Very low and low density lipoprotein synthesis and secretion by the human hepatoma cell line Hep-G2: effects of free fatty acid

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Abstract The liver is a major source of the plasma lipoproteins; however, direct studies of the regulation of lipoprotein synthesis and secretion by human liver are lacking. Dense monolayers of Hep-G2 cells incorporated radiolabeled precursors into protein ([^{35}S]methionine), cholesterol ([^{3}H]mevalonate and [^{14}C]acetate), triacylglycerol, and phospholipid ([^{3}H]glycerol), and secreted them as lipoproteins. In the absence of free fatty acid in the media, the principal lipoprotein secretory product that accumulated had a density maximum of 1.039 g/ml, similar to serum low density lipoprotein (LDL). ApoB-100 represented greater than 95% of the radiolabeled apoprotein of these particles, with only traces of apoproteins A and E present. Inclusion of 0.8 mM oleic acid in the media resulted in a 54% reduction in radiolabeled triacylglycerol in the LDL fraction and a 324% increase in triacylglycerol in the very low density lipoprotein (VLDL) fraction. Similar changes occurred in the secretion of newly synthesized apoB-100. The VLDL contained apoB-100 as well as apoE. In the absence of exogenous free fatty acid, the radiolabeled cholesterol was recovered in both the LDL and the high density lipoprotein (HDL) regions. Oleic acid caused a 50% decrease in HDL radiolabeled cholesterol and increases of radiolabeled cholesterol in VLDL and LDL. In general, less than 15% of the radiolabeled cholesterol was esterified, despite the presence of cholesteryl ester in the cell. Incubation with oleic acid did not cause an increase in the total amount of radiolabeled lipid or protein secreted. We conclude that human liver-derived cells can secrete distinct VLDL and LDL-like particles, and the relative amounts of these lipoproteins are determined, at least in part, by the availability of free fatty acid. — **Ellsworth, J. L., S. K. Erickson, and A. D. Cooper.** Very low and low density lipoprotein synthesis and secretion by the human hepatoma cell line Hep-G2: effects of free fatty acid. *J. Lipid Res.* 1986. **27:** 858–874.

Supplementary key words liver • lipids • apoproteins • lipoproteins • cholesterol • triglyceride

The lipoproteins of human serum transport hydrophobic compounds, including cholesterol, triglyceride, and phospholipid, through the aqueous plasma compartment to sites of utilization or catabolism. In normal man, the plasma low density lipoproteins (LDL) are the major carriers of plasma cholesterol, whereas the very low density lipoproteins (VLDL) transport endogenous triglycerides. Both of these lipoproteins contain the high molecular weight form of the B-apoprotein. The origin, metabolic fate, and the interrelationships of these lipoprotein classes have been under extensive investigation for some time (1, 2). Production of VLDL by the liver has been demonstrated in studies utilizing the perfused rat (1–7) and monkey (8–10) liver, rat liver slices (1), and hepatocytes grown in short-term culture (11, 12). In addition, electron microscopic evidence (13) and studies by subcellular fractionation of the liver (14) have demonstrated that precursors of plasma VLDL (300–1000 Å diameter particles) are present in the Golgi region of the hepatocyte. Although the majority of plasma VLDL is synthesized in and secreted from the liver, the intestine also produces lipoproteins similar in size and lipid composition to plasma VLDL (15, 16) which contain a low molecular weight form of apoprotein B (16).

Through the activity of extrahepatic lipoprotein lipase, VLDL lose most of their content of triglyceride to form particles poor in triglyceride and rich in cholesterol termed intermediate density lipoproteins (IDL). Studies on the fate of ^{125}I-labeled VLDL in humans (17–20) and rats (21) have indicated a precursor-product relationship in the B apoprotein of VLDL and IDL, although the evidence for a simple precursor-product relationship has been questioned (22).

Plasma LDL appear to be derived from two sources, by conversion of IDL to LDL and by direct secretion of LDL by the liver. Under most circumstances, the LDL in the circulation can be accounted for by the conversion of VLDL (19). However, in patients with familial hypercholesterolemia (23–25) and in some hypertriglyceridemic

Abbreviations: VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; EBSS, Earle's balanced salt solution; EMEM, Eagle's minimal essential medium; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline.

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subjects (26), the plasma LDL apoprotein B pool size appears to exceed the amount derived by flux from VLDL through the IDL pathway. In this case, direct secretion of particles in the density range of LDL by some organ has been postulated. The direct secretion of LDL has been demonstrated in some (12) but not all (11) studies with rat hepatocytes, the perfused liver of pigs (27), monkeys (8-10), and rats (5), and LDL-like particles have been found in the secretory vesicles of the guinea pig Golgi apparatus (28). Although in these studies the lipoproteins were isolated in the LDL density range, their size and chemical composition were different from plasma LDL. The particles appeared relatively enriched in phospholipid, free cholesterol, and triglyceride and were deficient in esterified cholesterol. Furthermore, fat-feeding in several species has been associated with the production of apoE-rich LDL-like particles (29-32). Indirect evidence for additional synthetic pathways for LDL apoprotein B in rats (33), rabbits (34), and monkeys (35, 36) has been reported.

The purpose of the present study was to investigate regulation of lipoprotein synthesis and secretion in a defined system of human hepatic origin. The minimal deviation human hepatoma cell line Hep-G2 has been shown previously to synthesize and secrete apoproteins (37-39). It was used in the present study as a model to investigate several aspects of the regulation of hepatic lipoprotein production.

MATERIALS AND METHODS

The human hepatoma cell line Hep-G2 was obtained from Dr. Barbara Knowles of the Wistar Institute of Anatomy and Biology (Philadelphia, PA). [1,2,3-^{3}H]Glycerol (200 mCi/mmol, in sterile H_2O), [carboxyl-^{14}C]triolein (103.5 mCi/mmol), 1,2-diplamitoyl-*sn*-glycero-3-phospho-[methyl-^{14}C]choline (153.0 mCi/mmol), and acetic acid, sodium salt, [1-^{14}C] (56.0 mCi/mmol, in ethanol) were obtained from New England Nuclear (Boston, MA). L-[^{35}S]Methionine (1145 Ci/mmol in sterile H_2O) and DL-[2-^{3}H]mevalonic acid lactone (1.3 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). *cis*-9-Octadecenoic acid (sodium salt), egg yolk 3-*sn*-phosphatidylcholine, 3-*sn*-phosphatidylethanolamine, 3-*sn*-phosphatidic acid, 1-oleoyl-2-lyso-*sn*-glycerophosphocholine, mono-, di-, and trioleoylglycerol, phenylmethylsulfonylfluoride, and radioimmunoassay grade bovine serum albumin were purchased from Sigma (St. Louis, MO). Ethylenediaminetetraacetic acid (sodium salt) was obtained from Baker (Phillipsburg, NJ) and gentamycin sulfate (40 mg/ml) was obtained from Schering (Kenilworth, NJ). Fetal bovine serum, Earle's Balanced Salt Solution without $CaCl_2$ and $MgSO_4 \cdot 7\ H_2O$, Minimal Essential Media (Eagle's, with Earle's salts and L-glutamine), penicillin (10,000 U/ml)-streptomycin (10,000 μg/ml) solution, and trypsin-EDTA solution (10 $\times$) were obtained from Gibco (Santa Clara, CA). T-25 tissue culture flasks were purchased from Falcon (Cockeysville, MD). Goat anti-human serum β-lipoprotein, anti-human serum albumin, and non-immune goat serum were obtained from Antibodies Incorporated (Davis, CA). X-Omat AR-2 film was obtained from Kodak (Rochester, NY). Cholesterol, stigmasterol, and 3% OV-17 on Gas Chrom Q (80-100 mesh) were purchased from Applied Science (College Park, PA). Prosil-28 was purchased from PCR, Inc. (Gainesville, FL).

Tissue culture

Hep-G2 cells were cultured in T-25 flasks in 5.0 ml of Eagle's minimal essential medium containing 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (complete medium). The cells were grown at 37°C under a humidified atmosphere of 95% air, 5% CO_2. The cell medium was replaced on the third day with 5.0 ml of fresh complete medium. By day 7 each flask contained approximately 7.2×10^6 cells. The cells were subcultured as follows. The growth medium was removed and the adherent cells were detached by treatment with 0.5 ml of a trypsin (2.5 g/l)-EDTA (1 g/l) solution. After 3-4 min of incubation at room temperature, residual tryptic activity was inhibited by the addition of 2.5 ml of complete medium. The cell suspension was mixed vigorously and a 1.0-ml aliquot of the suspension was seeded into each of three T-25 flasks containing 4.0 ml of fresh complete medium. Cell viability, as measured by the exclusion of 0.04% trypan blue, was greater than 94% on each day of culture. In all experiments, unless stated otherwise, cells were used on the fourth or fifth day of culture by which time dense monolayers were formed.

