Characterization of lipoproteins produced by the human liver cell line, Hep G2, under defined conditions[1]

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Abstract Confluent monolayers of the human hepatoblastoma-derived cell line, Hep G2, were incubated in serum-free medium. Conditioned medium was ultracentrifugally separated into d < 1.063 g/ml and d 1.063–1.20 g/ml fractions since very little VLDL was observed. The d < 1.063 g/ml fraction was examined by electron microscopy; it contained particles of 24.5 ± 2.3 nm diameter, similar in size to plasma LDL; a similar size was demonstrated by nondenaturing gradient gel electrophoresis. These particles possessed apoB-100 only. The d < 1.063 g/ml fraction had a lipid composition unlike that of plasma LDL; unesterified cholesterol was elevated, there was relatively little cholesteryl ester, and triglyceride was the major core lipid. The d 1.063–1.20 g/ml fraction was heterogeneous in size and morphology. Electron microscopy revealed discoidal particles (14.9 ± 3.2 nm long axis and 4.5 ± 0.2 nm short axis) as well as small spherical ones (7.6 ± 1.4 nm diameter). Nondenaturing gradient gel electrophoresis consistently showed the presence of peaks at 13.4 11.9, 9.7, and 7.4 nm. The latter peak was conspicuous and probably corresponded to the small spherical structures seen by electron microscopy. Unlike plasma HDL, Hep G2 d 1.063–1.20 g/ml lipoproteins contained little or no stainable material in the $(HDL_{3a})_{gge}$ region by gradient gel electrophoresis. Hep G2 d 1.063–1.20 g/ml lipoproteins differed significantly in composition from their plasma counterparts; unesterified cholesterol and phospholipid were elevated and the mole ratio of unesterified cholesterol to phospholipid was 0.8. Cholesteryl ester content was extremely low. ApoA-I was the major apolipoprotein, while apoE was the next most abundant protein; small quantities of apoA-II and apoCs were also present. Immunoblot analysis of the d 1.063–1.20 g/ml fraction after gradient gel electrophoresis showed that apoE was localized in the larger pore region of the gel (apparent diameter greater than 12.2 nm); the apoA-I distribution in this fraction was very broad (7.1–12.2 nm), and included a distinct band at 7.4 nm. Immunoblotting after gradient gel electrophoresis of concentrated medium revealed that a significant fraction of apoA-I in the uncentrifuged medium was in a lipid-poor or lipid-free form. This cell line may be a useful model for investigating the metabolism of newly formed HDL. — **Thrift, R. N., T. M. Forte, B. E. Cahoon, and V. G. Shore.** Characterization of lipoproteins produced by the human liver cell line, Hep G2, under defined conditions. *J. Lipid Res.* 1986. **27:** 236–250.

Supplementary key words hepatoma • VLDL • LDL • HDL • gradient gel electrophoresis • electron microscopy • apolipoprotein

Rat hepatocytes in primary culture have been useful in studying lipoprotein synthesis and secretion (1); however, little is known about the corresponding functions of human liver cells. It has recently been demonstrated that the human hepatoblastoma-derived cell line, Hep G2, secretes must of the plasma proteins expected from liver cells, including apolipoprotein (apo) B (2). This finding stimulated investigations on apolipoprotein synthesis and, in fact, many of the plasma apolipoproteins have been identified in culture medium (3–5). Several laboratories have shown that these cells possess receptors for apoB (6, 7) and apoE (8). Utilizing this cell line, Zannis et al. (9) and Gordon et al. (10) showed that apoA-I is secreted as a proapolipoprotein, which possesses an additional six-amino acid propeptide as compared to the major plasma form of apoA-I. Zannis et al. (4) also found that apoE from culture medium contains more sialic acid groups than does the major plasma form. Intracellular and secreted forms of apoA-II (11) and A-IV (5) have been ascertained for Hep G2 cells. Although chromosomal abnormalities have been demonstrated, the line appears to be diploid (12) in the chromosomal regions thought to code for the B–E receptor, lecithin:cholesterol acyltransferase (LCAT), and apolipoproteins A-I, A-II, A-IV, C-II, C-III, and E (13–20). Hep G2 cells, however, produce little or no serum amyloid A (21), a protein of hepatic origin which under some conditions is associated with

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HDL (22). The cell line also lacks a specific protease involved in the intracellular processing of pro-complement Factor I (23).

The major emphasis thus far in lipoprotein studies with Hep G2 cells has been on the elucidation of newly synthesized apolipoprotein primary structure and the steps involved in intra- and extracellular protein processing. The studies of Rash, Rothblat, and Sparks (3) and Zannis et al. (4) suggested that the major apolipoproteins secreted by these cells are complexed with lipids; however, the complexes remain uncharacterized. These cells may be useful as a model for the study of newly secreted hepatic lipoproteins that have not undergone the modifications normally occurring in the circulatory system. We report on the composition, size distribution, and morphology of the major lipoprotein classes produced by the human liver-derived cell line, Hep G2, in chemically defined medium.

METHODS

Cell culture

The human hepatoblastoma-derived cell line, Hep G2, was kindly provided by Dr. B. B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA). Cells were propagated in Eagle's Minimum Essential Medium (MEM) (Gibco Laboratories, Santa Clara, CA) with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) in an atmosphere of 95% air/5% CO_2 and were subcultured weekly at a 1:3 ratio. To initiate experiments, cells (approximately $4-5 \times 10^6$ per flask) were seeded into 75-cm^2 tissue culture flasks (T75 flasks, Falcon, Oxnard, CA). One day later the MEM with FBS was replaced with 15 ml of MEM supplemented with 1.23 mg/l porcine insulin (Calbiochem-Behring, San Diego, CA) and 3 g/l glucose (referred to as supplemented medium) with 10% FBS. Three days later, approximately at confluency, monolayers were washed carefully with three changes of Hank's balanced salt solution (Gibco); each flask then received 15 ml of supplemented medium without FBS. "Conditioned" medium was harvested every 24 hr thereafter for lipoprotein isolation, and replaced with fresh supplemented medium without FBS.

Lipoprotein isolation

Conditioned medium harvested from 10–20 T75 flasks was pooled and cell debris was pelleted at 1000 g for 20 min at 4°C. EDTA (1 g/l) and gentamicin sulfate (1 g/l) were added and the medium was then adjusted to pH 7.4. The harvested medium was concentrated at 4°C under nitrogen pressure in Amicon stirred cells (Amicon, Lexington, MA) using Amicon PM-30 membranes. Possible loss of apoA-I and albumin into the ultrafiltrate or by

adsorption onto the membranes was tested by single radial immunodiffusion and dot blotting of samples of ultrafiltrate and extracted membranes. Using this procedure, no apoA-I or albumin was detectable; the assay would have detected loss of 3% of either protein. Lipoprotein fractions were isolated by sequential ultracentrifugation (24). Although artifacts due to loss of some apolipoproteins, particularly apoA-I, E, and Cs, during this isolation procedure have been reported (25, 26), ultracentrifugation allows direct comparison with the vast majority of published data on human plasma lipoproteins. As described below, little material was isolated in the d < 1.006 g/ml fraction; therefore, the concentrated medium was usually adjusted directly to 1.063 g/ml with NaCl/NaBr solution and centrifuged at 114,400 g in a Beckman 40.3 rotor at 17°C for 24 hr. The top 0.5-ml fraction followed by the next 1.5 ml were collected by pipetting. The infranatant was adjusted to 1.21 g/ml, again centrifuged for 24 hr, and the top 0.5-ml fraction (d 1.063–1.20 g/ml) was collected. All fractions were dialyzed to a d 1.006 g/ml NaCl solution containing 0.1 g/l EDTA.

