cholesteryl ester tranferred (%TG/%CE for the data shown in Fig. 7,A and B is 2.3 ± 0.2). For comparison, the lipid transfer activity of partially purified CETP was assayed in the same incubation system used to assess transfer activity in conditioned medium (**Fig. 8:** panel A, conditioned medium; panel B, CETP). The transfer activity of CETP differed from transfer activity observed for conditioned medium in that the percentage transfer of triglyceride was less than the percentage transfer of cholesteryl ester (%TG/%CE = 0.7). To determine immunochemically whether CETP plays any role in the lipid transfer activity observed in HepG2 conditioned medium lipid transfer, assays were performed in the presence of antibodies that specifically inhibit CETP activity. Antisera to CETP prepared in rabbits and monoclonal antibody TP2 were used in these studies. Both antibody preparations had little, if any, effect on the lipid transfer activity in HepG2 conditioned medium (Fig. 8; panel A). The activity of purified plasma CETP, however, was greatly diminished when either of the antibody preparations was used (Fig. 8; panel B); triglyceride transfer was completely abolished while cholesteryl ester transfer was reduced by as much as 98%. In addition, dot blot analysis using TP2 to probe conditioned medium failed to detect CETP.

The assays used to assess lipid transfer activity do not distinguish triglyceride hydrolysis from triglyceride trans-

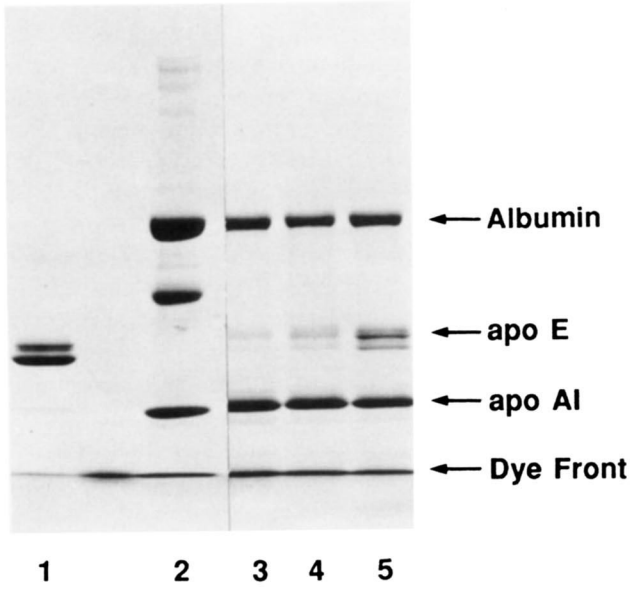

Fig. 6. SDS polyacrylamide gel (4–20%) electrophoresis of HepG2 HDL isolated from 24-h incubation mixtures. Incubations were performed with LCAT, 5 mM mercaptoethanol, HSA, and either conditioned medium stripped of lipid-poor apoA-I (control, lane 5; experimental, lane 4) or ultracentrifugally isolated HepG2 lipoproteins (experimental, lane 3). Standard proteins were electrophoresed in lane 1 (human apoE) and lane 2 (the three prominent bands from top to bottom are: human serum albumin (HSA), ovalbumin, bovine apoA-I). Samples were reduced prior to electrophoresis.