Lipoprotein preparation

Human serum was isolated from normolipemic fasting volunteers; it was allowed to clot at room temperature and the erythrocytes were removed by centrifugation at 6000 rpm for 6 min at 4°C in an International Centrifuge Model HR-1. The serum was removed by aspiration and adjusted to a final concentration of 0.04% EDTA, 0.04% NaN_3, and 0.05 mg/ml gentamycin. Lipoproteins were isolated by sequential ultracentrifugation as described by Havel, Eder, and Bragdon (40). Low density lipoproteins, $1.019 < d < 1.063$ g/ml were dialyzed against phosphate-buffered saline pH 7.4 (PBS) containing 0.04% EDTA, sterilized by passage through a 0.45-μm Amicon filter (Danvers, MA), and stored at 4°C until use.

Preparation of oleic acid/albumin complexes

Oleic acid complexed to bovine serum albumin was prepared essentially as described by Van Harken, Dixon, and Heimberg (41). Oleic acid (40.0 mg) was dissolved in 10.0 ml of 95% ethanol containing 1 mg of phenol-

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phthalein/ml, and the mixture was titrated to a phenolphthalein end-point with 1.0 N NaOH. The ethanol was evaporated under N_2, and residual alcohol was removed by drying in vacuo. The sodium oleate was dissolved with gentle heating in 4.0 ml of 0.9% NaCl. Bovine serum albumin (BSA) was delipidated as described by Goodman (42). A 1.0-g sample of defatted BSA was dissolved in 5.0 ml of 0.9% NaCl, the pH of the solution was adjusted to 7.4, and the solution was placed on ice. The pH adjustment was critical for the formation of stable, optically clear fatty acid/albumin complexes. The warmed fatty acid solution was added to the cold albumin solution, and the mixture was stirred overnight at room temperature. The next day, the complexes were sterilized by passage through a 0.45-μm filter and stored at room temperature. The ratio of oleic acid to albumin, measured as described below, after sterile filtering, was 9.0, mol/mol (mean, four preparations). For the control experiments, a 1.0-g sample of albumin was carried through the procedure in the absence of oleic acid. The residual fatty acid content of the albumin solution was 0.91 mol of fatty acid per mol of albumin. All complexes were used within 1 week of preparation.

Radiolabel incorporation into cells and media

On day 4, the medium from each flask of Hep-G2 cells was removed, and the cells were washed with 4.0 ml of Earle's Balanced Salt Solution (EBSS). The EBSS was removed, and 3.5 ml of serum-free EMEM was added to each flask. In those experiments that utilized [^{35}S]methionine, methionine-free EMEM was used. [^{35}S]Methionine (500 μCi) and/or [^{3}H]glycerol (100 μCi) or [^{3}H]mevalonic acid lactone (400 μCi) or [^{14}C]acetate (100 μCi), and the indicated amount of oleic acid/albumin complexes or the albumin control was added to each flask of cells. When [^{14}C]acetate was used, the ethanol solution was dried under N_2 and the radiolabel was resuspended in sterile water. Aliquots of this solution were added to the appropriate flasks of cells. The flasks were then incubated for 20 hr at 37°C in a humidified atmosphere of 95% air, 5% CO_2. After 20 hr, the radiolabeled cell medium was removed by aspiration and placed on ice, and any cell debris was removed by centrifugation of the cell medium at 2000 rpm for 10 min at 4°C. The supernatant fraction was dialyzed for 48 hr against six changes, of 1.0 liter each, of buffer A (150 mM NaCl, 20 mM Tris-HCl, 0.04% EDTA, 0.02% NaN_3, 2 mM phenylmethylsulfonylfluoride, pH 7.4) at 4°C. After removal of the cell medium, each monolayer was washed twice with 4.0 ml of serum-free EMEM and then twice with PBS. The cells were harvested by scraping with a rubber policeman into 1.5 ml of PBS. Aliquots were removed for determination of cell protein and extent of radiolabel incorporation. The remainder of the cell suspension was extracted with chloroform-methanol for total lipids (see below).

Analysis of lipoproteins from cell media

Density gradient ultracentrifugation of the Hep-G2 cell medium was performed as described by Redgrave, Roberts, and West (43). All operations were carried out at 4°C. In general, a 3.25-ml sample of the dialyzed cell medium was placed in a 13.5-ml Beckman (Palo Alto, CA) Ultraclear centrifuge tube, 0.5 ml of freshly isolated normolipemic human serum was added as carrier, and the density of the mixture was adjusted to 1.210 g/ml by the addition of solid KBr. A discontinuous KBr gradient was formed by layering 3.0 ml of PBS at d 1.063 g/ml above the sample followed by 3.0 ml of PBS at d 1.019 g/ml. The remainder of the tube was filled with 2-3 ml of PBS at d 1.006 g/ml. The samples were centrifuged for 24 hr at 4°C at 40,000 rpm in a Beckman SW41 rotor in a Beckman L2-65B ultracentrifuge. After centrifugation, the top 1.0 ml of each gradient containing the VLDL was removed by aspiration. The remainder of the density gradient was fractionated on a Buchler (Fort Lee, NJ) Auto Densi-Flow II fractionator at a rate of 0.7 ml/min and 0.35-ml fractions were collected. A 20-μl aliquot of each fraction was placed in a scintillation vial with 4.0 ml of Aquasol-2 (New England Nuclear, Boston, MA), and the radioactivity was measured in a Beckman liquid scintillation counter. The density was determined by measuring the refractive index of a 50-μl aliquot of each fraction using a Zeiss Opton refractometer (Nr 128464). The distribution of protein was estimated by measuring the absorbance at 280 nm. The density gradient fractions were combined as indicated and dialyzed overnight against two 1-liter changes of buffer A.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and radioautography

Samples of the fractionated cell medium containing [^{35}S]methionine were prepared for SDS-PAGE by two methods. A sample of each combined density gradient fraction was immunoprecipitated by antibodies directed against human serum β-lipoprotein. The sample was mixed with 40 μg of human serum LDL protein, 20 μl of goat anti-β-lipoprotein serum or an equivalent amount, based on protein, of non-immune goat serum, and brought to a final volume of 1.0 ml with PBS. Preliminary experiments demonstrated that 20 μl (522 μg of protein) of anti β-lipoprotein serum quantitatively immunoprecipitated 40 μg of LDL protein. In addition, immunoblot analysis of human serum VLDL, LDL, and HDL demonstrated that the anti-β-lipoprotein antibody was specific for apoB and did not recognize apoA, C, E or albumin. After a 24-hr incubation at 4°C, the immunoprecipitates were isolated by centrifugation for 3 min at 4°C in a Beckman microfuge. The immunoprecipitates were washed twice with 1.0 ml of ice-cold PBS, followed by a single wash with 1.0 ml of ice-cold water. The pellets were resuspended in

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100 μl of SDS-PAGE sample buffer (0.063 M Tris-HCl, pH 6.8, 1% SDS, 1% β-mercaptoethanol, 15% glycerol, and 0.001% bromophenol blue). Another sample of each combined density gradient fraction was delipidated overnight at −20°C with nine volumes of ethanol–acetone 1:1 (v/v). The protein precipitates were collected by centrifugation at 2000 rpm for 10 min at 4°C. The organic phase was removed by aspiration, residual solvent was removed by drying under a gentle stream of N_2, and the protein pellets were resuspended in 100 μl of the SDS-PAGE sample buffer.

Prior to electrophoresis, all samples were heated at 60°C for 15 min. Sodium dodecyl sulfate–PAGE on 7.5% and 3.0% polyacrylamide gels was performed as described by Laemmli (44). The stacking gels contained 3% acrylamide. After electrophoresis, the gels were fixed overnight with isopropanol–acetic acid–water 25:10:65 (v/v/v). The next day, the gels were stained for 2 hr in methanol–acetic acid–water 50:10:40 (v/v/v) containing 0.05% Coomassie Brilliant Blue-G and destained in isopropanol–acetic acid–water 10:10:80 (v/v/v). The apoproteins were identified by comparison of their apparent molecular weights with those of the apoproteins of authentic human serum lipoproteins that were electrophoresed on 7.5% polyacrylamide gels under identical conditions. The apparent molecular weights ± SD were: 33,983 ± 598 (n = 5) for apoE; and 23,433 ± 423 (n = 6) for apoA-I. ApoC migrated with the dye front. ApoB migrated close to the origin in this system, consequently its apparent molecular weight could not be accurately measured.

For radioautography, the gels were dried, overlaid with X-ray film, and placed at −80°C. The radioautograms were developed and the radioactivity distribution in the gels was quantitated by excising the protein bands and placing the gel slices in scintillation vials. They were rehydrated by the addition of 0.1 ml of water and, after overnight incubation at room temperature, 10 ml of a solution of Lipofluor–Solulyte–water 10:1:0.1 (v/v/v) was added. The incubation was continued for 1 week, and radioactivity was measured as described above. The values shown represent the difference between total radioactivity and the radioactivity measured in background areas of each gel.