Electrophoretic analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Weber and Osborn (27) was performed in 3% polyacrylamide gels for analysis of apoB (28) and in 10% polyacrylamide gels for analysis of other apolipoproteins. For 3% gels, adult human apoLDL and the high molecular weight standard kit of Pharmacia (Pharmacia Fine Chemicals, Piscataway, NJ) were used as standards. For 10% gels, the standards used included bovine serum albumin, human apoE (gift of Dr. Karl H. Weisgraber), bovine apoA-I, and lysozyme. Gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Richmond, CA).

Particle size distribution was determined by nondenaturing electrophoresis of whole lipoproteins in precast 2-16% (for d < 1.063 g/ml fraction) or 4-30% (for d 1.063-1.20 g/ml fraction) polyacrylamide gradient gels (Pharmacia) as described (29, 30). Standards used to determine particle diameter were 38.0-nm diameter carboxylated latex beads (Dow Chemical Co., Indianapolis, IN), and the high molecular weight proteins thyroglobulin, apoferritin, lactate dehydrogenase, and BSA (Pharmacia) which have Stokes radii of 8.50 nm, 6.10 nm, 4.08 nm, and 3.55 nm, respectively. For comparison with published work, molecular weights were calculated according to the manufacturer's instructions, neglecting the difference in density between protein standards and lipoproteins. Gels were stained with Oil Red O (Sigma, St. Louis, MO), according to Bautovich et al. (31) or Coomassie Brilliant Blue G-250 (BioRad); unstained gels were used for immunoblotting. Densitometric scans of all

gels were obtained with a Transidyne RFT densitometer (Transidyne Corp., Ann Arbor, MI).

Chemical analyses

Protein concentrations and cell protein mass were determined by a modified Lowry procedure (32), using bovine serum albumin as standard.

Phospholipid (inorganic phosphorus) in dialyzed lipoprotein fractions was assayed according to Bartlett (33). Triglyceride was assayed with an enzymatic reagent kit (Gilford Diagnostics, Cleveland, OH). Esterified and unesterified cholesterol were determined by a gas–liquid chromatography method (34).

Electron microscopy

For negative staining electron microscopy, lipoprotein samples were dialyzed to 0.13 M ammonium acetate containing 0.1 g/l EDTA, pH 7.4. Samples were stained with 1% sodium phosphotungstate, pH 7.4, and examined in a JEM 100C electron microscope. Particle size was determined as previously described (35).

To examine cell structure, the cells were rinsed three times with Hank's balanced salt solution and fixed with 2.5% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.4, for 1 hr at room temperature. The monolayers were postfixed in 1% osmium tetroxide in 0.15 M sodium cacodylate, "in block"-stained with 2% uranyl acetate, dehydrated with ethanol, embedded in Spurr's resin (36), and sectioned.

In one experiment, an electron-dense intercellular tracer was used in order to delineate spaces continuous with the culture medium. The method used was essentially that of Handley and Chien (37). Rinsed monolayers were pre-fixed with 2.5% glutaraldehyde in 0.15 M sodium cacodylate, followed by fixation for 2 hr with oxidized ruthenium red-OsO_4 (0.05% and 1%, respectively) in 0.1 M sodium cacodylate; the ruthenium red-OsO_4 solution was allowed to oxidize for 3.5 hr prior to use. Samples were dehydrated and embedded as above.

Quantitation of apolipoproteins by single radial immunodiffusion

Concentrations of apoA-I and albumin were determined by single radial immunodiffusion (SRID) (38). Lower limits for quantitation of albumin and apoA-I were approximately 2.5 mg/dl and 1 mg/dl, respectively. Dilutions were made with isotonic saline. Samples and standards were added to 2-mm wells in an SRID plate containing 1.5% agarose and the appropriate monospecific antiserum. The plates were incubated in a humid box on a leveled plate at room temperature, 18 hr for albumin and 48 hr for apoA-I. The immunoprecipitin rings were measured to 0.1 mm diameter with a calibrated scale (QUIP, Helena Laboratories, Beaumont, TX), which

gave diameters-squared directly. Assays were run in duplicate or triplicate. Standards included purified albumin or apoA-I (quantitated by amino acid analysis), isolated HDL, and reference serum. Assays of intact (native), heated (2 hr at 53°C), urea-denatured, Tween 20-treated, and delipidated lipoproteins and serum indicated complete reactivity of the intact, undelipidated apoA-I-containing particles.

ApoB concentration was determined by competitive ELISA by a method similar to that of Fruchart et al. (39). Standards and samples were assayed in triplicate in 96-well Immunolon I microtiter plates (Dynatech Laboratories, Alexandria, VA). A fixed amount of purified LDL (apoB) was adsorbed to the wells (0.05–1.0 μg in 100 μl of 50 mM carbonate buffer, pH 9.6; overnight at 4°C). Nonspecific binding sites were then blocked with 2% bovine albumin and 0.05% Tween 20 in phosphate-buffered saline (PBS) (1 hr at room temperature). Two washes with PBS preceded and followed the blocking step. Standards and unknowns in the range 0.05–2.5 μg/100 μl were mixed with polyclonal anti-apoB in PBS–0.05% Tween 20 and added to the wells (2 hr at room temperature). The plates were washed three times with 0.05% Tween 20 in PBS before adding second antibody (horseradish peroxidase-labeled anti-rabbit immunoglobulins, United States Biochemical Corp., Cleveland, OH) diluted 1:10,000 in 100 μl of PBS, which was allowed to react for 1 hr at room temperature. The plates were washed again two times with PBS. After reaction with 100 μl substrate, O-phenylenediamine, for 30 min at room temperature, the reaction was stopped by adding 25 μl of 0.25 M H_2SO_4, and absorbances at 492 nm were recorded with an automated plate reader.

Immunoblotting

Western blotting (40) was used to determine the distribution of apoA-I and apoE after gradient gel electrophoresis. Duplicate gels were stained with Coomassie blue. Gel electrophoresis was followed by electrophoretic transfer to nitrocellulose sheets (Bio-Rad). Transfers were carried out at 500 ma and 10°C for 16 hr. Nonspecific binding sites were blocked with 1% bovine serum albumin and 0.05% Tween 20 in isotonic saline containing 0.01 M Tris–HCl at pH 7.4–7.5 (20 ml for a 10 × 15 cm nitrocellulose sheet in a plastic bag; 30 min at 37°C on a platform rocker, with occasional manual mixing as well). Mouse monoclonal anti-apoA-I and mouse monoclonal anti-apoE were used as primary antibodies. Blots were then developed with biotinylated anti-mouse IgG and avidin:biotin:horseradish peroxidase complex (Vectastain ABC, Vector Laboratories, Burlingame, CA) using 4-chloro-1-napthol and H_2O_2 as substrate. Specificity and reactivity of antibodies were verified after original screening of monoclonal antibodies by ELISA, by dot blotting of purified antigen standards and by blotting after SDS-

PAGE or isoelectric focusing of HDL, apoA-I, apoLDL, apoVLDL, apoB, apoE, and albumin.

