Assembly of Rat Hepatic Very Low Density Lipoproteins in the Endoplasmic Reticulum*

(Received for publication, July 20, 1992)

Antonio Rusinol*, Henkjan Verkade†, and Jean E. Vance‡

From the Lipid and Lipoprotein Group and Departments of †Medicine and ‡Biochemistry, University of Alberta, Edmonton, Alberta T6G 2S2, Canada

The intracellular site of assembly of hepatic very low density lipoproteins has been investigated. Two endoplasmic reticulum fractions and Golgi vesicles (relatively free from endosomal contamination) were isolated from rat liver and the luminal contents were released. The apoB-containing entities were separated from the lumen of the endoplasmic reticulum and Golgi vesicles by an immunoaffinity isolation procedure. The amount of each lipid moiety (triacylglycerols, cholesteryl plus cholesteryl esters and phospholipids) associated with a unit mass of apoB was shown to be very similar in the luminal contents isolated from each of the three fractions. Moreover, the apoB-containing particles that were isolated from the endoplasmic reticulum and Golgi were mainly present in a fraction of density <1.02 g/ml. The average diameter of these lipoprotein particles was shown by negative staining electron microscopy to be in the size range of very low density lipoproteins and low density lipoproteins. The size and composition of the intracellular lipoproteins were very similar to those of both very low density lipoproteins isolated from the culture medium from rat hepatocytes and plasma very low density lipoproteins. The conclusion from this study is that the full complement of lipids associate with apoB at an early stage during the secretory pathway, in the endoplasmic reticulum.

The concentration of low density lipoproteins (LDL) in the circulation is balanced by the rates of their entry into, and removal from, the circulation. The cell biological and biochemical details of how the liver regulates the secretion of VLDL (1), the direct precursor of LDL, have not been firmly established. Human VLDL consist of a core of hydrophobic lipids (mainly triacylglycerols with some cholesteryl esters) surrounded by a monolayer of phospholipids, cholesteryl, and one molecule of apo(protein) B-100 (2), as well as some other proteins, primarily apoproteins E and C. VLDL produced in the liver of rats are similar to those from humans except that rat VLDL contain a single molecule of either apoB-100, or apoB-48 (2), which constitutes the amino-terminal half of apoB-100 (3). Secretion of triacylglycerol-rich VLDL requires the presence of apoB; in the disease abetalipoproteinemia serum apoB is absent, and triacylglycerols cannot be secreted from the liver or intestine (4). Nor can apoB be secreted in the absence of bound lipids, presumably because the hydrophobic nature of this very large protein (Mr > 500,000) requires its association with amphipathic lipids for solubility in an aqueous environment.

Most available data suggest that under physiological conditions the rate of secretion of VLDL is not regulated by the rate of production of apoB. The rate of VLDL-apoB secretion in the human hepatoma cell line, HepG2, has been varied over a 7-fold range yet no differences in the levels of apoB mRNA were detected (5). The apoB-100 gene appears to be constitutively expressed and excess apoB that is not assembled with lipid to be secreted as VLDL may be degraded in the endoplasmic reticulum (6) or in a pre-Golgi compartment. Consequently, under most metabolic conditions the supply of lipid, rather than the supply of apoB, probably regulates the rate of hepatic VLDL secretion.

In the present study we have examined the intracellular site(s) at which apoB combines with each of its major lipid components (triacylglycerols, phospholipids, cholesterol, and cholesteryl esters) during formation of VLDL. The following three plausible models for this assembly process have been proposed, each of which is supported by some experimental evidence. In the first model, apoB is synthesized on ribosomes attached to the rough endoplasmic reticulum. The endoplasmic reticulum is also the principal site for synthesis of phospholipids, triacylglycerols, cholesterol, and cholesteryl esters. The lipids assemble with the apoB molecule in the endoplasmic reticulum, perhaps cotranslationally. The apoB-containing VLDL particles are thereby completely assembled in the endoplasmic reticulum and are secreted by the normal secretory route via the Golgi apparatus. This model is supported by immunoelectron microscopy (7) and by pulse-chase studies in cultured rat hepatocytes (8) and cultured human hepatoma cells (9). In the second model, apoB, which is synthesized on rough endoplasmic reticulum membranes, remains membrane-bound until the apoB reaches the Golgi, at which time apoB combines with triacylglycerols, phospholipids, and other lipid components and is released into the Golgi lumen in the form of VLDL. This model is supported by data from pulse-chase studies in estrogen-treated chicken hepatocytes (10) and rat liver (11, 12). In the third model, apoB combines with some lipid in the endoplasmic reticulum, and more lipid is sequentially added to the apoB-containing particles during their passage through the endoplasmic reticulum and Golgi stacks. Pulse-chase studies in chicken hepato-
toocytes (13) and HepG2 cells (14-16) have suggested that VLDL may be assembled sequentially during secretion. Moreover, Golgi membranes do have the capacity to synthesize phospholipids (17). However, the lack of immunoelectron microscopic evidence for the existence of apoB-containing particles of increasing size as apoB moves from the endoplasmic reticulum through the Golgi to secretion (7) makes the two latter models less attractive than the first.