Isolation of Hep-G2 cellular and media lipids

Samples of Hep-G2 cells or of the combined density gradient fractions were delipidated as described by Folch, Lees, and Sloane Stanley (45). In those experiments where [^{3}H]glycerol incorporation was determined, [^{14}C]triolein was added as internal standard and for [^{3}H]mevalonate incorporation, [^{14}C]cholesteryl oleate was added as internal standard. Recovery of both radiolabeled lipids was always greater than 95%. After standing at 4°C overnight, the chloroform phase was removed, the chloroform was evaporated with gentle heating under N_2, and the lipids were resuspended in a small volume of chloroform. An aliquot was removed for determination of total radioactivity. A second aliquot was analyzed by thin-layer chromatography on silica gel G plates that were activated by heating for 1 hr at 120°C. The polar lipids were separated by developing the plates halfway in chloroform–methanol–acetic acid–water 65:25:8:4 (v/v/v/v). The plate was removed from the tank, dried, and the neutral lipids were separated by development in hexane–diethylether–acetic acid 86:16:1 (v/v/v) to within 1.0 cm of the top of the plate. The plate was dried and the lipids were visualized with I_2 vapor. The lipids were identified by comparison of their R_f values to those of authentic lipid standards (phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, lysophosphatidylcholine, *cis*-9-octadecenoic acid, triolein, cholesteryl oleate, and unesterified cholesterol) which were chromatographed on each plate. The lipid-containing areas were scraped directly into vials and 10 ml of Aquasol-2 or toluene-based scintillation fluid (toluene–Liquafluor (New England Nuclear, Boston, MA) 24:1, v/v) was added to quantitate the radioactivity in the polar and nonpolar lipids, respectively. The mass of triacylglycerol secreted by Hep-G2 that accumulated in the cell medium was measured in the media of eighteen 75 cm^2 flasks of Hep-G2 cells (85 mg of total cell protein). Nine flasks received serum-free EMEM containing 0.8 mM oleic acid/albumin complexes and nine received the albumin control at equivalent protein concentrations (9.0 mg protein/ml). After incubation for 20 hr at 37°C as described above, the cell medium from each of the nine flasks was combined and centrifuged at 2000 rpm for 10 min at 4°C to remove any cell debris. The density was adjusted to d 1.210 g/ml with solid KBr, and the lipoproteins were isolated by centrifugation at 48,000 rpm for 24 hr at 4°C in a Beckman 50.2 rotor. The total triacylglycerol content of both the supernatant and infranatant fractions was measured on the dried chloroform extract as described. The cells from each incubation condition were collected and the total cell protein content was determined as described above.

Other methods

Protein was measured as described by Lowry et al. (46) using bovine serum albumin as standard. Long chain fatty acids were determined colorimetrically by the method of Duncombe (47). Cellular triglyceride was measured by the glycerol phosphate dehydrogenase enzyme assay (Sigma kit 320-UV, St. Louis, MO) after lipid extraction as described above. Phospholipids were determined colorimetrically as described by Bartlett (48) on an aliquot of the dried cellular lipid extract.

Unesterified cholesterol and total cholesterol mass were determined using a modification of the procedure of Ishikawa et al. (49). Stigmasterol was added as internal

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standard to each sample prior to lipid extraction. The chloroform phase of each lipid extraction was dried under N_2, resuspended in 2.0 ml of chloroform, and placed in a separate 6-ml conical centrifuge tube fitted with a ground-glass stopper. This sample was dried under N_2 and resuspended in 12 ml of tetrachloroethylene. A sample of this was analyzed directly by gas-liquid chromatography for unesterified cholesterol. The remainder of the sample was saponified by adding 100 μl of tetramethylammonium-hydroxide (24% in methanol)-isopropanol 1:4 (v/v) and heating at 80°C for 15 min. After allowing the sample to cool, 50 μl of tetrachloroethylene-methyl butyrate 1:3 (v/v) was added and each sample was vortexed. Phase separation was achieved by the addition of 200 μl of water; the samples were vortexed and then centrifuged for 10 min at 2000 rpm. A sample of the lower phase was taken for total cholesterol analysis by gas-liquid chromatography. The mass of esterified cholesterol was calculated as the difference between the measured total and unesterified cellular cholesterol mass. Gas-liquid chromatography was performed using a 6-ft glass column filled with 3% OV-17 on Gas Chrom Q. The injector, column, and detector temperatures were 260°C, 240°C, and 270°C, respectively. The carrier gas (helium) flow rate was 30 ml/min. The retention times for cholesterol and stigmasterol were 7.75 min and 12.00 min, respectively.

RESULTS

Lipid composition of Hep-G2 cells

The lipid composition of dense monolayers of Hep-G2 cells cultured under basal conditions was determined (**Table 1**). This was expressed per gram of dry weight to facilitate direct comparison to literature values for the lipid composition of human liver biopsy samples (Table 1). The data from the literature that were reported in terms of liver wet weight were converted to a dry weight basis with a correction factor of 0.69, as suggested by Martinnson, Sunzel, and Hood (50). The lipid content of Hep-G2 cells was similar to that reported for normal human liver.

Lipoprotein secretion by Hep-G2 cells

To determine the characteristics of the lipoproteins secreted by Hep-G2, dense monolayers of cells were incubated for 20 hr in serum-free media containing [^{3}H]glycerol or [^{35}S]methionine to label the intracellular pools of glycerolipids (triglyceride and phospholipid) and protein. The media were harvested, the unincorporated precursor was removed by dialysis, and the media were analyzed by density gradient ultracentrifugation. Maximal incorporation of both precursors was into particles that banded at a density of 1.039 g/ml (**Fig. 1,** upper panels). On this same gradient, human serum VLDL, LDL, and high density lipoprotein (HDL) banded at density maxima of 1.015 g/ml, 1.042 g/ml, and 1.122 g/ml, respectively. Addition of normal human serum (0.5 ml) to each sample immediately prior to ultracentrifugation had no effect on the density distribution of the radiolabels, but did result in a modest increase in the recovery of the radiolabeled lipoproteins from the medium (Fig. 1, lower panels). Carrier serum was routinely included in subsequent experiments. Radiolabels in the VLDL and LDL regions were well resolved using this density gradient; however, the radiolabeled protein in the HDL region was not well resolved from the large amount of label recovered in the d > 1.210 g/ml fraction.

Effects of fatty acid on intracellular lipid metabolism

To investigate the effects of fatty acid on the synthesis and secretion of lipoproteins by Hep-G2 cells, monolayers

TABLE 1. Comparison of the lipid content of Hep-G2 cells with literature values reported for normal human liver

Tissue Source	Reference	Lipid Component[a]				
		PL	TC	UC	CE	TG
		mg/g dry weight				
Human liver	50	82.4	7.7	–	–	3.3
Human liver	51	–	–	4.6-20.0	1.0-12.2	8.4-697.0
Human liver	52	94.3	–	11.3	2.0	23.7
Human liver	53	81.0	12.6	9.7	3.2	64.5
Human liver	54	85.0	10.0[b]	–	–	–
Human liver	55	–	–	5.5	1.4	–
Hep-G2 cells		131.3 ± 39.6	–	13.5 ± 4.7	4.4 ± 1.0	31.0 ± 6.5

On day 4 after passage, dense monolayers of Hep-G2 cells were harvested, and the cellular lipid content was determined as described in Experimental Procedures. The abbreviations are: PL, phospholipid; TC, total cholesterol; UC, unesterified cholesterol; CE, esterified cholesterol; TG, triacylglycerol; –, not determined.

[a]For Hep-G2 cells, the values represent the mean ± SEM for three separate experiments of triplicate flasks of cells.

[b]Stated in text that 19-32% of the total cholesterol was esterified.

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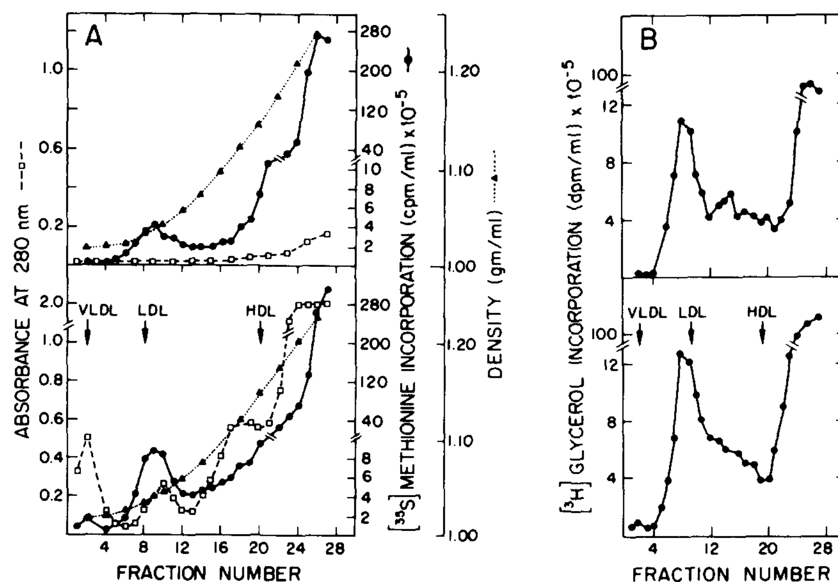