RESULTS

Morphology of cultured cells

The morphology of Hep G2 cells grown in the presence of supplemented medium with 10% FBS is shown in **Fig. 1A.** The free surface of the cell is studded with short microvilli. The cytoplasm typically contains numerous profiles of the rough endoplasmic reticulum, large Golgi complexes, lysosomes, multivesicular bodies, and lipid storage droplets not surrounded by membranes. Profiles of smooth endoplasmic reticulum are generally sparse. A higher magnification (Fig. 1B) of a Golgi complex reveals that the structure is composed of several flattened cisternae with associated vesicles and tubular structures. Some small vesicular structures have coated surfaces. Osmiophilic VLDL-like particles cannot be discerned within Golgi vesicles or cisternae of cells grown in the presence (Fig. 1) or absence (not shown) of serum.
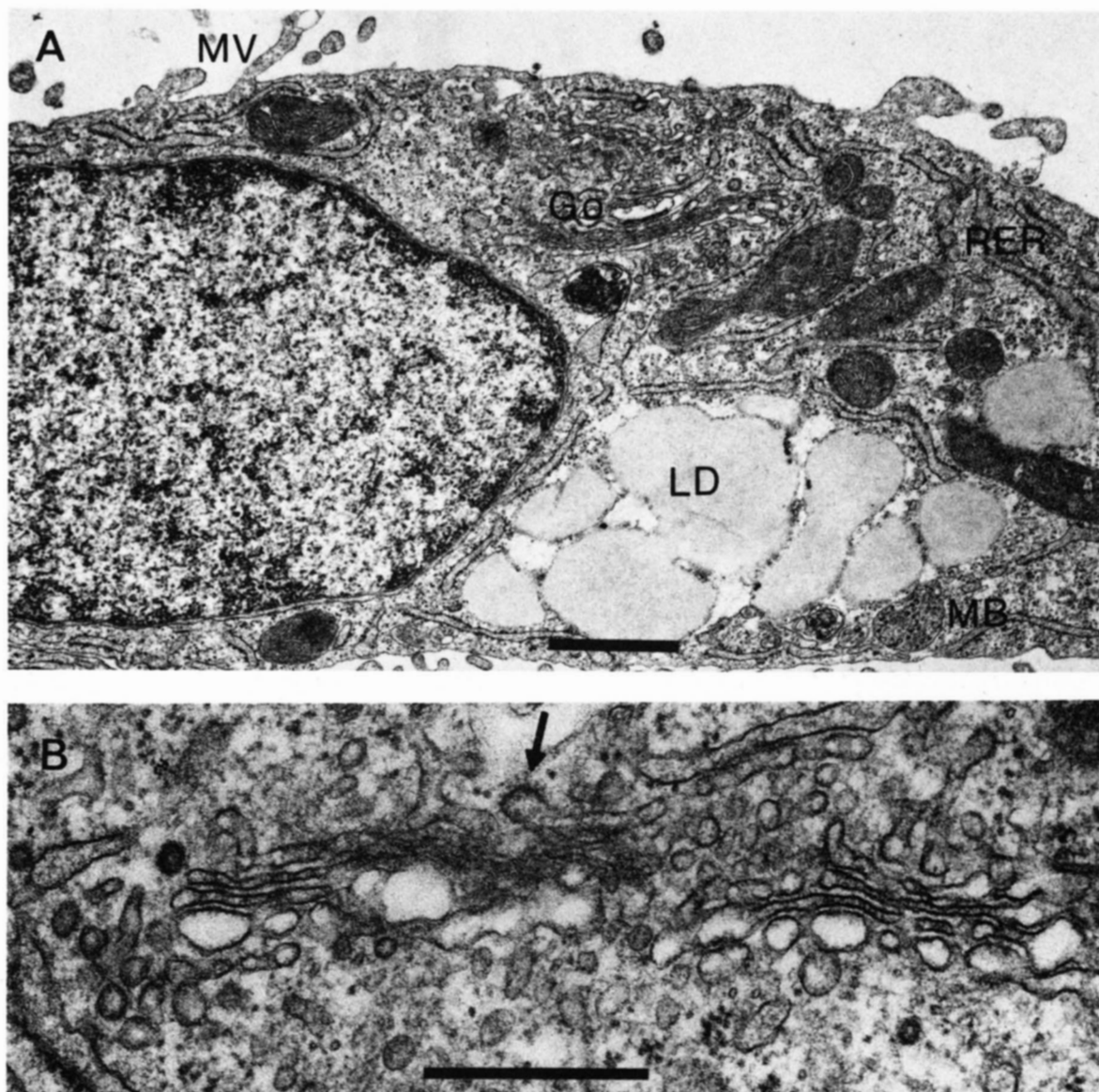

Fig. 1. Electron micrograph of a thin section cut tangentially through the monolayer. Cells were grown to confluency in supplemented medium containing 10% FBS. (A) A low magnification view of a representative Hep G2 cell. The cell has numerous short microvilli (MV) on its free surface. Along with mitochondria and the nucleus, the cell contains a large Golgi (Go), extensive rough endoplasmic reticulum (RER), storage lipid droplets (LD), and multivesicular bodies (MB). Bar = 1 micron. (B) A high magnification micrograph of a Golgi region of a Hep G2 cell. Large vesicular structures associated with the Golgi are electron lucent and do not contain osmiophilic particles. Arrow indicates a coated vesicle. Bar = 0.5 micron.

Hep G2 cells do not organize in trabeculae as do rat primary cultures (41) but form sheets of cells that do not exhibit contact inhibition of growth. The confluent cells, however, do appear to maintain elements of cellular polarity, for we could frequently observe structures that were morphologically similar to bile canaliculi (**Fig. 2**). The canaliculi are 1–3 μm in diameter and possess numerous microvilli. The bile canaliculi appear to be isolated from the bulk medium, for the extracellular tracer (oxidized ruthenium red) was unable to penetrate into the canalicular lumen although the free surfaces of cells and intercellular regions were clearly delineated by the tracer.

Lipoprotein composition

Hep G2 concentrated medium was ultracentrifugally separated into two lipoprotein fractions: d < 1.063 g/ml and d 1.063–1.20 g/ml. The d < 1.063 g/ml fraction was usually not subfractionated further because less than 1%

of the total protein in the d < 1.063 g/ml fraction floated at d 1.006 g/ml, the density of VLDL. The d < 1.063 g/ml fraction, therefore, predominantly represented lipoproteins in the density range of LDL. Compositions of the two major lipoprotein fractions isolated from Hep G2 medium are presented in **Table 1;** literature values (42) for plasma fractions are provided for comparison. The Hep G2 d < 1.063 g/ml fraction is distinctively different from plasma LDL in its lipid composition: triglyceride, not cholesteryl ester, is the major core lipid, and the proportion of unesterified cholesterol is twofold greater than in plasma LDL. The Hep G2 d 1.063–1.20 g/ml fraction has a lower protein-to-lipid ratio than plasma HDL. In addition, this fraction is deficient in core lipids, particularly cholesteryl ester, and is enriched in polar lipids, unesterified cholesterol and phospholipid. The molar ratios of unesterified cholesterol to phospholipid in the d < 1.063 g/ml and d 1.063–1.20 g/ml fractions are 1.0
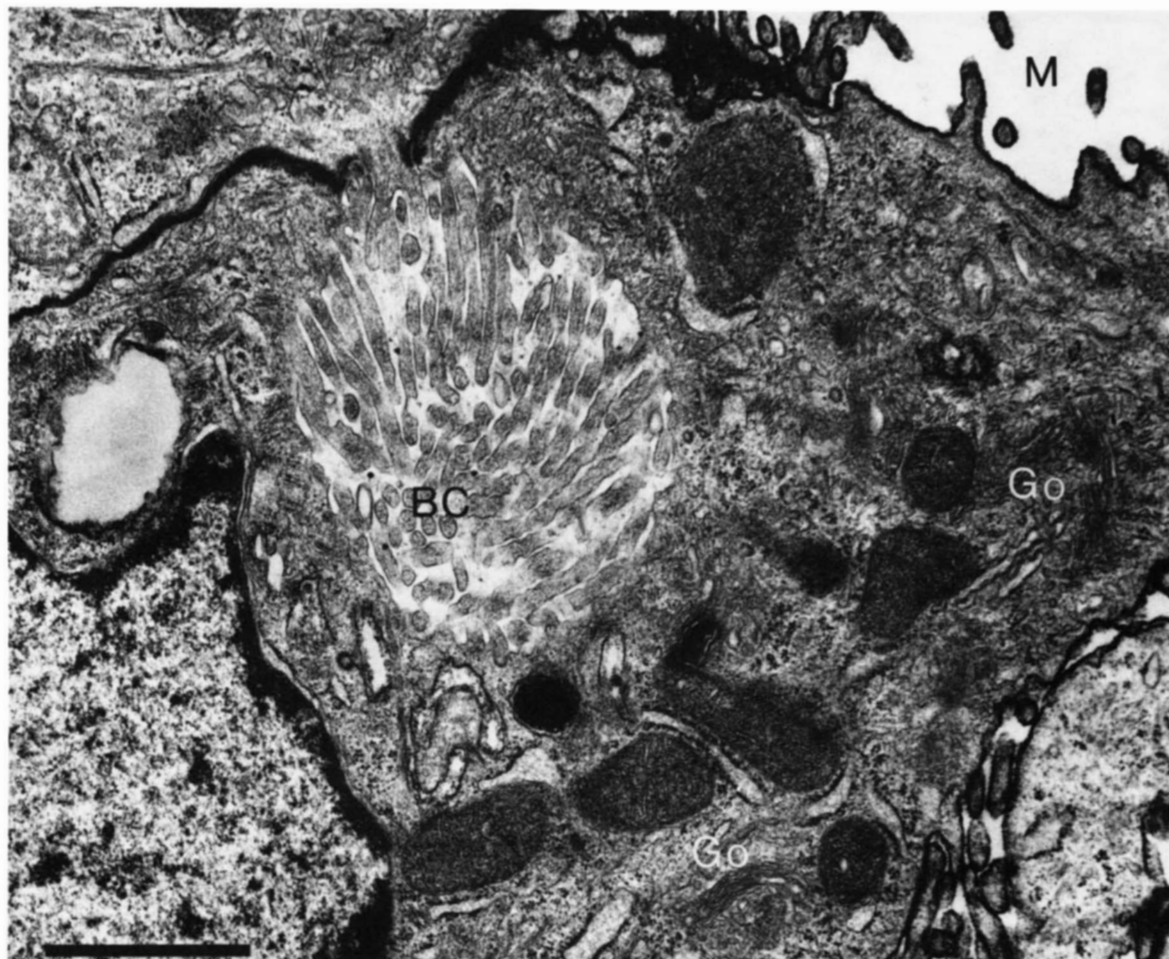