The reasons for the apparent discrepancies in available data concerning the intracellular site(s) of VLDL assembly are not entirely clear. The different model systems used (e.g., rat hepatocytes (8), human hepatoma cells (9, 14-16), rat liver (11, 12), and estrogen-treated chicken hepatocytes (10, 13)) may account for some of the differences. Most of the reported studies have applied similar techniques as those employed for subcellular studies coupled with subcellular fractionation of cultured cells. A large variability in the recovery, purity, and characterization of the rough and smooth endoplasmic reticulum and Golgi fractions among the different cultured cell types is apparent, which makes interpretation of the results problematic. Especially difficult has been separation of the Golgi apparatus from the endoplasmic reticulum in cultured cells and isolation of Golgi membranes that are not significantly contaminated by endosomal vesicles which also contain apoB (18). Subcellular fractionation of rat liver is more satisfactory than of cultured hepatocytes; more material is available and the purity of the organelle fractions is superior.

Our experiments were designed to distinguish among these three proposed models for VLDL assembly. ApoB-containing particles were isolated from the luminal contents of the endoplasmic reticulum and Golgi of rat liver by immunoaffinity chromatography. The number of molecules of each lipid component (triacylglycerols, phospholipids, and cholesterol/cholesteryl esters) per unit mass of apoB was almost identical in two endoplasmic reticulum fractions and in Golgi. In addition to the chemical composition, the density of the luminal lipoproteins and their size (as measured by electron microscopy) were also very similar from the three subcellular fractions. Comparison of these properties with those of VLDL secreted by cultured rat hepatocytes and VLDL of rat serum support the hypothesis that apoB-containing lipoprotein particles of composition, density, and size similar to those of newly secreted VLDL are present in the lumina of heavy and light endoplasmic reticulum fractions, as well as in Golgi. Therefore, the full complement of lipid moieties of VLDL appear to be assembled with apoB at an early stage of the secretory pathway, in the endoplasmic reticulum.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sheep monoclonal antibodies directed against human apoB were supplied by Boehringer Mannheim, as was NADPH, unlabeled, and apomycin, 2.2'-azino-di-[3-ethylbenzthiazoline] sulfonate peroxidase substrate, and endo-β-N-acetylglucosaminidase H (EC 3.2.1.96). The radiochemicals [H]leucine, UDP-[3H]acetylglucosamine, cystidine-5'-diphosphate-[methyl-3H] choline, and [methionyl-H]-S and adenosylmethionine were purchased from Amersham Canada, Oakville, ON, Canada, as were the enhanced chemiluminescence detection reagents. The conjugate anti-apoB IgG-horseradish peroxidase, CNBr-activated Sepharose 4B, concanavalin A linked to horseradish peroxidase, Cab-O-Sil (fumed silica), and the cholesterol assay kit (Cholesterol 20) were purchased from Sigma. The Triglycine G kit for quantitation of triacylglycerol was obtained from Wako Pure Chemical Industries Ltd., Japan. Microtiter Immulon immunoassay plates were from Dynatech Laboratories Inc., Chantilly, VA. All reagents for polyacrylamide gel electrophoresis and immunoblotting, including the polyvinylidine difluoride membrane, were from Bio-Rad. Thin layer chromatography plates (Silica Gel G, 0.55 mm thickness) were purchased from BDH Chemicals, and standard lipids were from either Avanti Polar Lipids, Birmingham, AL, or from Sigma. IODIGEN (1,3,4,6-tetrachloro-3,6-dipyphenyldicyochlor) was obtained from Pierce Chem. Co. All other reagents were from Sigma or Fisher.