Fig. 1. Density gradient ultracentrifugation profiles of the radiolabeled products accumulating in the culture medium of Hep-G2 cells. Dense monolayers of Hep-G2 cells were incubated in serum-free media containing [^{35}S]methionine (panel A) or [^{3}H]glycerol (panel B) as described in Experimental Procedures. After incubation, the cell media were removed, dialyzed, and analyzed by density gradient ultracentrifugation. Immediately prior to ultracentrifugation, each radiolabeled cell medium was divided into two fractions and either 0.5 ml of normal human serum (lower panels) or 0.5 ml of PBS (upper panels) was added. Fractions were collected and analyzed for protein by absorbance at 280 nm, for density by refractive index, and for radioactivity. The data are representative of a typical density gradient ultracentrifugation profile of three experiments; two of duplicate flasks and one of a single flask of cells for each incubation condition.

of cells were incubated with oleic acid complexed to bovine serum albumin. Although the free fatty acid to albumin mole ratio used was unphysiologically high, this allowed the addition of the smallest amount of albumin and least volume while still delivering adequate free fatty acid. The presence of oleic acid in the cell media increased intracellular triacylglycerol mass in a dose-dependent manner (**Fig. 2**). An intracellular triacylglycerol content of about 200 μg of triacylglycerol/mg cell protein was achieved when the extracellular oleic acid concentration was 0.8 mM. When monolayers of Hep-G2 cells were examined by light microscopy, increasing numbers of large intracellular lipid inclusions were evident as the oleic acid concentration in the media was increased. Cell viability remained greater than 95% over this range of oleic acid concentrations, but incubation with a concentration of 1.4 mM oleate resulted in massive intracellular lipid accumulation, progressive detachment of the cells from the substratum, and cell death. Therefore, an oleic acid concentration of 0.8 mM was used for all subsequent experiments. This concentration is within the physiological free fatty acid range for human serum.

In the absence of exogenous fatty acid there was approximately equal incorporation of [^{3}H]glycerol into the triacylglycerol and phospholipid fractions (**Table 2**). Oleate induced a 4.5-fold stimulation of [^{3}H]glycerol incorporation into cellular triacylglycerol and a 3.2-fold stimulation into lower glycerides. There was no significant effect of incubation with oleic acid on [^{3}H]glycerol incorporation into the cellular phospholipid fraction or on the distribution of radiolabel between cellular phosphatidylcholine and phosphatidylethanolamine.

In one experiment, oleic acid also altered the relative amounts of [^{3}H]mevalonate incorporated into unesterified

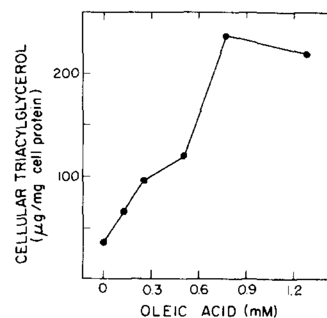

Fig. 2. Induction of intracellular triacylglycerol accumulation by oleic acid. Hep-G2 cells were incubated for 20 hr at 37°C in serum-free medium containing the indicated concentrations of oleic acid/albumin complexes. The cell media were then removed, and the cells were washed and harvested into 1.5 ml of ice-cold PBS by scraping with a rubber policeman. A 20-μl aliquot was used for determination of cell protein and the remainder was extracted for total lipid as described in Experimental Procedures. Cellular triacylglycerol was determined on the lipid extract as described. Each point represents one flask of cells.

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TABLE 2. Effect of oleic acid on the incorporation of [^{3}H]glycerol, [^{3}H]mevalonate, and [^{14}C]acetate into Hep-G2 cellular lipids

Incubation Conditions	[^{3}H]Glycerol Incorporation[a]				[^{14}C]Acetate Incorporation[b] Experiment 1		[^{3}H]Mevalonate Incorporation[b] Experiment 2	
	PC	PE	MDG	TG	FC	CE	FC	CE
	dpm/mg cell protein ($\times 10^{-4}$)				dpm/mg cell protein ($\times 10^{-5}$)		dpm/mg cell protein ($\times 10^{-3}$)	
+ 0.8 mM Oleic acid	198.0 ± 33.5	162.4 ± 18.0	265.8* ± 56.0	2222.9* ± 669.0	9.84** ± 0.77	4.28* ± 0.29	28.58 ± 0.69	7.12* ± 0.43
+ Albumin control	212.6 ± 22.4	158.5 ± 10.4	82.8 ± 36.2	490.9 ± 112.9	7.06 ± 0.53	0.55 ± 0.03	26.06 ± 0.84	1.32 ± 0.20

Hep-G2 cells were incubated for 20 hr at 37°C in 4.0 ml of serum-free medium containing 0.8 mM oleic acid complexed to BSA (20 mg of protein) or the albumin control (20 mg of protein). The incorporation of [^{3}H]glycerol into glycerolipids and [^{3}H]mevalonate and [^{14}C]acetate into cholesterol and cholesteryl esters was determined as described in Experimental Procedures. The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; MDG, mono-diglyceride. All other abbreviations are as described in the legend to Table 1. Difference significant (* $P < 0.01$, ** $P < 0.05$) as compared to the albumin control incubations. For [^{3}H]glycerol incorporation, the data were analyzed by matched pair two-tailed t-test. For [^{3}H]mevalonate and [^{14}C]acetate incorporation, the data were analyzed by a two-tailed t-test.

[a] The data represent the mean ± SEM for three separate experiments, two of duplicate flasks and one of a single flask of cells for each incubation condition.

[b] The data represent the mean ± SEM for one experiment of triplicate flasks of cells.

and esterified cholesterol (Table 2). The intracellular ratio of labeled free to esterified cholesterol decreased in the presence of oleic acid. In a second experiment, similar changes were seen for [^{14}C]acetate incorporation into cellular cholesterol (Table 2). These changes in the relative amounts of radiolabeled cholesteryl ester reflected oleic acid-induced changes in cellular cholesteryl ester mass. The cellular content of unesterified cholesterol was not changed by oleic acid administration (12.60 ± 1.36 and 12.05 ± 0.21 μg/mg of cell protein, mean ± SD, n = three flasks of cells, in the presence and absence of oleic acid, respectively), but in this experiment, the cellular cholesteryl ester content increased from 3.93 ± 0.26 to 7.56 ± 1.53 μg/mg of cell protein (mean ± SD, n = three flasks of cells, $P < 0.02$) with oleate in the medium.

The incorporation of [^{35}S]methionine into total cellular protein was not affected by oleate administration. In the absence of oleate, the specific activity of cellular protein was $1.11 \pm 0.34 \times 10^8$ dpm/mg cell protein (mean ± SD, n = five flasks of cells) as compared to $1.15 \pm 0.41 \times 10^8$ dpm/mg cell protein (mean ± SD, n = five flasks of cells) in the presence of oleic acid. Furthermore, incubation with oleic acid did not affect the cell protein content per flask. In summary, the presence of oleic acid in the cell medium induced changes in the cellular content of triacylglycerol and cholesteryl ester without affecting unesterified cholesterol or protein.

Effect of oleic acid on lipoprotein secretion

Free fatty acid administration is known to stimulate VLDL secretion. To determine whether the oleic acid-induced alterations in intracellular lipid metabolism had an effect on the density distribution of the secreted lipoproteins, Hep-G2 cells were incubated in media containing radiolabeled precursors in the presence or absence of oleic acid. After 20 hr, the media were removed, dialyzed exhaustively, and analyzed by density gradient ultracentrifugation. In control (no free fatty acid) incubations, the major incorporation of [^{35}S]methionine (**Fig. 3A**) and [^{3}H]glycerol (Fig. 3B) was into a lipoprotein with a density maximum of 1.039 g/ml. In the experiment with [^{3}H]-mevalonate as precursor, two distinct peaks of radiolabel incorporation at density maxima of 1.039 and 1.100 g/ml were evident (Fig. 3C). Inclusion of 0.8 mM oleic acid in the serum-free media resulted in a profound change in the density distribution of newly synthesized lipid and protein in the media (Fig. 3, lower panels). The amounts of radiolabeled glycerolipids and proteins that accumulated in the density range of LDL decreased while there was an increase in the amount of these components recovered in the density range of VLDL. In the [^{3}H]mevalonate experiment, there was an increase in radiolabel associated with the VLDL, while the radiolabeled cholesterol associated with particles of density similar to human serum