Fig. 2. An electron micrograph of a section cut perpendicular to the plane of the Hep G2 monolayer. Note the bile canalicular (BC) structure formed by two cells; numerous microvilli extend into its lumen. The monolayer was treated with oxidized ruthenium red-OsO₄ as an intercellular tracer (see Methods). The dense tracer outlines the free border of the cells and also the intercellular spaces; it does not penetrate into the lumen of the bile canaliculus, which is thus not in communication with the culture medium (M). Golgi complexes are indicated as Go. Bar = 1 micron.

TABLE 1.   Composition of the major fractions isolated from Hep G2 medium[a]

	Hep G2 d < 1.063 g/ml	Plasma[b] LDL	Hep G2 d 1.063–1.20 g/ml	Plasma[b] HDL
	% ± SEM			
Protein	27 ± 4	18–22	38 ± 3	45–55
Phospholipid	28 ± 3	16–25	40 ± 3	26–32
Unesterified cholesterol	14 ± 2	5–8	16 ± 3	3–5
Cholesteryl ester	6 ± 1	45–50	3 ± 1	15–20
Triglyceride	25 ± 6	3–9	2 ± 1	2–7

[a]Values represent the percent of the summed mass of the assayed components, mean ± SEM (where n = 7).
[b]Plasma values (42) are shown for comparison.

and 0.8, respectively, as compared with plasma values of 0.6 and 0.3, respectively (42). Based on total protein determinations, the apparent rates of secretion for proteins in the d < 1.063, d 1.063–1.20, and d > 1.21 g/ml fractions are 1.2 ± 0.4, 3.3 ± 0.3, and 170 ± 17 mg/g cell protein per day, respectively (data from an average of 32 flasks pooled and assayed each day for 3 consecutive days).

Electrophoresis of the d < 1.063 g/ml fraction on 3% SDS-PAGE revealed a major band in the region of apoB-100 when compared with plasma LDL (**Fig. 3**). The variants B-74, B-48, and B-26 were not seen on 3% gels of culture medium d < 1.063 g/ml lipoproteins. In unreduced samples a larger molecular weight band was noticeable above the B-100 position, but upon reduction with β-mercaptoethanol, only apoB-100 was evident. The participation of apoB in intermolecular disulfide bonds is reminiscent of the behavior of Lp(a) (43), although we observed no bands other than apoB upon reduction. To rule out the presence of Lp(a), concentrated medium was tested by radioimmunoassay but no Lp(a) was detected (J. Albers, T. Forte, and B. Cahoon, unpublished results). The larger band thus appears to be an oligomer of apoB.

Fig. 4 reveals the apolipoprotein electrophoretic patterns on 10% SDS-PAGE of the d 1.063–1.20 g/ml fraction and, for reference, normal plasma HDL. The predominant apolipoprotein in both Hep G2 and plasma d 1.063–1.20 g/ml fractions is apoA-I. ApoE is the second most abundant apolipoprotein in the Hep G2 fraction as opposed to apoA-II in plasma HDL. A small quantity of apoE in the Hep G2 fraction is present in the form of the apo(E–A-II) complex. Based on densitometry, and assuming equal chromogenicity for the proteins, the estimated percent distribution for the reduced apolipoproteins was: apoA-I, 72 ± 7%; apoE, 16 ± 4%; apoA-II, 8 ± 4%; and apoC, 4 ± 2% for Hep G2 (n = 7; mean ± SEM), while those for plasma HDL were 76 ± 2%; 1.0 ± 0.8%; 21 ± 3; and 3 ± 0.7% (n = 3), respectively.

Electron microscopic structure of lipoproteins

The morphology and size distribution of isolated Hep G2 fractions determined by electron microscopy are

shown in **Fig. 5.** Although lipoproteins in the d < 1.006 g/ml fraction are extremely low in concentration, particles can be seen if the sample is permitted to concentrate on the grid by drying without blotting the sample (Fig. 5A). The particles are heterogeneous with respect to size (range, 24–92 nm) and shape, and are frequently not round. The interiors of some particles appeared to contain negative stain, similar to observations with lipolyzed chylomicrons (44) and with VLDL from LCAT-deficient patients (35). In contrast to the d < 1.006 g/ml fraction, the d 1.006–1.063 g/ml fraction is homogeneous with respect to size and morphology, as illustrated in Fig. 5B. The particles are round and have a mean diameter of 24.5 ± 2.3 nm, which is similar to that of plasma LDL. The d 1.063–1.20 g/ml fraction (Fig. 5C) is heterogeneous with respect to morphology. At least two populations of particles can be seen: one is discoidal and forms rouleaux, and the other consists of small, round particles. These two populations were sized separately and distributions for each are shown in Fig. 5C. The mean disc dimensions are
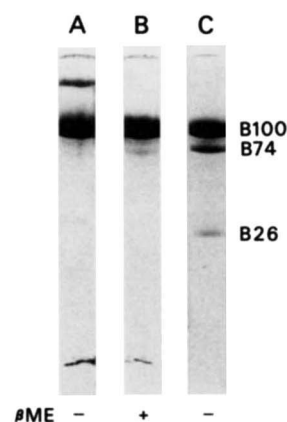

