Lipoprotein secretion by isolated rat hepatocytes: characterization of the lipid-carrying particles and modulation of their release

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Abstract Lipoprotein production by freshly isolated rat hepatocytes in suspension was studied during short (1–3 hr) incubation periods. The hepatocytes release very low density (d < 1.01 g/ml) lipoprotein (VLDL) particles, which as a group a) contain triacylglycerols, phospholipids, free cholesterol, and cholesteryl esters in molar proportions of 100:21:8:4; b) have a mean diameter 1.5-fold larger than those of plasma VLDL; c) have a similar electrophoretic mobility as plasma VLDL; and d) carry apoproteins B and E as major, and apoproteins AI and C as minor, protein components. These apoproteins in the secreted VLDL can be newly synthesized during the incubation, as indicated by the incorporation of [¹⁴C]leucine. The secretion of VLDL by the hepatocytes is inhibited by addition of glucagon or dibutyryl cyclic AMP, and stimulated by added palmitate; thus, as in the whole liver, the secretory process is under hormonal or substrate control also in the isolated cells condition. Phospholipids and free cholesterol are also released as components of particles with higher densities, ranging from 1.03–1.08 g/ml, and from 1.10 to 1.24 g/ml. Colchicine and cycloheximide, while strongly suppressing VLDL secretion, inhibit the release of these other particles to a lesser extent (d 1.03–1.08 g/ml) or not at all (d > 1.10 g/ml). These particles with higher densities have not been positively identified; the latter group is dissimilar to the high density lipoprotein, which occurs in rat liver perfusates, or to rat bile micelles.—**Kempen, H. J. M.** Lipoprotein secretion by isolated rat hepatocytes: characterization of the lipid-carrying particles and modulation of their release. *J. Lipid Res.* 1980. **21:** 671–680.

Supplementary key words lipid composition · apoprotein composition · apoprotein synthesis · density gradient centrifugation · bile

Lipoprotein synthesis and secretion by isolated liver parenchymal cells has been investigated previously, using either freshly isolated hepatocytes (1–3) or hepatocytes maintained for a few days in suspension (4), or monolayer (5, 6) cultures. In these studies, very low density lipoprotein (VLDL) was found to accumulate in the incubation medium; it differed to a varying degree from mature plasma VLDL in both lipid and apoprotein compositions. For instance, for rat hepatocytes the active synthesis and secretion of apoproteins B and E could be demonstrated, whereas that of apoprotein C was very low or absent (4, 6). This is in contrast to the situation in the perfused rat liver, which produces a VLDL with a complement of apoprotein C similar to that of rat plasma VLDL (7–9). Furthermore, the content of cholesteryl ester in the VLDL from cultured hepatocytes was found to be lower than that of plasma VLDL (6).

Although the secretion of HDL has been described for the perfused rat liver (8–10), documentation concerning HDL production by isolated hepatocytes is as yet lacking. In the study by Edwards, Lemongello, and Fogelman (3) the secreted lipid material was separated in two density regions (below and above 1.074 g/ml), but except for the cholesterol content the particles in the higher density region were not further characterized.

In the present study with freshly isolated rat hepatocytes, the nature of the released lipid-carrying particles has been assessed by lipid and apoprotein analysis, agarose electrophoresis, and density gradient centrifugation. In addition, the effect of various agents, known to influence the secretion of VLDL from the perfused liver, on the lipid release in the various density fractions was investigated.

MATERIALS AND METHODS

Animals

Male Wistar rats, weighing between 200 and 300 g, were obtained from the Centraal Proefdierenbedrijf

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; dibutyryl cyclic AMP, N⁶,2′-O-dibutyryl-adenosine 3′-5′-monophosphate, cyclic; SDS, sodium dodecyl sulfate; LCAT, lecithin:cholesterol acyltransferase.

TNO, Zeist, The Netherlands, and were housed in an artificially lighted (6 AM to 6 PM) and temperature-controlled (20°C) room. They had free access to water and to a commercial chow (RMH-pellets, Hope Farms, Woerden, The Netherlands). After at least one week of acclimatization, they were used as hepatocyte, plasma, or bile donors.

Hepatocytes isolation and incubation viability

Isolation of the hepatocytes of one liver was started at 9:30 AM, after induction of ether anaesthesia. The procedure of tissue dissociation was essentially that described by Seglen (11). The basal medium used for perfusions, washings, and incubation was composed according to Krebs and Henseleit (12), buffered at pH 7.4 with 20 mM HEPES in addition to the prescribed bicarbonate/carbon dioxide system (KHH-medium). It was kept at 37°C during all steps, and flushed with the carbogen gas mixture whenever possible. After cannulation of the portal vein, and during excision of the liver, non-recirculating perfusion was carried out at a flow rate of 10 ml/min with 250 ml of KHH-medium, from which calcium was omitted. A recirculating perfusion was then performed for 15 min at the same flow rate with 100 ml KHH-medium, which contained 1.3 mM calcium chloride, 1% (w/v) bovine serum albumin, and 0.05% collagenase. Thereafter the liver was bathed in 60 ml KHH-medium with 1.3 mM calcium chloride but without albumin and collagenase ("washing medium"). The Glisson capsule was torn away with forceps, and the cells were liberated by mild manual pressure and agitation of the liver lobes. The suspension was filtered through a nylon screen (60 μm pore width), and the filtrate centrifuged for 1 min at 100 g. The supernatant was discarded and the pellet was resuspended in 50 ml of the washing medium. After three further centrifugation-resuspension cycles (the last resuspension to 15 ml), 1.0-ml portions of the final cell suspension were pipetted in 100 × 30 mm cylindrical glass tubes, equipped with screw caps, for incubation. The fluid contents of the tubes had a final volume of 2.5 ml, and routinely contained 2% bovine serum albumin. Other substances (drugs, radioactive precursors, to be specified in the Results section) were always added as a solution in washing medium. For addition of palmitic acid, it was first complexed to 10% bovine serum albumin in washing medium, according to the method of Spector and Hoak (13), and the final concentration was determined by the method of Regouw et al. (14). Glucagon was dissolved to 10^{-4} M in washing medium, brought to pH 10 with 1 N NaOH, and diluted to the required concentration with washing medium.