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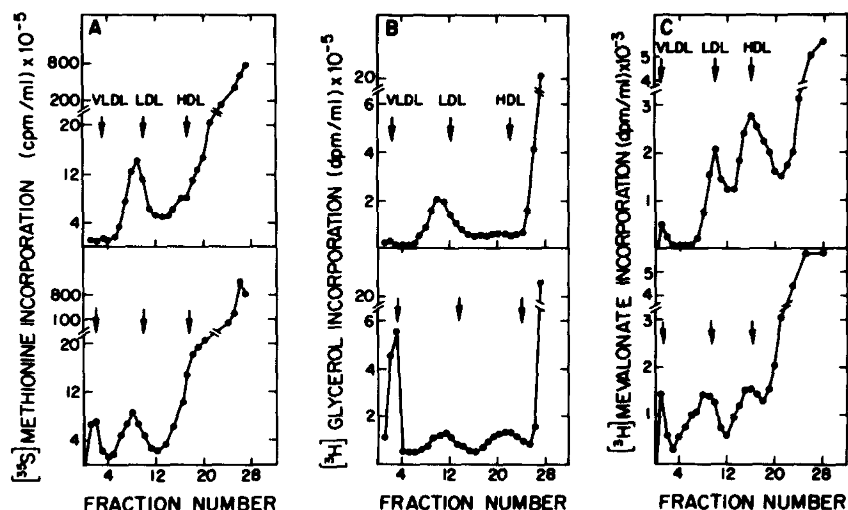

Fig. 3. Effect of oleic acid on the density gradient ultracentrifugation profiles of the radiolabeled products accumulating in the culture medium of Hep-G2 cells. Hep-G2 cells were incubated in serum-free medium containing [^{35}S]methionine, (panel A); [^{3}H]glycerol, (panel B); or [^{3}H]mevalonate, (panel C) as described in the legend to Fig. 1 in the absence (upper panels) or presence (lower panels) of 0.8 mM oleic acid complexed to bovine serum albumin. The culture media contained equivalent concentrations of albumin (4–6 mg of protein/ml). The lipoprotein fractions were isolated by density gradient ultracentrifugation after addition of 0.5 ml of human serum and analyzed as described in the legend to Fig. 1. The arrows (left to right) mark the buoyant density maxima of human serum VLDL, LDL, and HDL added as carriers prior to ultracentrifugation. For [^{35}S]methionine and [^{3}H]glycerol incorporation, each figure represents the data from a single flask of cells and is typical of three separate experiments: two of duplicate flasks and one of a single flask of cells for each incubation condition. For [^{3}H]mevalonate incorporation, the figure is representative of data from a single experiment of triplicate flasks of cells.

HDL was decreased by approximately 50%. A similar distribution of radiolabel was obtained when [^{14}C]acetate was used as a cholesterol precursor (data not shown).

The secretion of VLDL from cultured rat hepatocytes (56) and Hep-G2 cells (38) is reported to be inhibited by physiological concentrations of albumin. To determine whether lipoprotein secretion by Hep-G2 cells was influenced by extracellular albumin at the concentrations used in the present study, the accumulation of [^{35}S]methionine- and [^{3}H]glycerol-labeled components was measured in the presence and absence of albumin (4–6 mg/ml). Over this range of concentrations, albumin had no effect on the amount or the density distribution of radiolabeled lipoproteins (data not shown). Thus oleic acid induces the secretion of a lipoprotein with a density similar to VLDL and causes a reduction in the amount of radiolabeled lipid and protein in the LDL and HDL density range.

Effect of oleic acid on the lipid composition of the secreted lipoproteins

The lipoprotein fractions from the previous experiments were collected and analyzed. The total secretion of radiolabeled triacylglycerol into the cell medium did not change despite the approximately fivefold increase in the intracellular content of triacylglycerol induced by incubation with 0.8 mM oleic acid. In the absence of oleic acid, 56.7×10^4 dpm as triacylglylcerol/mg cell protein was secreted into the medium as compared to 50.1×10^4 dpm/mg cell protein in triacylglycerol secreted into the medium when oleic acid was included in the incubation. The cellular glycerolipid specific activity did not change in response to oleic acid supplementation. However, oleic acid induced a change in the density distribution of the lipoproteins secreted without increasing the amount of lipoprotein secretion in the total d $\leq$ 1.054 g/ml density range. The results of these studies are summarized in **Table 3** and **Table 4.** To confirm that there was no change in the amount of triacylglycerol secreted, the actual mass of lipoprotein triacylglycerol secreted was measured in two separate experiments. In the presence of oleic acid, the rate of triacylglycerol accumulation in the d $\leq$ 1.210 g/ml fraction was 3–8 μg of triacylglycerol/mg cell protein per 20 hr as compared to 4–6 μg of triacylglycerol/mg cell protein per 20 hr in its absence. In each case, this represented 80% of the total media triacylglycerol with the remainder recovered in the d > 1.210 g/ml fractions. These values were in agreement with the rates of triacylglycerol accumulation measured as described above by isotope incorporation (range = 3–14 μg of triacylglycerol/mg of cell protein per 20 hr).

In the absence of exogenous oleate, nearly 60% of the radiolabeled triacylglycerol in the medium was recovered in the density range of LDL with less than 10% in the density region of VLDL. Inclusion of 0.8 mM oleic acid in the culture medium produced a 54% reduction in radiolabeled triacylglycerol in the LDL region and a 324% in-

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TABLE 3. The effect of oleic acid on the density distribution of [^{3}H]glycerol incorporation into Hep-G2 cell media lipoprotein lipids

Lipoprotein Density Region	Condition	[^{3}H]Glycerol Incorporation[a] PL	MDG	TG
g/ml		*dpm/mg cell protein* ($\times 10^{-4}$)		
d ≤ 1.020	Control	1.28 ± 0.45	0.19 ± 0.05	5.44 ± 2.45
	+ Oleate	2.05 ± 0.64	0.67 ± 0.19*	17.65 ± 4.54**
1.026 ≤ d ≤ 1.054	Control	6.50 ± 2.47	1.76 ± 0.87	32.57 ± 9.28
	+ Oleate	7.30 ± 3.59	1.16 ± 0.50	14.94 ± 3.90***
1.091 ≤ d ≤ 1.143	Control	4.52 ± 1.27	0.71 ± 0.28	8.30 ± 2.69
	+ Oleate	4.89 ± 1.08	1.01 ± 0.49	8.00 ± 3.27
d > 1.210	Control	29.69 ± 14.58	4.09 ± 0.70	10.44 ± 4.78
	+ Oleate	18.98 ± 7.07	6.98 ± 5.10	9.46 ± 4.32

The cell media from monolayers of Hep-G2 cells incubated in the presence or absence of 0.8 mM oleic acid and the radiolabeled lipid precursor as described in Table 2 were removed, dialyzed, and the radiolabeled cell media lipoproteins were isolated by density gradient ultracentrifugation. The lipoprotein-containing fractions were collected and analyzed by thin-layer chromatography after total lipid extraction as described in Experimental Procedures. Difference significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.02$) from albumin control incubation. Statistical analyses were performed using a matched pair two-tailed *t*-test.

[a]The data represent the mean ± SEM for three separate experiments as described in the legend to Table 2.

crease in the triacylglycerol label found in the VLDL. No significant changes were observed in the content of radiolabeled mono-, di-, or triacylglycerol in the HDL or the d > 1.210 g/ml fraction.

In contrast to the density distribution of radiolabeled triacylglycerol, in control incubations (no free fatty acid) 45-60% of the cell medium radiolabeled unesterified cholesterol was recovered in the density range of HDL and 25-40% was recovered in the LDL region. Addition of oleic acid to the incubation media resulted in a reduction of radiolabeled unesterified cholesterol recovered in HDL and increased recovery of radiolabeled sterol in the VLDL and LDL fractions. Similar changes were seen in the distribution of radiolabeled lipoprotein cholesteryl ester. Only 3-7% of the total radiolabeled cholesterol that accumulated in the cell medium was in the ester form. The amount of ester cholesterol secreted relative to free cholesterol as determined by both radioactivity and mass (W. Craig and A. D. Cooper, unpublished observations) was low compared to that in serum lipoprotein. The apparent increase in cholesteryl ester secretion seen with free fatty acid is of unknown significance. Similar changes were seen in the experiment with [^{14}C]acetate (Table 4). Thus, oleic acid induces the secretion of a triglyceride-rich VLDL-like particle by causing redistribution of lipids from the higher density lipoprotein fractions.

TABLE 4. The effect of oleic acid on the density distribution of [^{3}H]mevalonate and [^{14}C]acetate incorporation into Hep-G2 cell media lipoprotein lipids

Lipoprotein Density Region	Condition	[^{14}C]Acetate Incorporation[a] Experiment 1 UC	CE	[^{3}H]Mevalonate Incorporation[b] Experiment 2 UC	CE
g/ml		*dpm/mg cell protein* ($\times 10^{-3}$)		*dpm/mg cell protein* ($\times 10^{-2}$)	
d ≤ 1.020	Control	0.82 ± 0.14	0.14 ± 0.02	1.36	0.20
	+ Oleate	6.30 ± 0.33*	0.77 ± 0.16**	4.10	0.43
1.026 ≤ d ≤ 1.054	Control	20.00 ± 1.41	2.04 ± 0.81	14.91	0.30
	+ Oleate	25.64 ± 4.51	3.06 ± 0.66	21.65	1.76
1.091 ≤ d ≤ 1.143	Control	22.51 ± 2.46	1.15 ± 0.16	38.70	0.75
	+ Oleate	15.04 ± 2.87	1.41 ± 0.53	10.94	0.49
d > 1.210	Control	6.02 ± 1.77	1.61 ± 0.48	3.83	0.29
	+ Oleate	1.87 ± 0.50	0.52 ± 0.05	4.72	0.20

The cell media from monolayers of Hep-G2 cells incubated in the presence or absence of 0.8 mM oleic acid and the radiolabeled lipid precursor as described in Table 2 were removed, dialyzed, and the radiolabeled cell media lipoproteins were isolated by density gradient ultracentrifugation. The lipoprotein-containing fractions were collected and analyzed by thin-layer chromatography after total lipid extraction as described in Experimental Procedures. Difference significant (*, $P < 0.01$; **, $P < 0.02$) from the albumin control incubation. Statistical analyses were performed using a two-tailed *t*-test.