Fig. 3.   SDS-polyacrylamide gel (3%) electrophoresis of d < 1.063 g/ml fractions. (A) Hep G2 fraction, unreduced; (B) Hep G2 fraction reduced with β-mercaptoethanol; (C) adult plasma d 1.02–1.063 g/ml fraction unreduced. The unreduced Hep G2 fraction possesses primarily apoB-100; however, an additional larger molecular weight band is also visible. This band disappears when β-mercaptoethanol is added to the sample. The plasma fraction is one that demonstrates not only the presence of B-100 but also the smaller molecular weight fragments, B-74 and B-26. Approximately 40 μg of protein was applied to each gel.
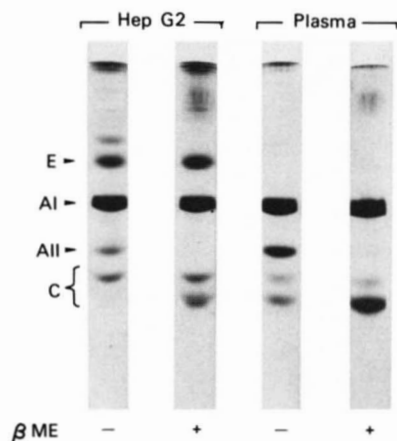
Fig. 4. SDS-polyacrylamide gel (10%) electrophoresis of Hep G2 d 1.063–1.20 g/ml lipoproteins compared with the same density fraction from plasma. Unreduced Hep G2 lipoproteins (– βME) possess mainly apoA-I and, in decreasing order of importance, apoE, apoA-II, and apoC. Reduction (+ βME) of the sample leads to the disappearance of the small band above apoE in the unreduced sample; thus, this band represents apo(E–A-II) complex. Unreduced and reduced plasma fractions possess apoA-I and apoA-II as major apolipoproteins. Twenty-five to thirty μg of protein was applied to each gel, and gels were stained with Coomassie R250.

14.9 ± 3.2 nm and 4.5 ± 0.3 nm (long and short axis, respectively); the small, round particles are 7.6 ± 1.4 nm in diameter.

Gradient gel electrophoresis

Nondenaturing electrophoresis in polyacrylamide gradient gels is an extremely sensitive technique for determining particle size distribution. **Fig. 6A** shows a typical scan of the Hep G2 d < 1.063 g/ml fraction electrophoresed on a 2–16% polyacrylamide gradient gel; for comparison, a plasma LDL profile is depicted in Fig. 6C. The ultracentrifugally isolated Hep G2 fraction exhibits two sharp peaks that stain with Coomassie G250: a major one at 25.6 ± 0.2 nm and a minor one at a position corresponding to 31.4 ± 0.4 nm (n = 8). No band was observed at the position expected for VLDL. In order to test for artifacts that may have resulted from the ultracentrifugation step, concentrated medium was applied directly to the gel, electrophoresed, and stained with the lipid stain, Oil Red O. Under these conditions, only the 25.6-nm peak was observed (Fig. 6B). In the ultracentrifugally isolated fraction, the appearance of the minor 31.4-nm peak could be prevented by the addition of 1% dithiothreitol to the sample prior to electrophoresis (not shown). Since the volume of a 31.4-nm sphere is twice that of a 25.6-nm sphere, it is likely that the particle banding at the 31.4-nm position is a disulfide-bonded dimer of 25-nm particles. Presumably the newly secreted apoB becomes oxidized upon exposure to the cystine-containing medium, and some interparticle disulfide bond exchange occurs during ultracentrifugation while

the particles are highly concentrated. Thus the lipoproteins of d < 1.063 g/ml produced by Hep G2 cells are primarily a single triglyceride-rich species of 25.6 nm by gradient gel electrophoresis. Plasma LDL (Fig. 6C), by comparison, tend to show size heterogeneity, and typically two or more peaks or shoulders are present. Moreover, peak position for the major LDL component from plasma varies from subject to subject (30).

The size distribution of the Hep G2 d 1.063–1.20 g/ml fraction electrophoresed on 4–30% gradient gels is shown in **Fig. 7**. Two different densitometric scans are shown in Fig. 7A and B, illustrating typical variability in relative peak height. Although relative heights vary, peak positions in the d 1.063–1.20 g/ml fraction are quite consistent. Peaks are observed at 13.4 ± 0.2 nm, 11.9 ± 0.2 nm, 9.7 ± 0.1 nm, and 7.4 ± 0.1 nm (n = 14). In addition, variable numbers of other minor peaks are observed. Plasma HDL size distribution, represented in Fig. 7C, is distinctly different from that of the Hep G2 product. Most notably, plasma HDL almost always have a pronounced peak in the 8.4-nm region [(HDL$_{3a}$)$_{gge}$] where there is a minimum in the Hep G2 scan. Additionally, plasma HDL contain little or no material in the 7.4-nm region or in the size range greater than 12 nm (29).

The scan pattern of the d 1.063–1.20 g/ml fraction is complex; however, there were no major changes upon addition of the LCAT inhibitor p-hydroxymercuriphenyl-sulfonic acid (2 mM) to the freshly harvested medium or upon addition of 1% dithiothreitol to the sample before electrophoresis (data not shown). Since HDL size distribution is not significantly altered and discoidal particles are observed both in the presence or absence of LCAT inhibitor, it is presumed that LCAT is not active in the medium during concentration and isolation steps. In one experiment, conditioned medium was harvested daily and cells were refed with fresh supplemented medium over a 5-day period; no major change was seen in the size distribution of either d < 1.063 g/ml or d 1.063–1.20 g/ml fractions evaluated by gradient gel electrophoresis (data not shown). This suggests that Hep G2 cells produce lipoproteins that are reproducible in size and are not derived from contaminating fetal bovine serum lipoproteins.

In evaluating 4–30% gels it must be remembered that the gels are calibrated on the basis of Stokes' radius, which assumes spherical geometry, but that Hep G2 d 1.063–1.20 g/ml lipoproteins contain numerous nonspherical particles (discs), as determined by electron microscopy. Discoidal lipoproteins are known to migrate farther than spherical particles of the same diameter (45). The sizes assigned in Fig. 7, therefore, represent actual particle diameter only when the particle is spherical. When the shape of the particle responsible for a given peak is unknown, the assigned "diameter" merely indicates the peak's position in the gel with respect to the standards. Since a spherical 7.6-nm particle was observed in the
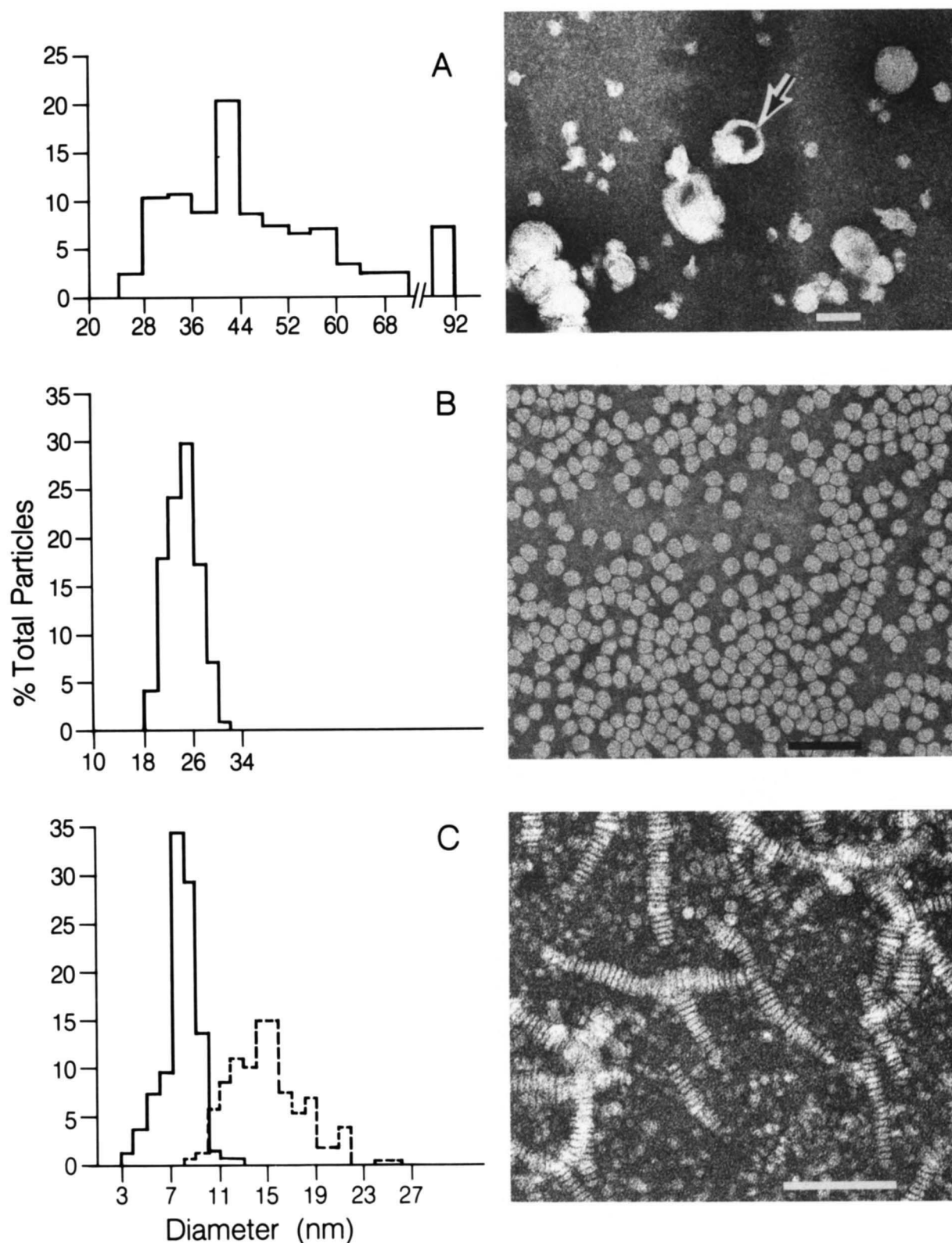
Fig. 5. Electron micrographs of lipoproteins isolated from the medium and their corresponding size distributions. (A) The d < 1.006 g/ml fraction. This fraction has an extremely wide range of particle size and is heterogeneous with respect to particle morphology. (B) The d 1.006–1.063 g/ml fraction. These are apoB-containing particles that are similar in size and morphology to plasma LDL. (C) The d 1.063–1.20 g/ml fraction. At least two distinct populations are visible: discoidal ones, in rouleaux (size distribution designated by the dashed line), and individual, small, round ones (size distribution shown as solid line). Bar marker in all micrographs represents 100 nm.