The tubes were filled with carbogen gas, closed, placed in a water bath maintained at 37°C, and shaken at a rate of 90 strokes per min during periods of 60, 120, or 180 min. After incubation the tubes were centrifuged for 2 min at 1000 g; the supernatants were carefully aspirated and again centrifuged in polystyrene tubes for 5 min at 5000 g. The cell pellets (resuspended in 2.4 ml of distilled water) and aliquots of the final supernatants ("incubation media") were extracted according to Bligh and Dyer (15) for lipid analysis.

More than 85% of the hepatocytes were viable after 180 min incubation, as judged by exclusion of Trypan Blue. As described before (16), the cells were able to perform various biosynthetic functions (gluconeogenesis from lactate and pyruvate; fatty acid synthesis from endogenous glycogen), at rates that indicated a high degree of metabolic integrity.

Plasma and bile collection

Rat plasma was prepared from aortic blood, collected under ether anaesthesia, in a syringe with EDTA (4 mM final concentration) as anticoagulant. Bile was collected during a 3-hr period under pentobarbital anaesthesia, by means of a PT-10 cannula in the common bile duct, placed at the hepatic hilus. At the start of the bile collection, the rat was given an intravenous dose of 1 μCi of [1-^{14}C]oleate, complexed to 4% bovine serum albumin in saline.

Density gradient centrifugation

Rat plasma, bile, or hepatocyte incubation medium was subjected to density gradient centrifugation as described by Redgrave, Roberts, and West (17). For that purpose, a 3.0-ml sample was transferred to a polypropylene tube fitting in the 6 × 14 swing-out rotor MSE number 43127-111. The background density of the sample was brought to 1.24 by addition of 975 mg solid KBr; after complete dissolution the tubes were filled by the consecutive layering of 3.0-ml portions of 9%, 3%, and 1.2% (w/v) NaCl solutions, respectively. The rotor was operated for 18 hr at 39,000 rpm in a MSE-75 centrifuge at 20°C. After termination of the run, the contents of the tubes were collected in fractions of 1.0 ml by pipetting from the meniscus. An aliquot was taken for measurement of the refractive index at 20°C; the remainder was extracted by the method of Bligh and Dyer (15) without any prior desalting treatment. The density (ρ) of the gradient fractions was read from a graph, obtained by measuring both refractive index (Abbott refractometer) and density (DMA-40, Paar, Graz, Austria) at 20°C in fractions from separate runs, carried out for that special purpose. Different graphs were obtained for either a 2% solution of bovine serum albumin in KHH-medium or a rat plasma sample. Recovery of lipids from the centrifuge tubes

was checked routinely, and usually found to be better than 90%.

Agarose electrophoresis

Two hundred μl of rat plasma or hepatocyte incubation medium were mixed with 200 μl 80% sucrose, and applied on top of a vertical agarose gel (0.5%, in a cassette of 8 cm width and 3 mm thickness). Electrophoresis was carried out with 0.1 M Tris-glycine buffer, present in both the electrode chambers and the gel. A constant current of 30 mA per gel cassette was applied for 45 min. The gel was then cut in transverse sections (5 mm height), which were lyophilized, crushed, and extracted with chloroform–methanol 1:1 (by vol) for lipid analysis. Recovery of triacylglycerol from the gel was between 60 and 70%.

Lipids and bile acid determinations

The lipid extracts of the cell pellets, incubation media, centrifugation fractions, or agarose gel sections, were applied on a thin layer of silica, which was developed as described by Van Gent (18). After iodine staining, the various lipid classes were identified and scraped from the plate. The scrapings were either suspended directly in 10 ml "Insta-Fluor" (Packard) for scintillation counting, or extracted with organic solvents for chemical assay of the lipid mass. Triacylglycerols were determined as glycerol (19) after extraction with hexane–isopropanol 7:4, phospholipids as phosphate (20) after extraction with chloroform–methanol–water 75:25:4, free cholesterol by an enzymatic-fluorimetric method (21) after extraction with ethanol, and cholesteryl esters by the same method after extraction with chloroform and saponification in methanolic KOH. Recovery of each of these lipids, from the complete sequence of extractions and separations, was found to be greater than 90%.

The content of bile acids in the centrifuge fractions of rat bile was determined in the upper phase of the Bligh and Dyer extraction (found to contain at least 85% of both free and conjugated primary bile acids, also in the presence of high KBr concentrations). These upper phases were dried, and the residues were extracted with methanol. Aliquots of these extracts were assayed enzymatically for total bile acids (22).

SDS polyacrylamide gel electrophoresis

The apoprotein compositions of lipoproteins from hepatocyte incubation medium or plasma were analyzed by electrophoresis on 10% polyacrylamide gels in the presence of 0.1% SDS (23). The fractions obtained from the density gradient centrifugation were pooled, as indicated in the Results section.