[a]The data represent the mean ± SEM for one experiment of triplicate flasks of cells.

[b]The data represent the values for one experiment in which the lipoprotein density fractions from media of three flasks were combined and analyzed.

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Effect of oleic acid on apoprotein secretion

Concomitant with the redistribution of radiolabeled lipid among the media lipoproteins, oleic acid also induced a change in the amount of ^{35}S-labeled protein in the lipoprotein fractions (**Table 5,** Fig. 3). The recovery of radiolabeled protein in the d ≤ 1.015 g/ml fraction (VLDL) increased by 400%, while that in the LDL density range decreased by 50%. As with radiolabeled triglyceride, the net apoprotein accumulation in the d ≤ 1.054 g/ml fraction was not increased by oleic acid treatment. In the absence of oleic acid < 12% of the radiolabeled apoprotein in the d ≤ 1.054 g/ml fractions accumulated in the density range of VLDL, whereas 89% of the radioactivity of the d ≤ 1.054 g/ml fraction was found in the density region of LDL (**Fig. 4**).

To determine whether the failure of oleic acid to increase the net radiolabeled apoprotein content of the d ≤ 1.054 g/ml fraction was due to a generalized decrease in protein secretion, equivalent volumes of the cell media from monolayers of cells radiolabeled with [^{35}S]methionine in the presence and absence of oleic acid were immunoprecipitated with specific antisera directed against human serum albumin. In the absence of oleic acid, the total dpm immunoprecipitated from 30 μl of cell media was 1.34 × 10^5 dpm as compared to 1.26 × 10^5 dpm of radiolabeled albumin immunoprecipitated from 30 μl of media from cell monolayers that were incubated in the presence of oleate. Thus, the lack of increased radiolabeled apoprotein accumulation in the presence of 0.8 mM oleic acid was unlikely to be due to reduced cell viability or generally reduced synthetic or secretory capacity.

To quantitate possible free fatty acid-induced redistribution of the radiolabeled apoproteins, equivalent volumes of each lipoprotein fraction from the density gradients of media from cells incubated in the presence or absence of oleic acid were analyzed by SDS-PAGE and radioautography. Samples were prepared for electrophoresis by two methods: specific immunoprecipitation with antibodies directed against human serum β-lipoprotein or by direct delipidation of each lipoprotein fraction followed by solubilization of the lipid-free apoproteins prior to electrophoresis. Radioautograms were prepared of each SDS-PAGE gel and the radioactivity in protein bands in gels from three separate experiments was quantitated. The major apoprotein component of the VLDL and LDL co-migrated with authentic apoB-100 (16) of human serum LDL (Fig. 4). In the LDL, apoB-100 was virtually the only apoprotein (> 95%). On some radioautograms of LDL there was no detectable apoE, while in others, there was a trace. On average of the three radioautograms quantified, apoE accounted for 2% of the radiolabeled protein in the absence of oleate and 1.7% in its presence. Incubation of Hep-G2 cells with oleic acid resulted in a 54% reduction in radiolabeled apoB which accumulated in the LDL range (Fig. 4 and **Fig. 5**). In these experiments, there were always traces of radiolabeled apoA-I and radiolabel at the dye front. Taken together, it appears that these cells secrete some LDL that contain apoB as the only apoprotein.

In contrast, the VLDL always contained apoE as well as radiolabel at the dye front, where apoC migrates in this system, and occasionally a small amount of apoA-I. Inclusion of 0.8 mM oleic acid in the cell media resulted in a profound increase of radiolabeled apoB-100 (312%) and radiolabel at the dye front (345%) of the VLDL (Figs. 4 and 5). The recovery of radiolabeled apoE in the VLDL density range was variable. In the presence of oleate, on average, 7% of the total radioactivity was in apoE. The ratio of apoE to apoB in VLDL secreted by these cells is relatively similar to their ratio in VLDL from human serum (57). The similarity of the gel profiles of the lipoprotein fractions that were immunoprecipitated and those that were delipidated suggests that these apoproteins were present on the same lipoprotein particles.

Although the amount of radiolabel recovered as VLDL increased, the relative radiolabeled apoprotein composition of the particles from the media from the control and the oleic acid-induced cells was similar as can be seen in **Fig. 6** where the same number of counts from media of treated and untreated cells was applied to the gels. Based on these data we suggest that the LDL and VLDL are distinct particles and that the VLDL is not simply a triacylglycerol-enriched LDL or vice versa. The observation that recovery of radiolabeled apoB in LDL decreased when that in VLDL increased suggests, however, that these lipoproteins share a common apoB pool.

To further characterize the apoprotein B secreted, the radiolabeled apoproteins of the Hep-G2 cell media VLDL and LDL isolated from cells incubated in the

TABLE 5. The effect of oleic acid on the density distribution of [^{35}S]methionine incorporation into the apoproteins of Hep-G2 cell media lipoproteins

Lipoprotein Density Region	Condition	[^{35}S]Methionine Incorporation[a]
g/ml		*dpm/mg cell protein* ($\times 10^{-4}$)
d ≤ 1.020	Control	6.1 ± 3.1
	+ Oleate	24.2 ± 7.5
1.026 ≤ d ≤ 1.054	Control	126.7 ± 34.5
	+ Oleate	58.9 ± 13.4
1.091 ≤ d ≤ 1.143	Control	127.6 ± 40.4
	+ Oleate	178.8 ± 41.1
d > 1.210	Control	3764.6 ± 1031.8
	+ Oleate	4617.0 ± 949.0

Monolayers of Hep-G2 cells were incubated with [^{35}S]methionine in the presence or absence of 0.8 mM oleic acid, and the cell medium lipoproteins were separated and analyzed as described in the legend to Fig. 3. Radiolabel incorporation was determined by counting an aliquot of each lipoprotein fraction as described in Experimental Procedures.

[a]The data represent the mean ± SEM for three separate experiments, two of duplicate flasks and one of a single flask of cells for each incubation condition.

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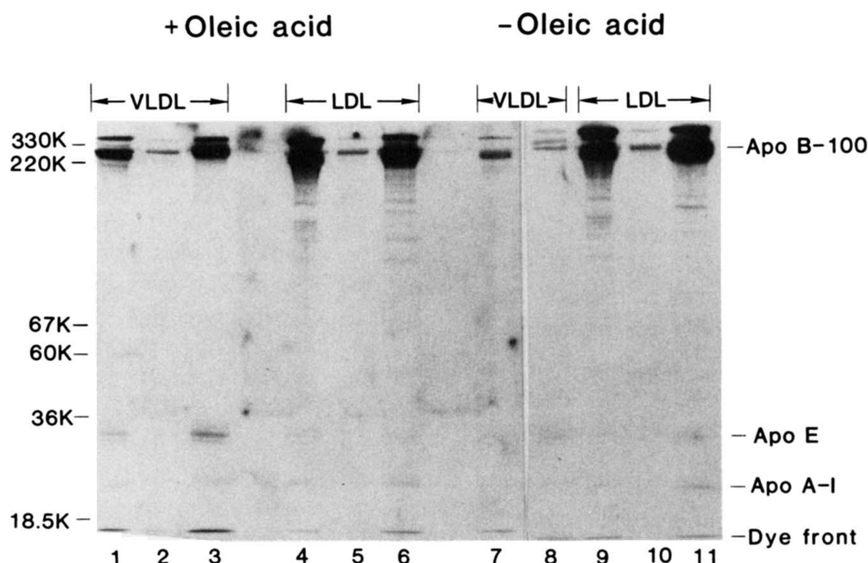

Fig. 4. Effect of oleic acid on the apoprotein profiles of Hep-G2 cell medium VLDL and LDL. Hep-G2 cells were incubated in serum-free medium containing [^{35}S]methionine in the presence (lanes 1–6) or absence (lanes 7–11) of 0.8 mM oleic acid. The radiolabeled cell medium lipoproteins were isolated as described in the legend to Fig. 1. They were analyzed by SDS-PAGE on 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate after immunoprecipitation or direct delipidation as described in Experimental Procedures. The samples were as follows: lanes 1, 4, 7, 9, immunoprecipitation with anti-β lipoprotein antiserum; lanes 2, 5, 10, control immunoprecipitation with non-immune serum; lanes 3, 6, 8, 11, delipidation with ethanol/acetone. The molecular weight markers are thyroglobulin (330,000), ferritin half-unit (220,000), bovine serum albumin (67,000), catalase (60,000), and lactate dehydrogenase (36,000). The figure shown is a photograph of the radioautogram and is typical of three separate experiments.

presence and absence of oleic acid were also examined by electrophoresis on 3% polyacrylamide gels followed by radioautography (**Fig. 7**). The major radiolabeled apoprotein component of the VLDL and LDL comigrated with human serum LDL apoB-100. Oleic acid induced a redistribution of apoprotein B between the lipoprotein fractions; radiolabeled apoB in LDL decreased while that in the VLDL increased. In some instances, lower molecular weight forms of apoB were also observed that migrated with R_fs similar to the apoproteins B-74 and B-26 of human serum LDL (Fig. 7, lanes 10, 12). Their appearance did not correlate with any specific manipulation of the cells or media. There was never any radiolabel comigrating with apoB-48, consistent with a previous report (38).