**Preparation and Characterization of Subcellular Fractions**—Female Sprague-Dawley rats (200–250 g) were fed standard chow and water ad libitum. The rats were anesthetized by subcutaneous injection of sodium pentobarbital, and the livers were perfused with cold phosphate-buffered saline through the portal vein. The livers were rapidly removed and subcellular fractions (two endoplasmic reticulum fractions, designated ER1 and ER2, and Golgi vesicles) were isolated according to the method of Croze and Morré (19) as modified by Hamilton et al. (18) to minimize endosomal contamination of the Golgi membranes. The ER1 fraction was isolated from the final discontinuous sucrose gradient at the interface between the sucrose solutions of 1.5 and 2.0 M, whereas ER2 was isolated from the interface between the sucrose solutions of 1.3 and 1.5 M from the same gradient. The isolated membrane fractions were assayed for NADPHcytochrome c reductase (endoplasmic reticulum marker enzyme) (17) and UDP-galactose: N-acetylglucosamine galactosyltransferase (trans-Golgi marker enzyme) (16). The cis-Golgi marker enzyme, UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase, was assayed according to a published method (16) using UDP-[3H]N-acetylglucosamine (40 Ci/mmol) and α-methylmannoside as acceptor. Luminal contents were released from vesicles of endoplasmic reticulum and Golgi fractions by treatment with sodium carbonate at pH 11.3, as described previously (14).

**Immunoadfinity Chromatography**—Sheep anti-human apoB antisera containing IgG (190 ml) was linked to CNBr-activated Sepharose 4B (20 ml) according to the directions of the manufacturer. Luminal contents of the endoplasmic reticulum and Golgi vesicles were adjusted to pH 8.3, after which the luminal contents were gently shaken with anti-human apoB-Sepharose for 16 h at 4°C in 100 mM NaHCO3 buffer, pH 8.3, containing 500 mM NaCl, 1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (buffer A). The beads were pelleted by centrifugation for 5 min at 1,000 × g and resuspended in 5 ml of buffer A. The beads were washed four more times. This procedure resulted in binding of apoB-containing entities to the beads. Lipids associated with the bound apoB were extracted by the method of Bligh and Dyer (20). Lipid extracts were dried and aliquots were used for quantitation of triacylglycerols, cholesterol, plus cholesteryl esters, and phospholipids (21).

Apoproteins bound to the beads were extracted by heating at 85°C for 15 min in a sample buffer containing 2% sodium dodecyl sulfate, 6 M urea, and 0.5% β-mercaptoethanol. The proteins were separated by electrophoresis on a gradient polyacrylamide gel (8–15%) containing sodium dodecyl sulfate (22). The protein bands were localized by staining with Coomassie Blue G-250. Individual apoproteins were identified by comparison with apoproteins of rat plasma lipoproteins isolated by sequential centrifugation as previously described (23). The apoB-containing bands were excised and digested with 100 μg of a mixture of 30% hydrogen peroxide and 60% perchorlic acid, 2:1 (v/v), for 16 h at 60°C. Radioactivity was measured in a gamma counter or a liquid scintillation counter, as appropriate. The recovery of apoB was greater than 85%. The recovery of lipids of rat serum VLDL after immunoadfinity chromatography was greater than 90% for triacylglycerol and greater than 90% for total cholesterol and phospholipids.