These pools were dialyzed twice for 24 hr against 300 volumes of distilled water, and then lyophilized. The residue was suspended without prior delipidation in 200 μl of 0.01 M phosphate buffer, containing 0.1% SDS, 0.001% bromphenol blue, and 10% glycerol. This suspension was heated for 5 min at 100°C before determining the protein content in a 50-μl portion according to the method of Bradford (24). The gels were loaded with 30 μg of protein in 100 μl of the above buffer, electrophoresed for 16 hr at 20 mV and stained with Coomassie Brilliant Blue. In order to obtain sufficient apolipoprotein material from hepatocyte incubations, the medium from six parallel incubation tubes was combined. The fractions with d > 1.10 g/ml contained large amounts of serum albumin, interfering with the electrophoretic analysis. In order to remove this albumin, these fractions were adjusted to d = 1.25 g/ml by addition of solid KBr, overlayered with KBr solution of d = 1.25 g/ml, and centrifuged for 24 hr as described above. The top 1.0-ml fraction was then used for gel electrophoresis.

When hepatocytes were incubated with [^{14}C]leucine, the gels were stained, photographed, and then cut in transverse sections (3 mm). These sections were dissolved in alkaline peroxide (25) prior to addition of 10 ml of scintillation fluid (dioxan scintillator, Packard) for radioactivity determination. Recovery of added radioactivity from the gel was 70% by this method.

Electron microscopy

Density gradient centrifugation fractions were dialyzed against 300 volumes of 0.15 M NaCl, containing 0.01% Na$_2$ EDTA, and concentrated by centrifugation in conical ultrafilters (Amicon CF-25). Aliquots of the concentrated lipoprotein fractions were examined after negative staining with uranyl acetate (26; method 1) under a Philips EM 300 electron microscope in the transmission mode.

Materials

Collagenase was Type II from Worthington; bovine serum albumin (fatty acid-free) was from Sigma; radioactive compounds were from the Radiochemical Centre; thin-layer silica plates were from Merck (art.nr. 2050); dibutyryl cyclic AMP, colchicine, and cycloheximide were from Boehringer; glucagon was from Eli Lilly.

RESULTS

Lipid content of hepatocytes and incubation medium

Lipid levels in rat hepatocytes and their incubation medium after 60 min of incubation are given in

TABLE 1. Lipid content of isolated rat hepatocytes and their incubation medium after 60 min incubation

	N	Cells	Medium	% in Medium
		nmol/mg dry cell weight		
Triacylglycerols	5	21.1 ± 5.6	1.54 ± 0.42	8.1 ± 2.7
Phospholipids	4	115.9 ± 17.1	6.0 ± 1.1	4.6 ± 1.3
Free cholesterol	5	14.1 ± 3.4	0.65 ± 0.20	5.0 ± 1.7
Cholesteryl esters	4	1.4 ± 0.7	0.19 ± 0.16	7.8 ± 4.8

Results are given as nanomoles of each lipid class per mg dry cell weight in the cell pellet ("cells") or supernatant ("medium") after incubation and centrifugation as described under Methods. Data are means ± SD, for the indicated (N) number of separate incubations with different hepatocyte preparations.

Table 1. As can be seen, the percent triacylglycerol and cholesteryl ester found in the medium is greater than that of phospholipids and free cholesterol, indicating that the various lipids appear in the medium by different mechanisms. During the second and third hour of the incubation the cellular lipid contents do not change except for a decrease in cholesteryl ester; the lipid contents in the medium continue to rise, although at a slower rate than during the first hour (**Fig. 1**). It can be concluded from Fig. 1 that the total amount of glycerolipids in the whole incubation increases during the 3-hr incubation, which reflects the de novo synthesis of these lipids from endogenous glycogen, as shown to occur in previous studies (16).

Characterization of triacylglycerol-containing particles

When the hepatocyte medium is subjected to density gradient centrifugation, the triacylglycerol is found predominantly in the fractions with d < 1.01 g/ml (**Figs. 2A and 2B,** upper panel). The same behavior is displayed by the triacylglycerol in rat plasma (Fig. 7, upper panel). Particles in this density region are designated as VLDL.

In **Table 2,** the lipid composition of the hepatocyte VLDL is compared to that of plasma VLDL. The former tends to possess relatively less phospholipid and free and esterified cholesterol with respect to triacylglycerol although the differences are not statistically significant.

Upon electron microscopic examination after negative staining, the particles of hepatocyte VLDL are found to have on the average larger diameters than those of plasma VLDL: 63.6 ± 20.2 nm versus 40.8 ± 18.0 nm (means ± SD of 200 particles of each kind). Further resemblance between hepatocyte VLDL and its plasma counterpart is found in their electrophoretic behavior on agarose gel (**Fig. 3**): the tri-

acylglycerol-containing particles in the hepatocyte incubation medium show a similar mobility (pre-beta) to those in rat plasma. However, a greater part of the triacylglycerol from the hepatocytes does not penetrate into the gel matrix, which may be related to the bigger mean size of the hepatocyte VLDL particles.

The apoprotein composition of hepatocyte and plasma VLDL, as studied by SDS-polyacrylamide gel electrophoresis, is shown in **Fig. 4.** Plasma VLDL possesses apoproteins with apparent molecular weights agreeing with those of apoprotein B (>150,000), apoprotein E (35,000) and apoproteins C (between 8,000 and 13,000), as reported to occur in plasma VLDL by other authors (27). On hepatocyte VLDL apoproteins B and E are found as major apoproteins, while apoproteins with apparent molecular weights of 28,000 and 15,000 are present as minor components. The 28,000 molecular weight apoprotein may be identical to apoprotein A-I, the major apoprotein of rat plasma HDL.