It was possible that the lipoprotein distribution after 20 hr of incubation did not reflect that of newly synthesized lipoprotein but was due to postsecretory modification. To test this, confluent monolayers of Hep-G2 cells were incubated in serum-free medium containing [^{35}S]methionine in the presence and absence of 0.8 mM oleic acid for 3 hr rather than 20 hr. With the shorter incubation, oleic acid did not induce an increase in VLDL secretion, as measured by the recovery of radiolabeled apoprotein. Ninety-three percent of the apoprotein radioactivity of the d ≤ 1.054 g/ml fraction was recovered in LDL, with more than 95% of the apoprotein radioactivity in apoB-100 (data not shown). This radiolabeled apoprotein distribution in LDL was identical to the pattern observed with 20 hr of incubation and suggests that the apoprotein patterns observed are not due to modifications caused by prolonged incubation in the cell medium. In addition, these data indicate that, under the described culture conditions, the incubation period required for fatty acid induction of VLDL synthesis and secretion is longer than 3 hr.

DISCUSSION

The details of the regulation of hepatic lipoprotein synthesis, secretion, and catabolism have been investigated extensively in nonhuman models. However, less is known about these processes in human liver. Because there are major limitations in the ability to acquire, maintain, and study normal human liver, the human hepatoma-derived cell line Hep-G2 is potentially a good system for the study of the regulation of hepatic lipoprotein metabolism. These cells have been reported to retain many normal hepatic metabolic functions including the expression of receptors for asialoorosomucoid, insulin, transferrin (58) and human plasma low density lipoproteins (59). They synthesize and secrete a wide variety of plasma proteins (60) including apolipoproteins A-I, A-II, A-IV, B, C-II, C-III, E, (39) as well as bile salts (61), and they incorporate apoproteins into lipoprotein particles that accumu-

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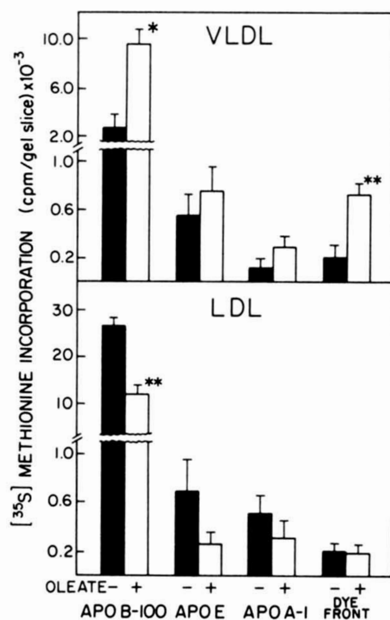

Fig. 5. Quantitation of the oleic acid-induced redistribution of radiolabeled apoproteins between VLDL and LDL. Lipoproteins radiolabeled with [^{35}S]methionine were isolated from the medium of cells incubated in the presence and absence of 0.8 mM oleic acid. They were analyzed by SDS-PAGE and radioautography as described in the legend to Fig. 4. Apoprotein bands were excised from the dried gels and the apoprotein radioactivity was quantitated as described in Experimental Procedures. The solid bars represent the apoprotein radioactivity from cells incubated in the absence of oleic acid and the striped bars, the radioactivity in the presence of oleate. Each value represents the mean ± SEM of three separate experiments. Differences significant at *$P < 0.05$ or **$P < 0.01$ by a matched-pair two-tailed t-test.

late in the cell media (37, 38). Thus, although one cannot extrapolate directly from malignant tissue to normal liver, this cell line seems a reasonable model for the human hepatocyte. The present study identified and characterized the lipoprotein particles that accumulated in the cell media and demonstrated the effects of free fatty acid availability on the distribution of newly synthesized lipid and apoprotein in these particles.

Hep-G2 cells secreted lipoprotein particles in the VLDL, LDL, and HDL density ranges as shown by comigration of radiolabeled lipids and apoproteins with human plasma lipoproteins. In contrast to the data reported for lipoprotein production by preparations of rat (11, 62, 63) or avian (64) hepatocytes and perfused primate liver (8–10), where the major secretory triglyceride-rich lipoprotein is VLDL, in the present study, the major triglyceride-rich lipoprotein that accumulated in Hep-G2 cell media had a density maximum of 1.039 g/ml, similar to that of serum LDL. Direct secretion of lipoproteins in the density range of LDL has been noted in primate liver perfusion studies (8–10). The apoprotein composition of the Hep-G2 LDL-like particles closely resembles that of human serum LDL; more than 95% of the apoprotein radioactivity was contained in the B-100 apoprotein. Electrophoresis on 3.0% polyacrylamide gels confirmed the observation that all of the B-protein was in apoB-100, with less than 1–2% of the radiolabel found in lower molecular weight B-apoproteins. These data are consistent with the concept that, in man, apoB-100 is the only form of apoB synthesized and secreted by liver (16) and that the generation of lower molecular weight forms of LDL apoB results from postsecretory processing or arises as an artifact, possibly induced by proteolytic or oxidative degradation of intact apoB-100 during the isolation procedures (65). The small and variable amounts of the other radiolabeled apoproteins present could easily be accounted for by HDL contamination as judged by the presence of apoA-I. The low recovery of radiolabeled apoE in the LDL density fraction suggests that at least some of the particles contain only apoB. Taken together, these results, along with those of Zannis, Kurnit, and Breslow (66) using fetal human liver, demonstrate that human liver-derived tissue is able to directly secrete LDL-like particles. These data potentially provide a cellular basis for the kinetic observation that, under certain circumstances, human liver may directly secrete an apoB-containing particle into the LDL density range.

The incorporation of [^{3}H]glycerol, [^{3}H]mevalonate, and [^{14}C]acetate into the lipids of the lipoproteins secreted by Hep-G2 paralleled, in general, the distribution of the radiolabeled apoproteins. Fifty-eight percent of the radiolabeled cell medium triacylglycerol was isolated in the density range of LDL. Of the triacylglycerol in the $d \leq 1.054$ g/ml fraction, 14% was found in the VLDL

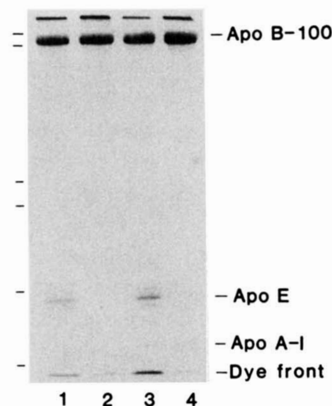

Fig. 6. Effect of oleic acid on the radiolabeled apoprotein content of VLDL and LDL. Samples of the [^{35}S]methionine-labeled Hep-G2 cell medium VLDL and LDL, isolated as described in the legend to Fig. 4, were delipidated with ethanol–acetone and analyzed by electrophoresis on 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. An equivalent amount of radioactivity (10,000 dpm) of each [^{35}S]methionine radiolabeled lipoprotein fraction was applied to the gel. Lanes 1 and 3 contained the VLDL fraction from cells incubated in the presence and absence of oleic acid, respectively. Lanes 2 and 4 represent the LDL fraction from cells incubated in the presence and absence of oleic acid, respectively. The molecular weight markers are as described in the legend to Fig. 4. The figure shown is a photograph of the radioautogram.