d 1.063–1.20 g/ml fraction by electron microscopy, the gradient gel peak at 7.4 nm probably represents a spherical particle. The other peaks may represent subspecies of discoidal particles, but their morphology has not been determined conclusively.

Gradient gels of lipoproteins from the Hep G2 d 1.063–1.20 g/ml fraction stained poorly with Oil Red O even when large amounts of protein were applied to the gels. Thus, consistent with the overall composition of this fraction, it is likely that none of the major components of this fraction contains much nonpolar lipid. Because of this lack of staining, the size distribution of these lipoproteins
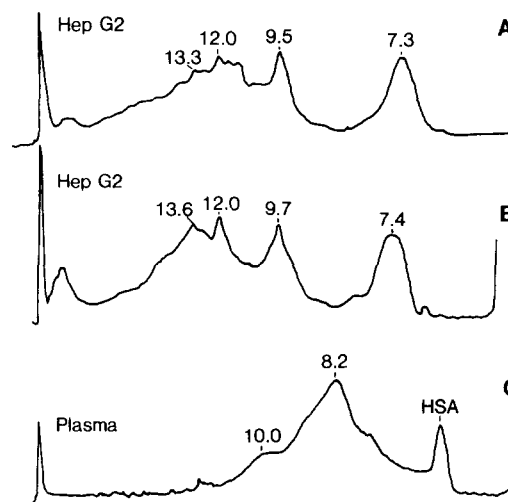

Fig. 7. Four to thirty percent gradient gel electrophoretograms. (A) and (B) are d 1.063–1.20 g/ml fractions from two different experiments. Major peaks are indicated; although there are minor differences from one scan pattern to the next, peaks are consistently seen at approximately 13.3, 12.0, 9.5, and 7.5 nm. (C) Plasma d 1.063–1.20 g/ml fraction shown for comparison. Plasma HDL have a major peak at 8.2 nm [(HDL$_{3a}$)$_{gge}$, according to Nichols, Blanche, and Gong (29)] where Hep G2 HDL have a minimum. HSA denotes the human serum albumin peak; numbers over the peaks are apparent Stokes' diameters in nm. All gels were stained with Coomassie G250.

could not be determined directly by electrophoresis of uncentrifuged medium.

Apolipoprotein quantitation and immunolocalization

As shown in **Fig. 8,** albumin, apoA-I, and apoB production is roughly linear with time. Apparent secretion rates for these proteins are 31.7 ± 8.9, 4.5 ± 0.3, and 2.8 ± 1.6 mg/g of cell protein per day, respectively (n = 4 experiments). ApoE is produced at 0.57 ± 0.07 mg/g of cell protein per day (n = 3 experiments).[3] These rates do not take into account endocytosis or degradation of secreted protein.

The distribution of apoA-I and apoE in all ultracentrifugal fractions was determined immunochemically; recoveries were 84–93% and 79–108%, respectively. Less than 2% each of apoA-I and apoE was recovered in the d < 1.063 g/ml fraction. The d > 1.063 g/ml fraction centrifuged at d 1.21 g/ml was pipetted as three fractions: the top ml, the intermediate (second) ml, and the bottom 4 ml. The percent distribution of apoA-I throughout the fractions was: top, $38 \pm 4\%$; intermediate, $4 \pm 0.5\%$; bottom, $58 \pm 4\%$ (n = 3). For apoE, the distribution was: top, $88 \pm 3\%$; intermediate, $3 \pm 0.4\%$; bottom, $9 \pm 3\%$ (n = 4).[3] Thus, while most of Hep G2 medium apoE floats at d 1.21 g/ml (presumably complexed to lipid), much of the apoA-I does not.

Fig. 6. Two to sixteen percent gradient gel electrophoretograms. The peak at left is the origin. (A) Hep G2 fraction of d ≤ 1.063 g/ml isolated by ultracentrifugation. The gel was stained with Coomassie G250. The major peak is at 25.6 nm, but a small sharp peak is also visible at 31.4 nm. Although not shown, the same pattern is seen in gels stained with Oil Red O. (B) Hep G2 medium was isolated, concentrated (see Methods), and applied to the gel without ultracentrifugation. Gel was stained for lipid with Oil Red O. Note the very sharp peak at 25.6 nm and the lack of material at 31.4 nm. (C) Plasma LDL shown for comparison; gel stained with Coomassie G250. In this subject at least three peaks are visible.

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[3] ApoE immunoassay was kindly performed by Dr. Joyce C. Gibson, University of Miami School of Medicine, Miami, FL.
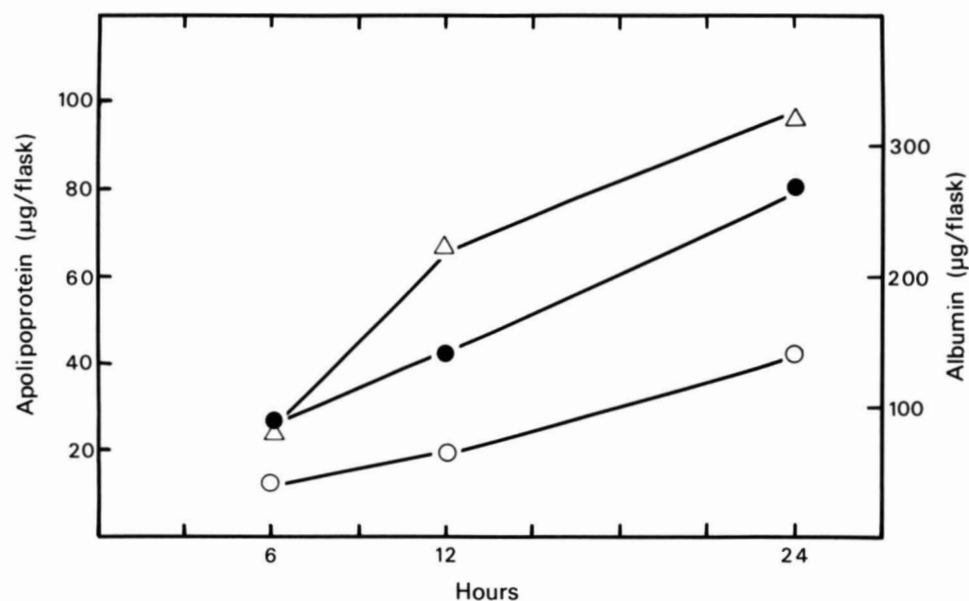
Fig. 8. Protein production with time. For each time point, medium from two 175-cm^2 flasks was harvested, pooled, concentrated 20-fold, and assayed by SRID for albumin ($\triangle$), apoA-I ($\bullet$), and apoB ($\circ$).