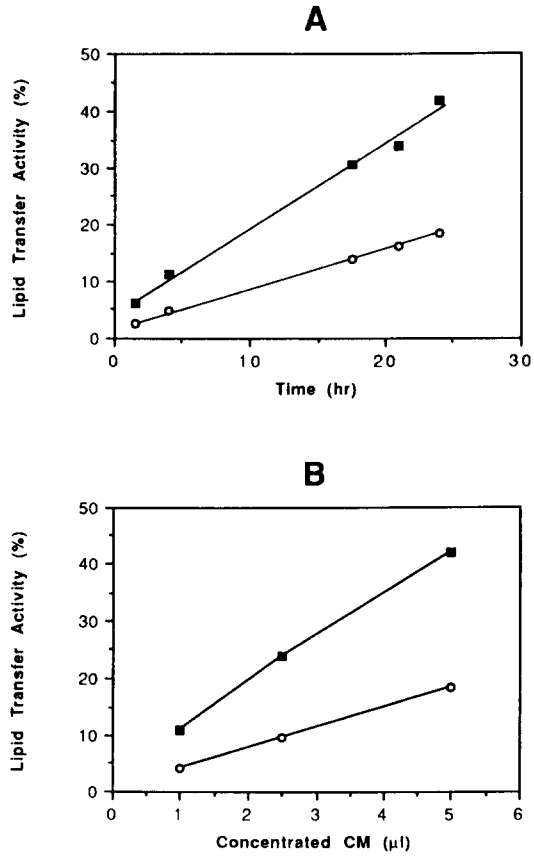

Fig. 7. Lipid transfer activity. Panel A, time dependence of HepG2 conditioned medium-mediated triglyceride (closed squares) and cholesteryl ester (open circles) transfer. Radiolabeled donor particles (phosphatidylcholine-liposomes labeled with [^{3}H]triglyceride and [^{14}C]cholesteryl ester) and unlabeled acceptor particles (LDL) were incubated with 5 µl of 160-fold concentrated conditioned medium for up to 24 h. Assays were terminated by affinity adsorption of LDL by ConA Sepharose. The fraction of radiolabeled donor lipid transferred to the acceptor was determined by liquid scintillation counting and is reported as the percent triglyceride or cholesteryl ester transferred. No measurable transfer activity was observed in buffer or media blanks. Data represent the mean of duplicate incubations and are consistent with experiments performed with conditioned media from three different cell passages. Panel B, concentration dependence of HepG2 conditioned medium-mediated lipid transfer (triglyceride, closed squares; cholesteryl ester, open circles). Incubations were for 24 h and contained 1, 2.5, or 5 µl of 160-fold concentrated HepG2 conditioned medium. Incubations were performed as described above.

fer, therefore an experiment was performed to determine if some unknown lipolytic activity contributed to the high triglyceride transfer activity present in conditioned medium. These experiments were performed using the radiolabeled liposome assay system and conditioned medium. Triglyceride and cholesteryl ester transfer activity was calculated using cpm data obtained from the isolated (TLC) transferred lipids as well as by the method used above. Both transfer activity measurements were similar (data not shown) suggesting that lipolysis did not contribute to the high %TG/%CE ratios observed in incubations containing conditioned medium.

DISCUSSION

The present study demonstrates that a factor (or factors) present in HepG2 conditioned medium modulates the particle size distribution of nascent lipoproteins undergoing transformation by LCAT. LCAT incubations performed with HepG2 conditioned medium, which contains lipoproteins as well as other secreted nondialyzable protein components, result in a particle size distribution in which HDL_{2b} sized particles predominate (*HDL_{2b} pattern*). We have previously shown that incubations performed with ultracentrifugally isolated lipoproteins,

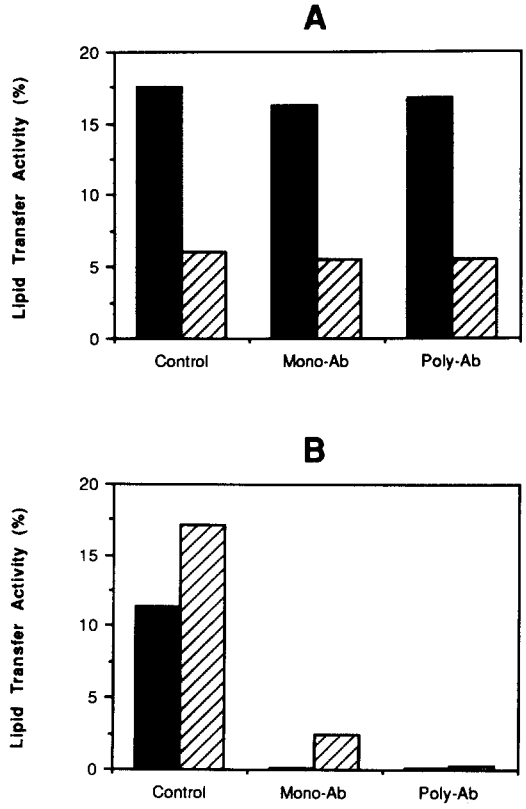

Fig. 8. Lipid transfer activity mediated by HepG2 conditioned medium (A) or partially purified CETP (B) in the absence (control) and presence of monoclonal and polyclonal antibodies (Mono-Ab and Poly-Ab, respectively) that inhibit CETP activity. Radiolabeled donor particles (plasma LDL labeled with [^{3}H]triglyceride and [^{14}C]cholesteryl ester) and unlabeled acceptor particles (HDL) were incubated with 5 µl of 160-fold concentrated conditioned medium or with partially purified CETP for 24 h. Monoclonal and polyclonal antibodies (TP2 and rabbit antisera, respectively) were included in some incubations. Assays were terminated by selectively precipitating LDL (24, 25). The fraction of radiolabeled donor lipid transferred to the acceptor was determined by liquid scintillation counting and is reported as the percent triglyceride or cholesteryl ester transferred. No measurable transfer activity was observed in buffer or media blanks. Data represent the mean of duplicate incubations and are consistent with experiments performed with conditioned media from three different cell passages. ■, triglyceride; ◪, cholesteryl ester.