When the hepatocytes are incubated in the presence of [¹⁴C]leucine, and the labeled VLDL is isolated by density gradient centrifugation and analyzed by SDS-polyacrylamide electrophoresis, radioactivity is present in each of the gel zones stained with Coomassie Brilliant Blue (**Fig. 5**).

Characterization of ρ > 1.02 particles

It can be seen from Fig. 2 that, whereas triacylglycerols and cholesteryl esters are found nearly only in the VLDL region, free cholesterol and phospho-
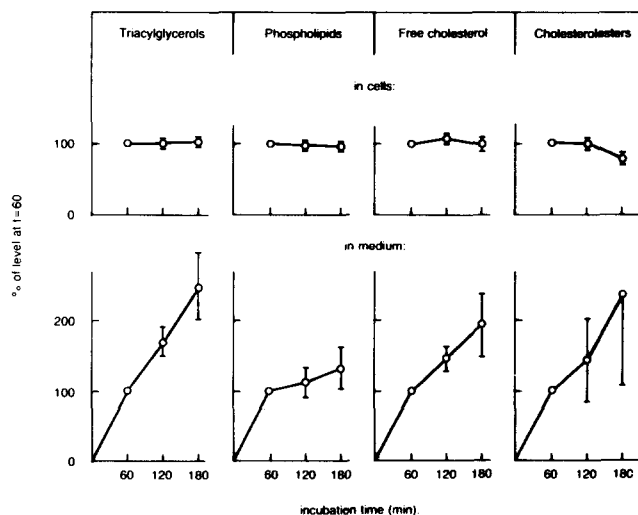
Fig. 1. Changes in lipid levels in cells and medium as a function of the incubation time. Results are given as % of the values after 60 min incubation, and are means ± SD of three to five separate incubations. Absolute lipid levels after 60 min incubation are given in Table 1.
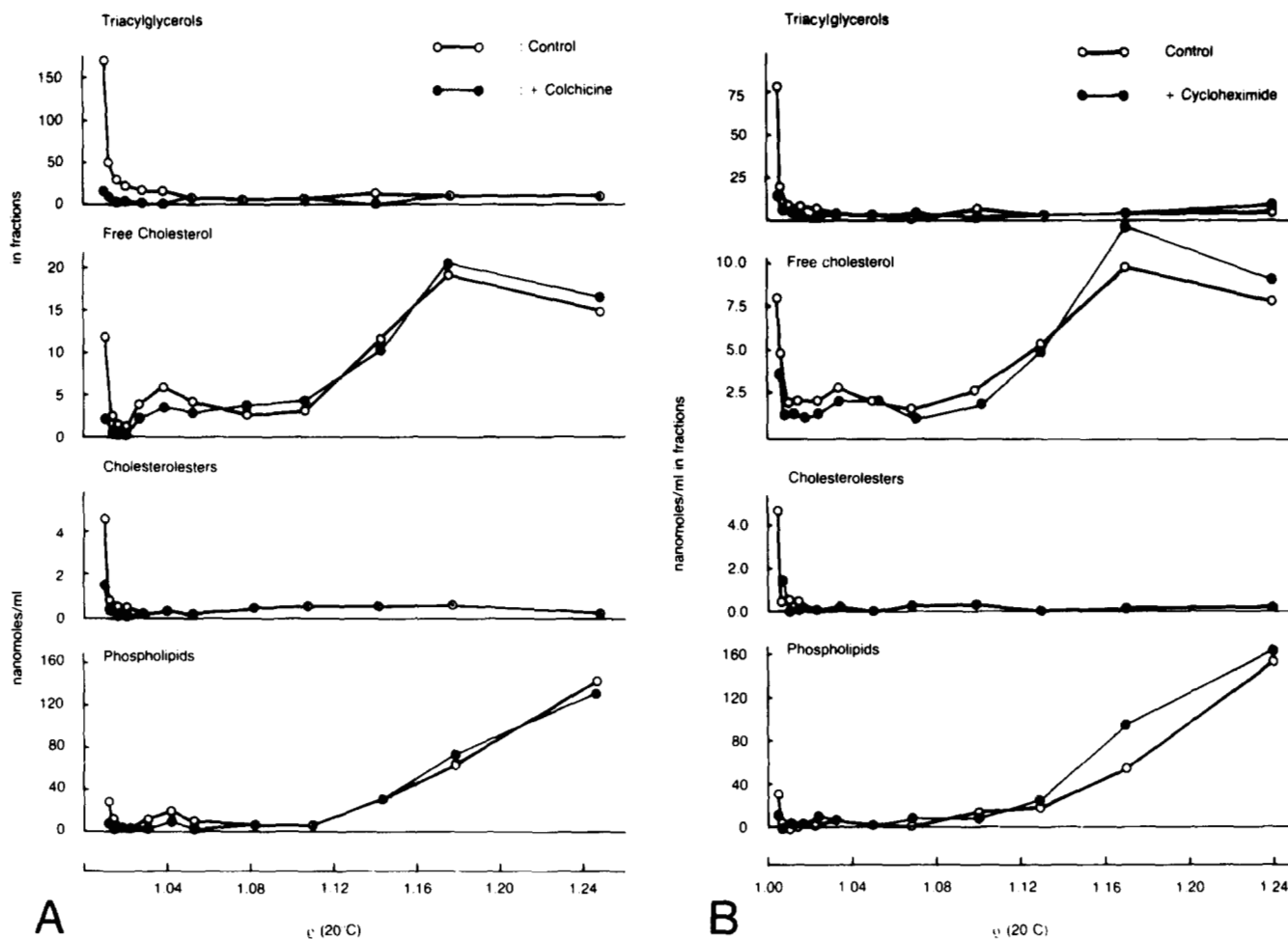
Fig. 2. Density gradient centrifugation of rat hepatocyte incubation medium. Incubations were for 180 min in the absence (○) or presence (●) of 2.10^{-5} M colchicine (A), or 7.10^{-5} M cycloheximide (B). Similar results were obtained with two other hepatocyte preparations for each of both drugs.

lipids[1] occur also in two other density regions, one between 1.03 and 1.08 g/ml (peak at 1.04), and another between 1.10 and 1.24 g/ml (free cholesterol peaking at 1.18).