In order to determine whether non-lipoprotein-bound apoA-I is present in medium prior to ultracentrifugation, Hep G2 medium and fractions were concentrated, electrophoresed in 4–30% polyacrylamide gradient gels, electroeluted onto nitrocellulose paper, and probed with antibody. In the d 1.063–1.20 g/ml fraction, apoE (**Fig. 9C**) appears mainly in complexes larger than ferritin (Stokes' diameter 12.2 nm), while the majority of apoA-I (Fig. 9D) is distributed between ferritin and bovine serum albumin (Stokes' diameter 7.1 nm). A distinct apoA-I band occurs just above albumin (Fig. 9D), corresponding to the 7.4-nm band detected by protein stain (Fig. 9B). The d > 1.20 g/ml fraction (Fig. 9E) shows apoA-I primarily in the non-lipoprotein region smaller than albumin; moreover, apoA-I in concentrated medium (Fig. 9F) also shows intense staining below albumin. This suggests the presence of lipid-poor or lipid-free apoA-I in uncentrifuged medium.

DISCUSSION

Hep G2 cells maintain many of the morphological and biochemical characteristics of hepatocytes. However, in contrast to results with primary rat hepatocytes (41, 46), no VLDL-sized osmiophilic particles were demonstrable within smooth endoplasmic reticulum or Golgi vesicles of Hep G2 cells, and very little material of the size or density of VLDL was observed in the medium as judged by electron microscopy. Less than 1% of the total protein of the d < 1.063 g/ml fraction floated at 1.006 g/ml. Based on this figure, and assuming a typical plasma VLDL com-

position, it can be calculated that, at most, 6% of Hep G2 d < 1.063 g/ml triglyceride could be due to the presence of VLDL.

The paucity of d < 1.006 g/ml lipoproteins we observed is in general agreement with the preliminary results of Ellsworth, Erickson, and Cooper (47). In contrast, Tam, Archer, and Deeley (48) detected more apoB in the d < 1.006 g/ml fraction than in the d 1.006–1.063
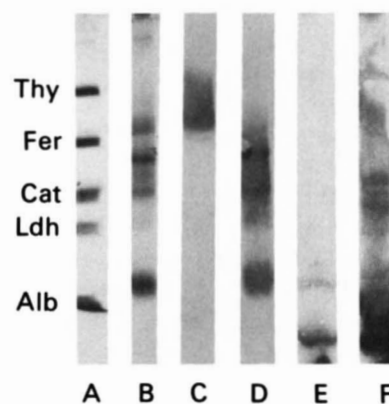

Fig. 9. Immunoblots of Hep G2 samples electrophoresed in 4–30% polyacrylamide gradient gels. (A) Gel lane showing standards (albumin, Alb; lactate dehydrogenase, Ldh; catalase, Cat; ferritin, Fer; thyroglobulin, Thy) stained with Coomassie G250. (B) Gel lane showing Hep G2 d 1.063–1.20 g/ml fraction stained with Coomassie G250. The apparent Stokes' diameters of the four major bands are: 7.3 nm (near albumin), 9.5 nm, 11.1 nm, and 13.1 nm (just larger than ferritin). (C) The d 1.063–1.20 g/ml fraction blotted to nitrocellulose and probed for apoE. (D) The d 1.063–1.20 g/ml fraction blotted and probed for apoA-I. (E) The d > 1.20 g/ml fraction blotted and probed for apoA-I. (F) Concentrated medium blotted and probed for apoA-I.

g/ml fraction of Hep G2 cells cultured in the presence of 10% lipoprotein-deficient FBS. This discrepancy may be due to LCAT and/or lipid transfer protein in the serum fraction used by the latter investigators, or it may be related to their indirect determination of apoB by precipitation of labeled antibodies, an assay which apparently was not calibrated against external standards. In a preliminary experiment we found that when cells were cultured with MEM plus 10% lipoprotein-deficient, heat-inactivated FBS, the density distribution of apoB (assayed with antibodies that do not cross-react with bovine apoB) was similar to that obtained with cells cultured under our standard conditions.

We, like Rash et al. (3), have found that B-100 is the only form of apoB present in the Hep G2 d < 1.063 g/ml fraction. Although lipoproteins of this fraction are similar in size and shape to plasma LDL, triglyceride is the major core lipid. Cholesteryl ester is only a minor lipid component and may have been derived from intracellular acyl coenzyme A:cholesterol acyltransferase activity, which has been demonstrated in Hep G2 cells (7). However, we have not ruled out the possibility of some contribution from very low levels of LCAT and lipid transfer activity in the medium. The chemical composition of the Hep G2 d < 1.063 g/ml lipoproteins is similar to that reported for the "small molecular weight" (22–25 nm diameter) plasma LDL fraction of LCAT-deficient patients (24% protein, 33% phospholipid, 20% unesterified cholesterol, 21% triglyceride, 2% cholesteryl ester; calculated for patient I.S. in ref. 49). Exposure of such triglyceride-rich particles to physiological levels of LCAT and lipid transfer activity is expected to normalize the particle composition, replacing much of the triglyceride with cholesteryl ester (50).

It has been suggested that in familial LCAT deficiency the triglyceride-rich small LDL are derived by lipolysis of VLDL (49). Although we have not completely ruled out the presence of lipase activity in the medium, several lines of indirect evidence suggest that the 25-nm particles we observed are not derived from lipolysis of secreted VLDL: 1) intracellular VLDL-sized particles were not observed; 2) conditioned medium incubated for 24 hr at 37°C showed no significant change in size of the 25-nm particle, as detected by gradient gel electrophoresis; 3) human plasma VLDL (at a concentration similar to that of secreted apoB) incubated for 24 hr with conditioned medium also showed no major change in size as determined by gradient gel electrophoresis; 4) medium conditioned by Hep G2 cells for as little as 3 hr exhibited the 25-nm peak but no VLDL-sized band after gradient gel electrophoresis and Oil Red O staining (B. Cahoon, T. Forte, and R. Thrift, unpublished observations).

Our results with Hep G2 cells suggest that the liver under some conditions may be capable of direct secretion of an apoB-containing lipoprotein which has the density of plasma LDL but is triglyceride-rich. It has been proposed from metabolic studies with pigs (51), monkeys (52), and humans (53, 54) that a portion of plasma low density apoB may be derived by direct synthesis and secretion by the liver, but the composition of putative de novo synthesized LDL has not been determined.