however, result in an HDL particle size distribution that approximates that of plasma HDL (*HDL_{2a-3a} pattern*) (14). These two very different particle size distributions do not appear to be the result of the preliminary ultracentrifugation step used to isolate HepG2 lipoproteins, because incubations using conditioned medium or reconstituted conditioned medium (i.e., recombining the d < and d > 1.235 g/ml fractions obtained from conditioned medium) result in particle size distributions that are nearly identical. It is possible however that the factor(s) responsible for HDL conversion are loosely bound to lipoproteins, dissociating during ultracentrifugation and reassociating when the two fractions are recombined.

It has been reported that HepG2 cells release LCAT (12) and CETP (11) into the culture medium and that much of the apoA-I produced by this cell line is secreted in a lipid-poor form (2). These proteins are likely to be found in the d > 1.235 g/ml fraction of conditioned medium and can potentially influence the maturation of nascent HepG2 lipoproteins. Incubations performed with conditioned medium, conditioned medium stripped of lipid-poor apoA-I, and conditioned medium from which the d < 1.063 g/ml lipoproteins were removed all resulted in virtually identical HDL particle size distributions (*HDL_{2b} pattern*). These data suggest that *a)* lipid-poor apoA-I is not required to stabilize the larger HDL_{2b} sized particles formed in the active LCAT-conditioned medium incubations; and *b)* CETP-mediated lipid movement between HepG2 LDL (possesses triglyceride core) and transformed nascent HDL (possesses cholesteryl ester core) is not requisite for the formation of the *HDL_{2b} pattern.* Our data are also inconsistent with the contention that HepG2-generated LCAT activity influences core formation to such an extent that the large HDL_{2b} sized particles are formed. In fact, the chemical composition data shown in Table 3 indicate that although *HDL_{2a-3a}* and *HDL_{2b} patterns* possess particles that differ considerably in size their average chemical compositions are similar.

Our data permit only speculation on the mechanism involved in the formation of the two particle size populations associated with the *HDL_{2b} pattern.* We have observed that incubation of conditioned medium with 1.5% albumin and the LCAT inhibitor PHMPS results in very minor changes in the HDL particle size distribution (M. R. McCall and T. M. Forte, unpublished observation), suggesting that at least some LCAT-induced transformation of nascent HDL (i.e., generation of a cholesteryl ester core) is necessary before conversion to the *HDL_{2b} pattern* can occur. Presumably, conversion involves protein/lipid transfers between HDL particles and/or particle fusion induced by the factor(s) present in conditioned medium. The role of the 7.4 nm particle in the conversion process is difficult to assess as it is not clear if the small particle observed in the *HDL_{2b} pattern* represents: *a)* a nascent particle that has not been transformed by LCAT into a larger one; *b)* a recycled small particle; or *c)* a product generated by the transformation or conversion process that is distinct from the nascent small particle. We are currently investigating these possibilities. In any event our data suggest that the small particle from both control and experimental incubations possesses predominantly apoA-I. The large particles observed in the *HDL_{2b} pattern* are presumably formed from LCAT-modified nascent particles as the result of particle fusion and/or lipid/apolipoprotein transfers. The resulting particles possess apoA-I and apoA-II as suggested by the Western blots shown in Fig. 1. It is unknown at present whether these particles represent only HDL with apoA-I and A-II or whether other apo-specific HDL populations are present, e.g., HDL with apoA-I and without apoA-II (32).