Incubations were also performed in the presence of radioactive tracers to be incorporated in the various lipids. With [1-^{14}C]acetate, labeled triacylglycerols occur only at d < 1.02 g/ml, whereas labeled phospholipids and free sterols are present in all three density regions mentioned above (**Fig. 6,** circles). After incubation with [2-^{14}C]mevalonate, labeled sterol esters are found at d < 1.02 g/ml and in some experiments also in the d 1.03–1.08 g/ml region, but never in the fractions with d > 1.10 g/ml. In contrast, labeled free sterols are present again in all three density zones (not shown).

The apoprotein contents of the d > 1.02 g/ml particles were studied by SDS-polyacrylamide gel electrophoresis. Particles in the 1.03–1.08 g/ml density range have proteins with apparent molecular weights of >150,000, of 68,000 and of 35,000 (minor component), as seen after staining. After incubation with [^{14}C]leucine, labeled proteins are found in the same zones as described for hepatocyte VLDL, however without the peak at 28,000 (not shown).

TABLE 2. Lipid composition of VLDL in rat plasma and in rat hepatocyte incubation medium after 180 min incubation

	Hepatocyte VLDL	Plasma VLDL
Triacylglycerols	100	100
Phospholipids	20.5 ± 1.7	23.9 ± 3.2
Free cholesterol	7.9 ± 2.1	9.8 ± 0.5
Cholesteryl esters	3.8 ± 2.1	7.7 ± 4.5

Data are molar proportions of the various lipid classes, normalized with respect to triacylglycerols (set at 100), and represent means ± SD for five different plasma or hepatocyte VLDL preparations.
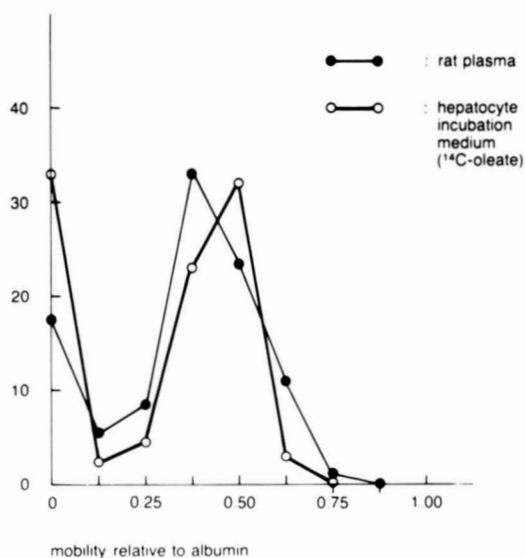
[1] Further separation of phospholipids showed that lecithin was nearly the only phospholipid in VLDL, while sphingomyelin and lysolecithin were also detected in the density region 1.10–1.24 g/ml.

Fig. 3. Agarose electrophoresis of rat plasma (●) or hepatocyte incubation medium (○). Hepatocytes (43 mg dry weight) were incubated for 60 min with 0.5 μCi[1-¹⁴C]oleate. The amount of triacylglycerol (plasma) or ¹⁴C in triacylglycerols (hepatocyte medium) in each gel section is given as % of the total amount recovered from the gel; mobility is given relative to that of albumin (detected by its refractive front).