It is not clear why Hep G2 cells produce triglyceride-rich, LDL-like particles rather than VLDL. Since we have shown that these cells are capable of secreting the major constituents of plasma VLDL, albeit in the form of a smaller particle, the deficiency of intracellular and medium VLDL in our culture system is probably not due to a gross genetic defect. The possibility remains that specific factors or growth conditions may be able to induce true VLDL synthesis. Although the inclusion of oleic acid (0.8 mM, with 0.2 mM albumin) in the supplemented medium caused Hep G2 cells to accumulate numerous cytoplasmic lipid storage droplets, it did not induce the appearance of significant amounts of VLDL-sized particles in the Golgi or medium (B. Cahoon, T. Forte, and R. Thrift, unpublished observations). This suggests that lipoprotein triglyceride content, and thus size, are not directly related to the partition between the oxidation and esterification of intracellular fatty acids. We speculate that under our culture conditions the rate of association of triglyceride with apoB may be limiting. The existence of such a limiting step has been suggested for rat hepatocytes (55, 56), but the mechanism is not known. Insulin has been shown to increase cellular triglyceride while decreasing the size of VLDL secreted by rat hepatocytes (57); however, the omission of insulin from our cultures did not affect the size distribution of Hep G2 d < 1.063 g/ml lipoproteins (B. Cahoon, T. Forte, and R. Thrift, unpublished observations). It is conceivable that levels of endoplasmic reticular proteins involved in triglyceride transfer (e.g., ref. 58) may limit the ultimate size attainable by a nascent particle. Alternatively, the accumulation of triglyceride may be limited by the particle's residence time in the endoplasmic reticulum. Both these proposed explanations may be related to the observation that in our cultures the cells possess relatively small amounts of smooth endoplasmic reticulum. Further study of triglyceride-rich lipoprotein production by Hep G2 cells may provide new insights into the intracellular processes involved in lipoprotein assembly.

The Hep G2 d 1.063–1.20 g/ml fraction, which contains both discoidal particles and small spherical ones, is indistinguishable by electron microscopy from the HDL of familial LCAT-deficient patients (59). We have not excluded the possible existence of larger spherical particles in the Hep G2 fraction, but the difficulty of staining this fraction with Oil Red O after separation on polyacrylamide gradient gels suggests that relatively little nonpolar lipid accompanies any of the bands detected by protein staining. The discoidal morphology and lipid-staining

properties of the d 1.063–1.20 g/ml fraction are consistent with the overall composition, i.e., elevated amounts of phospholipid and unesterified cholesterol and low amounts of cholesteryl ester and triglyceride. This composition is, in fact, remarkably similar to that of plasma HDL from LCAT-deficient patients (60) (**Table 2**). As summarized in Table 2, at least three other sources of hepatic HDL have compositional characteristics in common with Hep G2 d 1.063–1.20 g/ml lipoproteins; phospholipid and unesterified cholesterol are typically high, while cholesteryl ester is low. With such low amounts of neutral lipid, discoidal structures would be expected. Discs have been observed in LCAT deficiency (59) and in perfusate from LCAT-inhibited rat livers (61). Discs were not described but were probably present in African green monkey perfusate HDL, since the equivalent fraction from livers of cholesterol-fed monkeys contained discs and had a similar composition (62). Discoidal structures were not observed in lipoprotein fractions from perfused rhesus livers, but this was attributed to the dilute nature of the fractions (63).

It is known that LCAT is secreted and functional in rat liver perfusate (61); thus it is surprising that in monkey liver perfusate and Hep G2 cultures there is little evidence of cholesterol esterification. We have recently shown in a preliminary report (64) that LCAT is secreted by Hep G2 cells. It therefore appears that the enzyme is inhibited under our culture conditions. The nature of this inhibition is presently under investigation.

The observation of sharp reproducible peaks upon gradient gel electrophoresis of the Hep G2 d 1.063–1.20 g/ml fractions is consistent with the concept, demonstrated with apoA-I–phosphatidylcholine complexes, that in the absence of LCAT activity, discoidal particles tend to occur in discrete classes having characteristic sizes (45, 65). Relatively sharp peaks that resemble those of Hep G2 are also apparent in published gradient gel electrophoresis patterns of HDL from LCAT-deficient patients (66, 67). The 7.4-nm peak observed in the Hep G2 d 1.063–1.20 g/ml fraction has an apparent molecular weight of

90,000 (estimated by gradient gel electrophoresis), which is similar to that of the major apoA-I-containing fraction from primary mouse hepatocyte cultures (85,000) (68), and to that reported for the small spherical particles isolated from LCAT-deficient plasma HDL (100,000, ref. 67; 85,000, ref. 69). It has been suggested that, in the latter case, the small spherical particles are derived from the intestine (69); indeed, it has been shown in the rat that the intestine secretes spherical 7.8-nm particles (70). Our results indicate that the human liver may also be a source of small molecular weight HDL particles.

ApoE in the Hep G2 d 1.063–1.20 g/ml fraction occurs primarily in complexes larger than ferritin, as demonstrated by immunoblots following gradient gel electrophoresis. A similar size distribution has been shown for apoE in HDL from LCAT-deficient patients (66, 67). Only 9% of Hep G2 apoE is found in the d > 1.20 g/ml fraction, which is similar to results with LCAT-inhibited rat liver perfusate (71), although values obtained with cultured rat hepatocytes have varied between 15–70% (57, 72, 73). Our observation is consistent with that of Mitchell et al. (66), who found little loss of apoE from LCAT-deficient plasma HDL to the d > 1.20 g/ml fraction. They suggested that apoE is not readily dissociated from discoidal structures. A similar explanation could account for the small quantity of apoE noted in the Hep G2 d > 1.20 g/ml fraction.

In contrast to apoE, apoA-I-associated complexes in the d 1.063–1.20 g/ml fraction of Hep G2 form a broad spectrum of presumably discoidal particles smaller than ferritin, as well as spherical particles 7.4 nm in diameter. Large amounts of apoA-I were also present in both uncentrifuged medium and the d > 1.20 g/ml fraction in a lipid-poor or lipid-free form smaller than albumin. High recoveries of apoA-I in the ultracentrifugal d > 1.20 g/ml fraction have also been described for rat primary hepatocyte cultures (73) and rat and monkey liver perfusates (63, 71). In human LCAT deficiency, at least 75% of plasma apoA-I was recovered in this fraction (50), although the corresponding value for normal human plasma is only

TABLE 2. Comparison of composition of HDL from Hep G2, LCAT-deficient plasma, non-human primate liver perfusates, and DTNB-treated rat liver perfusate

	Components (% by weight)					
	Protein	Phospholipid	Unesterified Cholesterol	Cholesteryl Ester	Triglyceride	Reference
Hep G2	38	40	16	3	2	
LCAT-deficiency	30	38	21	1	10	60
African green monkey (control perfusate d 1.07–1.21 g/ml)	34	49	7	4	6	62
Rhesus monkey (perfusate II, middle)	58	33	8	1.5	nd	63
Rat perfusate (DTNB)	38	40	12	4	5	61

10% (74). It has been suggested that there is a lower affinity of apoA-I for discoidal particles than for spherical ones, hence apoA-I dissociation during ultracentrifugation is enhanced in LCAT deficiency (50). Although ultracentrifugation may contribute to the formation of lipid-poor apoA-I from Hep G2 medium, we have shown that it is not the sole source. Immunoblots of uncentrifuged medium provide evidence that a significant fraction of apoA-I may in fact have been secreted in a lipid-poor form. Similar results have recently been described for monkey liver perfusate apoA-I (63).

Hep G2 lipoproteins of d 1.063–1.20 g/ml in many respects resemble their counterparts from other model systems as well as those of LCAT-deficient patients, and provide further evidence that plasma HDL are the products of extensive post-secretory modification. The results obtained with these cells suggest that the liver may produce not only large apoE-rich discoidal HDL (61), but also apoA-I-rich discoidal particles, small spherical apoA-I-containing particles and lipid-poor apoA-I. We conclude that the Hep G2 cell line is potentially a valuable human-derived model for investigating newly formed HDL and its subsequent metabolism, particularly with respect to the roles played by specific enzymes and factors in the conversion of precursor lipoproteins into the major circulating forms. ▉

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