Conversion of plasma HDL and ultracentrifugally isolated HDL_3 to large and small particles with sizes comparable to those observed in the *HDL_{2b} pattern* of the present study has been described previously (16, 33–37). These conversions have been attributed to various factors including CETP, LCAT, and HDL "conversion factor" depending on the incubation conditions used. The early studies of Rye and Barter (33, 36) are of particular interest as they suggest that a factor independent of LCAT and CETP can convert ultracentrifugally derived plasma HDL_3 into a population of both larger and smaller particles. Subsequent studies by these investigators, however, suggested that the plasma-derived HDL conversion factor was, in fact, CETP (34, 35). We have examined HepG2 conditioned medium for the presence of this transfer protein. Immunoblots performed with a monoclonal antibody to CETP (TP2 (38), generously provided by Dr. Yves Marcel) have been unable to demonstrate the presence of this protein in our culture media. In addition, our lipid transfer data clearly demonstrate that the transfer activity present in HepG2 conditioned medium differs from that of CETP. The transfer activity in conditioned medium has an unusually high triglyceride to cholesteryl ester transfer ratio compared to CETP and this activity is not affected by antibodies that specifically inhibit CETP.

Because it has been reported that CETP is secreted by the HepG2 cell line (11) it is not clear why we are unable to demonstrate its presence in our culture media. Differences among HepG2 cell lines in various laboratories have been reported (39), however; these disparities could simply reflect differences in the clones of cells used or in the conditions of culture. In any event, our data clearly show that the HepG2 cell line used in these experiments (original low passage stock obtained from Dr. Barbara Knowles) produces a factor, which is not CETP, capable of generating the *HDL_{2b} pattern* during LCAT-induced transformation of nascent HDL particles.

In a recent abstract it has been reported that phospholipid transfer protein in plasma can promote the for-

mation of larger HDL (40). In the present study the presence of phospholipid transfer protein cannot be ruled out as phospholipid transfer activity was not assessed; the triglyceride and cholesteryl ester transfer activity observed, however, is not consistent with the activity of phospholipid transfer protein which promotes transfer of phospholipid but not of triglyceride and cholesteryl ester (41). If a single transfer factor is responsible for the HDL particle size rearrangements observed in incubations containing LCAT and conditioned medium, our lipid transfer data suggest that it is not phospholipid transfer protein or CETP.

Nondenaturing gradient gel analysis of LDL showed that these products, like their HDL counterparts, had different particle size distributions depending on the whether the $d > 1.235$ g/ml fraction of conditioned medium was included in the incubation. Whereas our previous studies on LCAT incubations with HepG2 $d < 1.235$ g/ml lipoproteins showed the formation of a heterogeneous smaller LDL (14), the present study (where conditioned medium was incubated with LCAT) demonstrated that LDL tended to become slightly larger and more homogeneous in size. Because of the small amounts of LDL available for analysis we can only speculate as to the mechanism for the shift in the LDL particle size distribution observed in LCAT incubations with conditioned medium. Western blot analysis indicates that LDL isolated from LCAT incubations containing conditioned medium acquire apoE (M. R. McCall and T. M. Forte, unpublished observation). As the addition of apoE to the surface of a lipoprotein would tend to increase its relative size, it appears that this movement of apoE to LDL from HDL plays some role in the modification of the LDL particle size distribution. Redistribution of apoE from HDL to lower density lipoproteins has also been observed when LCAT is incubated with plasma lipoproteins from patients with familial LCAT deficiency (42). It should also be noted that in some, but not all, incubations containing active LCAT and conditioned medium, LDL triglyceride was apparently exchanged for HDL cholesteryl ester. As previously discussed, this lipid transfer is likely to be mediated by a transfer factor other than CETP. In any event, development of a cholesteryl ester core in HepG2 LDL would tend to reduce rather than increase particle size.

In summary, we have investigated the role of the protein-rich $d > 1.235$ g/ml fraction of HepG2 conditioned medium on LCAT-induced transformation of nascent HepG2 lipoproteins. High density lipoprotein transformation products isolated from incubations containing LCAT and conditioned medium possess a particle size distribution in which HDL_{2b} sized particles predominate. This particle size distribution differs considerably from that observed when incubations contain LCAT and the $d < 1.235$ g/ml fractions isolated from HepG2 conditioned medium where HDL_{2a} and HDL_{3a} sized particles predominate. Our data suggest that the distinct differences in HDL particle size distribution, depending on whether LCAT incubation contains conditioned medium or isolated lipoproteins, are not the result of endogenous LCAT, CETP, or lipid-poor apoA-I in conditioned medium. Further studies are in progress to isolate and identify the factor (or factors) present in HepG2 conditioned medium that remodel nascent HDL undergoing transformation by LCAT.

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