The cholesterol-containing particles in the 1.16–1.20 g/ml density region were separated from the excess of serum albumin by flotation at d = 1.25 g/ml, yielding about 50% of the free cholesterol in the top fraction. In three experiments, the recovered material had a variable apoprotein composition, displaying up to nine polypeptides (other than serum albumin) with molecular weights between 46,000 and 13,000. The major component in all preparations was a 28,000 molecular weight protein (not shown).
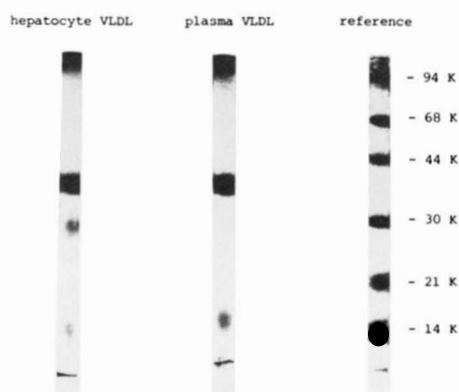
Fig. 4. SDS-polyacrylamide gel electrophoresis of hepatocyte or plasma VLDL, carried out as described in Methods and Materials. Hepatocyte VLDL was isolated after 180 min of incubation. The reference gel contained (from above) phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (2.5 μg of each), as molecular weight standards.
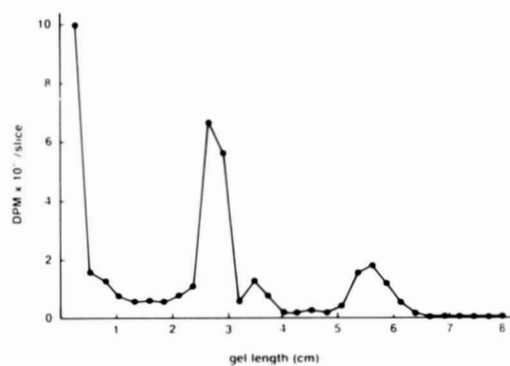
Fig. 5. Incorporation of ¹⁴C from [U-¹⁴C]leucine in apoproteins of hepatocyte VLDL. Hepatocytes (438 mg dry weight) were incubated with 30 μCi of [¹⁴C]leucine in 15 ml (six tubes) for 180 min. The isolated hepatocyte VLDL contained 210 μg of protein, of which 29 μg was applied on the gel. Similar results were obtained for two other hepatocyte incubations.
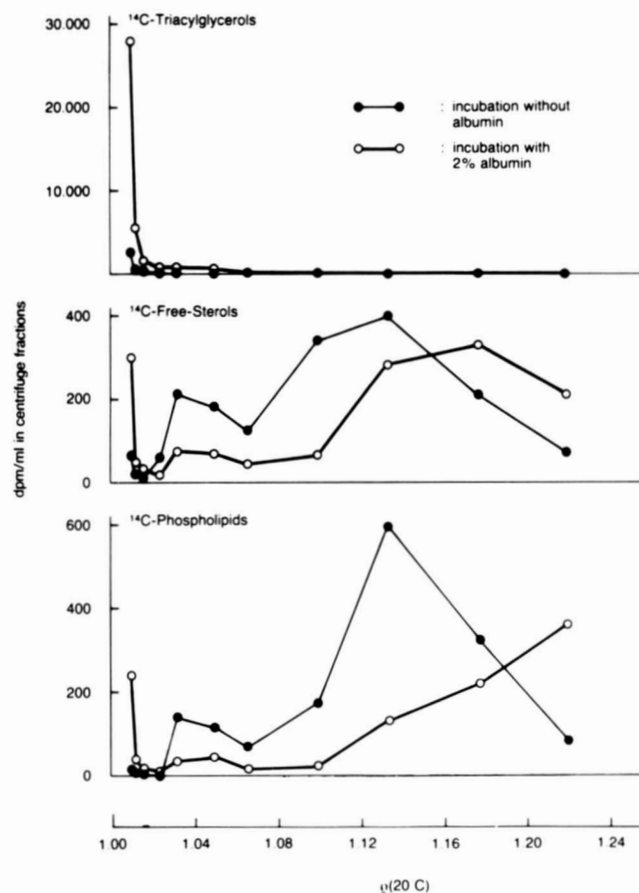
Fig. 6. Effect of serum albumin on the density distribution of the lipid-containing particles in the incubation medium. Hepatocytes (35 mg dry weight) were incubated for 180 min with 0.5 μCi of [1-¹⁴C]acetate in the presence (○) or absence (●) of 2% serum albumin. The recovery of [¹⁴C]triacylglycerols from the centrifugation tube was only 35% when albumin was absent (compared to 93% with albumin present), indicating strong adsorption of hepatocyte VLDL to the tube wall in that condition.

The identity of the d > 1.02 g/ml particles is uncertain. As shown in **Fig. 7,** rat plasma also contains phospholipids and free cholesterol in the 1.03–1.08 g/ml density region, but only as a "shoulder" before the greater amount of these lipids in the 1.08–1.17 g/ml density region. The particles in these regions correspond to the LDL and HDL, respectively, as characterized in rat plasma by others (28). In view of the virtual absence of cholesteryl esters in the d > 1.02 g/ml particles from the hepatocyte medium, the hepatocytes clearly do not secrete LDL and HDL as present in rat plasma.