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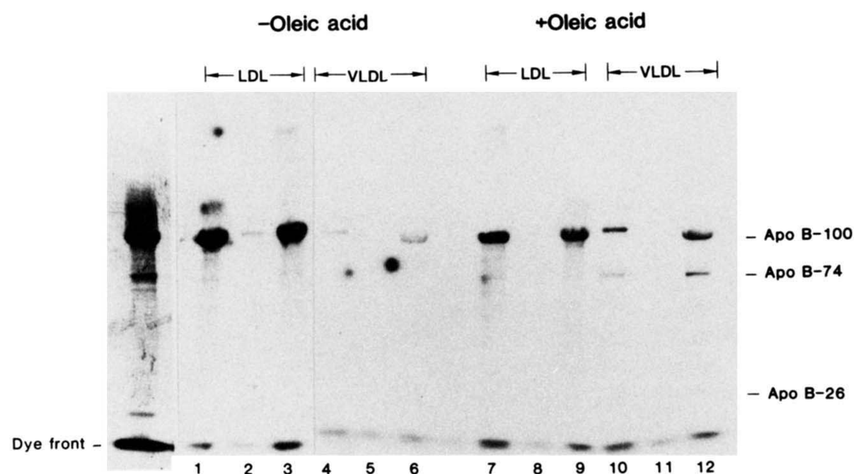

Fig. 7. Effect of oleic acid on the radiolabeled B-apoprotein distribution between VLDL and LDL. [^{35}S]methionine-labeled Hep-G2 cell medium VLDL and LDL were isolated as described in the legend to Fig. 1. Samples were analyzed by SDS-PAGE on 3% polyacrylamide gels containing 0.1% sodium dodecyl sulfate after immunoprecipitation or delipidation as described in Experimental Procedures. Lanes 1-6 represent the lipoproteins isolated from the medium of cells incubated in the absence of oleate and lanes 7-12 are those from cells incubated in the presence of 0.8 mM oleic acid. Lanes 1, 6, 9, 12: immunoprecipitation with anti-β-lipoprotein antiserum; lanes 2, 5, 8, 11: immunoprecipitation with non-immune antiserum; lanes 3, 4, 7, 10: delipidation with ethanol-acetone. The figure shown is a photograph of the radioautogram and is typical of three such experiments. The left lane shows the migration of the proteins of human serum LDL that were immunoprecipitated, electrophoresed, and stained with Coomassie Brilliant Blue-G.

and 86% was found in LDL. In reports of the composition of lipoproteins secreted into the LDL density range by both the perfused rat (5) and monkey liver (8-10), it was also noted that the particles were relatively enriched in phospholipid, unesterified cholesterol, and triglyceride as compared to plasma LDL. These data contrast with the results of Davis et al. (11), who demonstrated that in isolated rat hepatocytes greater than 95% of newly synthesized triacylglycerol was found in VLDL after 3 hr of incubation with [^{3}H]glycerol, but were consistent with the reports of Bell-Quint and Forte (12) and Patsch, Tamai, and Schonfeld (63) which described secretion of LDL-like particles by rat hepatocytes under certain metabolic conditions. These differences also suggest that the precise conditions of culture and incubation are important determinants of the nature of the particles secreted.

It is possible that the lipoproteins that accumulate in the cell media after 20 hr of incubation no longer represent nascent lipoprotein particles, but are representative of modified lipoproteins generated by prolonged incubation, particularly since Hep-G2 cell media showed cross-reactivity with antiserum prepared to human hepatic lipase (J. L. Ellsworth and A. D. Cooper, unpublished observations). Thus, the precise nature of the particles that have accumulated in media may be different than that of the particle actually secreted. In fact, one might speculate that there is some lipolytic remodeling of triglyceride-rich LDL that occurs after secretion in vivo. This would result in a decrease in triglyceride and an increase in cholesterol, thus converting a nascent LDL particle into a mature serum LDL (67). Furthermore, because Hep-G2 cells express lipoprotein receptors (59), the lipoprotein particles present in the cell media may represent the end product of several postsecretory processes. However, the similarity of particles obtained after 3 and 20 hr of incubation argues that such changes occur very rapidly or to a limited extent.

In response to free fatty acid in the culture medium, there was accumulation of triacylglycerol in the cell and accumulation in the medium of a radiolabeled triacylglycerol-rich lipoprotein with the characteristics of VLDL. The radiolabeled lipid composition of the Hep-G2 cell medium VLDL was similar to that of serum VLDL and it contained B, E, and probably C apoproteins, as does normal VLDL. The latter finding suggests that it was not simply a triglyceride-rich LDL, since the LDL secreted by these cells contained little apoE or apoC. Thus, these radiolabeled apoproteins were either secreted with the particle or were acquired by the particle after secretion. Although we cannot distinguish between these possibilities, in either case the results support the hypothesis that the lipid composition of the particle determines the apoprotein composition. Negative-stain electron microscopy of the particles, isolated by ultracentrifugation in the absence of carrier serum, confirmed their lipoprotein nature (J. L. Ellsworth, E. P. Reaven, and A. D. Cooper, unpublished observations). Their size and shape were nearly identical with those of human serum lipoproteins. The size range of Hep-G2 VLDL was 286 ± 61 Å and for Hep-G2 LDL was 178 ± 30 Å, as compared to the

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values of 302 ± 88 Å for human serum VLDL and 194 ± 30 Å for human serum LDL (mean ± SD, n = 100 particles of each lipoprotein fraction).

It has been demonstrated for freshly isolated rat hepatocytes (62, 63) that the availability of apoproteins appears to limit the secretion of triacylglycerol in response to increased stimulation by free fatty acid. In those studies, increased free fatty acid led to an increase in triacylglycerol secretion into the media but no change in either apoB, E, C, or A-I secretion. The triacylglycerol appeared to be secreted in larger lipoprotein particles. In the present study, there was a redistribution of radiolabeled triacylglycerol and apoB-100 in response to free fatty acid, but in contrast to freshly isolated rat hepatocytes, there was no increase in net triacylglycerol secretion whether measured by mass or by recovery of radiolabeled lipid. These data are consistent with the current concepts of lipoprotein structure which suggest that the apoB content of VLDL and LDL is the same and that larger VLDL particles can be synthesized by the inclusion of additional core lipid with no change in the amount of apoprotein B per particle. In secreting a VLDL-like particle, the cell seems to be responding to an intracellular signal. The distribution of triacylglycerol between LDL and VLDL changed despite the fact that net triacylglycerol accumulation was not enhanced. This is a potentially important finding; however, it is not yet established whether this finding is a characteristic of Hep-G2 or can be generalized to normal liver. Based on these and other findings, one must conclude that free fatty acid availability and increased triacylglycerol synthesis alone are not adequate stimuli for apoprotein synthesis in Hep-G2. A variety of metabolic or hormonal factors may also be necessary for regulating lipoprotein production. In fact, lipoprotein production by rat hepatocytes is affected by insulin (68, 69), carbohydrate (69) and fat-feeding (70), thyroid status (71, 72), and glucocorticoids (73). Thus, future studies with Hep-G2 may help elucidate the mechanism of control of apoB synthesis. However, based on these and other preliminary studies (74), we would suggest the hypothesis that free fatty acid flux determines the class of lipoprotein (VLDL vs. LDL) while other factors, including the amount of cholesterol available, may determine the rate of lipoprotein secretion.

The amount of triglyceride accumulation in the media of monolayers of Hep-G2 cells cultured in the presence and absence of oleic acid was estimated from the data on [^{3}H]glycerol incorporation and the measured triacylglycerol mass and compared with data from man. In normal man, with a liver weight of 1500 g, of which 15% of the wet weight is protein and 78% of the cytoplasmic mass is parenchymal cells, the liver is equivalent to 175.5 g of hepatocyte protein (75). It has been estimated that the secretion of VLDL-triacylglycerol in humans in the nonfasted state is approximately 10-15 mg/hr per kg ideal weight or 400-800 mg/hr (76). Consequently, 1.0 mg of human hepatocyte protein secretes 2-5 μg of triacylglycerol/hr or 45-90 μg of triacylglycerol in 20 hr by human liver. This would be less in the fasted state. In comparison, whether cultured in the presence or absence of oleic acid complexed to bovine serum albumin, Hep-G2 cells were estimated to secrete a total of 5-10 μg of triacylglycerol/mg cell protein per 20 hr or approximately 20% of the in vivo estimates of triacylglycerol secretion by normal nonfasted human liver. These results are comparable to the rate of 4-30 μg of triacylglycerol secreted/mg cell protein by preparations of rat hepatocytes (11, 62, 63).

There are contrasts between the composition of the lipoproteins that accumulate in the medium of Hep-G2 cells and those of human serum. The ratios of radiolabeled free to esterified cholesterol in each lipoprotein class were substantially greater than the mass ratios of free to esterified cholesterol in serum lipoproteins (77). Although Hep-G2 cells contain a responsive cholesterol esterification system (78), as does normal human liver (79), relatively little cholesteryl ester seems to be secreted. About 2 μg of cholesterol per mg cell protein are secreted. Only about 25% of this is esterified (W. Craig and A. D. Cooper, unpublished observations). These data are consistent with current concepts on the role of the lecithin: cholesterol acyltransferase-lipid transfer protein activities in human plasma (8). The LDL and HDL fractions contained relatively more triacylglycerol than comparable plasma fractions. Triglyceride-rich LDL and HDL are seen in liver disease (81) and in hepatic lipase (82) and LCAT (83) deficiencies. This is also compatible with the proposed role of hepatic lipase, LCAT, and transfer protein complex in modifying secreted lipoproteins.

Taken together, these and other results suggest that Hep-G2 is a reasonable model for studying the regulation of lipoprotein synthesis and secretion. Based on our finding, one might speculate that under normal circumstances, where the liver has an adequate supply of fatty acids, VLDL is the major secretory product, but under circumstances where the liver has a restricted supply of fatty acids relative to other lipids, such as occurs in most cell culture systems, it secretes a triglyceride-containing particle in the LDL density range.

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