It was investigated further, whether the d > 1.02 g/ml particles could correspond to, or be derived from, bile micelles, normally present in rat bile, now being released into the incubation medium. The density distribution of bile acids, cholesterol, and phospholipids in native rat bile is shown in **Fig. 8** (●). The major part of these components is found in the density region between 1.10 and 1.24 g/ml. When bovine serum albumin is added to the bile in the same concentration (2%) as present in the hepatocyte incubation medium, some free cholesterol and phospholipid is now found in a density region around 1.04 g/ml (Fig. 8, ○). The formation of low density particles during in vitro incubation of bile with serum albumin has been reported by others (29); they seem
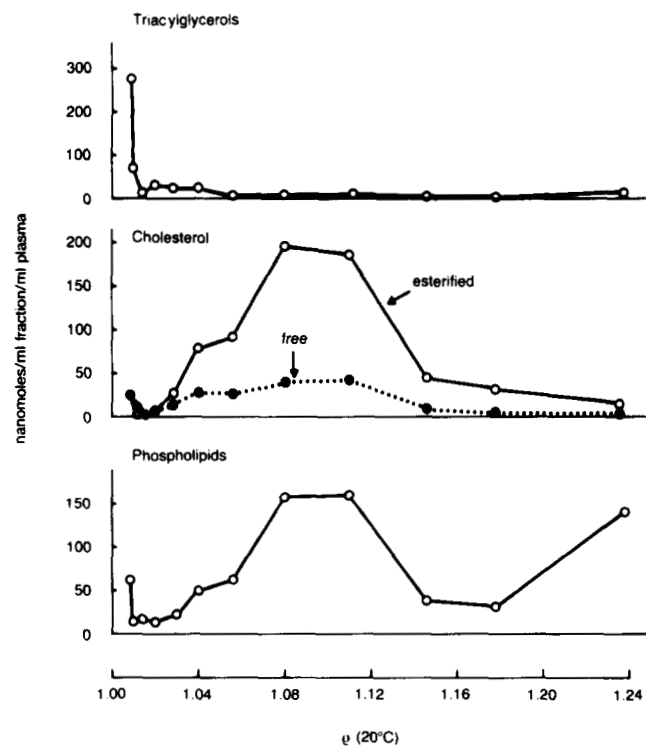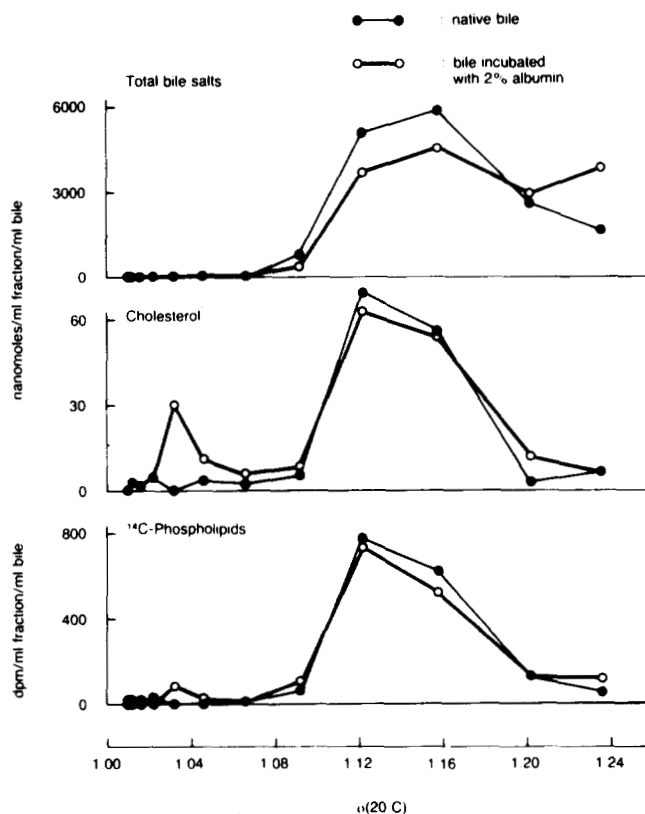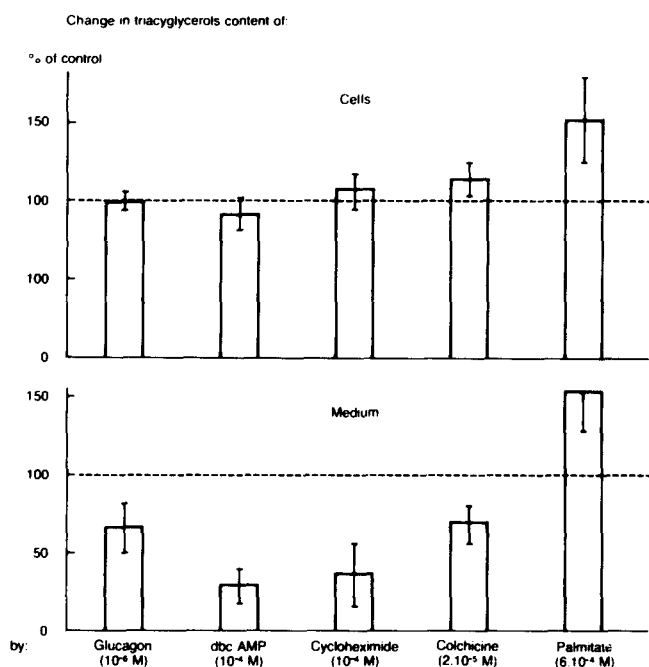

Fig. 8. Density gradient centrifugation of 3.0 ml of rat bile, collected as described in the Materials and Methods section, and incubated for 180 min with (○ —— ○) or without (● —— ●) 2% bovine serum albumin.

to be identical to lipoprotein-X occurring in plasma after bile duct obstruction. To see whether the hepatocyte particles with density around 1.04 g/ml could be the result of a similar mechanism, hepatocytes were incubated without albumin. As demonstrated in Fig. 6 (dots), this does not prevent the appearance of particles in the 1.03–1.08 g/ml density region, which argues against their being related to bile secretion by the hepatocytes. Furthermore, the density distribution of the particles in the 1.10–1.24 g/ml region is markedly shifted to the left by albumin omission (Fig. 6), free cholesterol now peaking around 1.13 g/ml. Therefore, these particles do not correspond to bile micelles, since the density distribution of the latter is invariant with respect to the presence of albumin (Fig. 8).

Effects of added substances on lipids in cell and medium

Addition of glucagon (10^{-6} M), dibutyryl cyclic AMP (10^{-4} M), colchicine (2.10^{-5} M) or cycloheximide (7.10^{-5} M) lowers the amount of triacylglycerols in the medium, while not significantly changing the intracellular content of that lipid (**Fig. 9**). In contrast, the triacylglycerol content of cells and medium is clearly

Fig. 7. Density gradient centrifugation of 3.0 ml of rat plasma, collected as described in the Materials and Methods section.

Change in triacylglycerols content of

Fig. 9. Changes in the content of triacylglycerols in rat hepatocytes and their medium after 180 min incubation, caused by addition of various substances to the medium. Results are given as % of control (incubation without added substances). Height of bars and lines represent means and SD for 4 to 6 different hepatocyte preparations.

increased by the addition of palmitate (0.6 mM) to the incubation.

The effects of colchicine and cycloheximide have been studied in more detail. It is apparent from Fig. 2 that both agents strongly suppress the release of all VLDL lipids, whereas the release of free cholesterol and phospholipids in the higher density regions are only weakly ($1.03 < d < 1.08$ g/ml) or not at all ($1.10 < d < 1.24$ g/ml) inhibited. The same pattern of inhibition has been noticed with these drugs, when the amount of radioactivity in the various lipids is measured after incubation with [^{14}C]acetate, [^{14}C]oleate and [^{14}C]mevalonate (not shown).

DISCUSSION

In this paper a global inventory is given of the lipid-releasing properties of isolated rat hepatocytes. Triacylglycerols, phospholipids, free cholesterol, and cholesteryl esters are secreted in the form of VLDL particles, where they occur in molar proportions of 100:21:8:4. The content of phospholipids and free cholesterol (as surface lipids) relative to triacylglycerols and cholesteryl esters is slightly lower than in plasma VLDL, consistent with their bigger mean particle size found with the electron microscope. It is notable that cholesteryl esters occur in the medium

only as a VLDL-component. Whether these are generated by the action of LCAT in the medium, or by intracellular esterification prior to their secretion, cannot yet be decided. LCAT secretion by isolated hepatocytes has been demonstrated (30), but this enzyme has HDL and not VLDL as the preferred substrate (31). On the other hand, the hepatocyte VLDL might be a significant substrate for LCAT, because it appears to contain apoprotein A-I (see below), known to activate LCAT.

The presence of apoproteins B and E on hepatocyte VLDL, as reported here,[2] has been established before, using hepatocytes in monolayer culture (6). The presence of an additional polypeptide with molecular weight of 28,000 in hepatocyte VLDL is a novel finding; this apoprotein was not detected in the VLDL secreted by rat hepatocytes maintained for longer periods in suspension (4) or monolayer (6) culture. It also is lacking in rat plasma VLDL (Fig. 4), and in VLDL from liver perfusates (7–10). However, chylomicrons and VLDL from rat intestinal lymph do possess this apoprotein, identified as apoprotein A-I (32, 33). The presence of apoprotein A-I on hepatocyte VLDL would be a further indication that similar mechanisms are involved in the synthesis and assembly of triacylglycerol-containing particles by liver and intestine.

The VLDL, released by freshly isolated (this study) or cultured (4, 6) rat hepatocytes, apparently carries lesser amounts of the C-apoproteins than the VLDL of rat plasma or liver perfusates (7–9). Nestruck and Rubinstein (34) have shown that VLDL particles extracted from liver Golgi vesicles have virtually no C-apoproteins, but are able to accept these peptides when offered in the form of plasma HDL. They speculate that the C-apoproteins may be secreted independent from VLDL, and join the latter only after their release in the space of Disse. Such an interaction conceivably becomes more difficult in the isolated hepatocyte system, because of the greater dilution with extracellular medium and the loss of the sinusoidal lining cell barrier.

As another explanation, the low apoprotein C content of the hepatocyte VLDL may be due to a secondary process, occurring in the medium after the secretion of a "complete" VLDL. Capuzzi, Sparks, and DeHoff (35) found that plasma VLDL containing ^{125}I-labeled apoprotein C was degraded during incubation with rat hepatocytes, which they ascribed to proteolytic

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[2] The presence of these apoproteins was also confirmed by immunodiffusion of hepatocyte VLDL using antisera, raised in rabbits against purified rat plasma apoprotein B or apoprotein E. (Kempen, H. J. M., and A. M. Havekes. Unpublished observation.)

enzymes retained by the cells after the collagenase perfusion. However, unlike their experiments, our incubations contained 2% serum albumin, which may protect the secreted VLDL against proteolysis. Further study is needed to settle this matter.

Inhibition of VLDL release by colchicine (1, 2, 36, 37), by cycloheximide (38), or by glucagon or dibutyryl cyclic AMP (39), as well as the enhancement by addition of palmitic acid (40, 41), has been demonstrated before in perfused livers or isolated hepatocytes from the rat. The mode of action of these agents does not need to be discussed here. The modulating effects, observed with these various agents, permit the conclusion that the release of VLDL by the hepatocytes is brought about by an active regulatable mechanism, and not by passive loss from dying cells.

In addition to VLDL, the hepatocytes release other lipid-containing particles into the medium. The particles in the 1.03–1.08 g/ml density region are similar in lipid and protein composition to the LDL material, secreted by perfused livers of cholestatic rats (42). The presence of albumin, apoprotein E, and C-apoproteins in that material was accounted for by the occurrence of lipoprotein-X, which did not occur in perfusates of control livers (42). If the low density particles from the hepatocytes indeed would prove to be lipoprotein-X, this would indicate that the isolated hepatocytes are "cholestatic" in the present conditions.

The particles in the d 1.10–1.24 g/ml region do not seem to correspond to the HDL secreted by the perfused rat liver (9, 10). The latter already contain a high amount of cholesteryl ester (due to the action of LCAT in the perfusate), whereas we find virtually none of this lipid in the hepatocyte high density particles. Furthermore, the HDL in liver perfusate has a defined apoprotein complement, while the hepatocyte particles had no constant protein spectrum. Finally, the release of these particles is not inhibited by colchicine and cycloheximide, suggesting that they are not secreted by an active process, but rather lost as microsomal membranous fragments by disintegrating cells. On the other hand, the particles have only a limited number of protein components (apoprotein A-I appearing to be the most predominant), which indicates that they are distinctly different from hepatocyte plasma membranes, shown to contain at least 15 protein bands in SDS-polyacrylamide gels (43). Obtaining further information on the nature and origin of these particles is the aim of our current research.

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