**Density Gradient Ultracentrifugation**—Sodium azide (0.02%, w/v), EDTA (0.02%, w/v), phenylmethylsulfonyl fluoride (0.1 mM), and NaCl (0.8%) were added to luminal contents derived from the endoplasmic reticulum and Golgi fractions. In some cases, polyethylene sulfone ultratirification membranes (low protein binding) from Filtron Technology Corporation, Clinton, MA were used for concentration of the luminal contents before ultracentrifugation. The suspension was placed on top of 0.4 g of solid sucrose in a polysilicon centrifuge tube (capacity 39 ml), and 1.3 ml of NaBr (density = 1.02 g/ml) was layered on top. The tubes were filled with 0.4 M NaCl. After centrifugation for 40 h at 150,000 × g in a Beckman Ti-70 rotor, the tubes were unloaded from the bottom and six 6-ml fractions were collected. The density of each fraction was determined by refractometry or weighing. For the electron microscopy study, the fractions were obtained using a method previously described (24) on a Hitachi H 7000 transmission electron microscope. Electronmicroscopy and Immunoblotting of Aipoproteins—Each fraction from the density gradient was added to 0.2 ml of an aqueous slurry of 3% low-speed supernatant of rat liver microsomes. The mixture was centrifuged for 15 min at 5,000 × g and the Cab-O-Sil pellet was washed three times with distilled water. The proteins were
were solubilized from the pellet by heating for 15 min at 85 °C in buffer containing 6 M urea and 2% sodium dodecyl sulfate. The proteins were separated by electrophoresis on a polyacrylamide gel (3-15%) containing 0.1% sodium dodecyl sulfate (22). Proteins were transferred to polyvinylidene difluoride membranes by modification of the method of Towbin et al. (25). The transfer buffer contained 25 mM Tris HCl, pH 8.3, 500 mM NaCl, 1 mM EDTA, and 75 mM Na2HPO4. The proteins were transferred for 20 h at 10 °C using 50 volts. Nonspecific binding sites were blocked by immersion of the membrane in a solution of Tris-buffered saline (TBS) (20 mM Tris, pH 7.5, and 0.5 M NaCl) containing 5% dried milk and 0.05% Tween 20 for 12 h at 4 °C or for 1 h at room temperature. The primary antibody to the polyclonal rabbit antisera was prepared in our laboratory against rat plasma VLDL, the secondary antibody was anti-rabbit IgG linked to horseradish peroxidase. Bound antibodies were visualized with enhanced chemiluminescence Western blotting reagents according to the manufacturer's instructions.

Endothelial Transferase H Treatment—Aliquots of fractions of density 1.02 g/ml were concentrated by the addition of Cab-O-Sil as described above and were extracted from the Cab-O-Sil pellet by heating for 15 min at 85 °C in a buffer containing 6 M urea and 2% sodium dodecyl sulfate. The solution was then added in a 1:1 (v/v) ratio to 100 mM acetate buffer, pH 5.0, containing 4% (v/v) Triton X-100. The samples were incubated in the presence or absence of 5 milliliters of endoglycosidase H for 21 h at 30 °C. The proteins were separated using polyacrylamide gel electrophoresis and subsequently transferred to a polyvinylidene difluoride membrane as described above. The membrane was blocked for 40 min in a solution of TBS containing 5% Tween 20. The membranes were washed twice with TBS containing 0.05% Tween 20. The blots were then incubated with TBS containing 0.05% Tween 20. Concanaavalin A linked to horse radish peroxidase (1 μg/ml in TBS containing 0.05% Tween 20) was added, and the sample was incubated for 20 min. The membranes were washed twice, for 10 min each time, with TBS containing 0.05% Tween 20, and the band limit was detected with the enhanced chemiluminescence reagents according to the manufacturer's instructions. The membranes were then incubated for 16 h in TBS containing 0.05% Tween 20, 5% (v/v) dry milk and 0.2 M α-methylmannoside, then replored with antibody directed against VLDL.

Quantitation of Apoprotein B—The amount of apoB in the luminal contents of the subcellular fractions was determined by enzyme-linked immunosay, as follows. For preparation of apoB for a standard curve, rat plasma VLDL was isolated (23) and apoB was purified by electrophoresis on a 3-15% polyacrylamide gel containing sodium dodecyl sulfate (22). The apoproteins B were electroeluted from the gel at 10 °C using 10 mM for 6 h. A standard curve for apoB was constructed each time an unknown quantity of apoB was analyzed. The sample of apoB (either standard apoB or luminal contents from the endoplasmic reticulum or Golgi) was diluted in a ratio of 1:1 (v/v) with coating solution (500 mM Na2CO3, pH 9.6, containing 0.2% NaN3). A total volume of 250 μl of diluted sample was applied to a well in the tray of a nitrocellulose transfer tank. The plate was incubated at room temperature, then at 4 °C for 16 h. Sheep anti-human apoB antisera (dilution 1:1000) and rabbit anti-sheep IgG antisera linked to horseradish peroxidase (dilution 1/3000) were separately dissolved in solutions containing 1% albumin and 1% Tween 20 and used as the first and second antibodies, respectively. 2,2'-Azino-di-[3-ethylthiazolone] sulphonate in a citrate-borate buffer was used as the color reagent for detection of binding to the second antibody (28). The absorbance of samples was read at 510 nm.

Other Procedures—VLDL was isolated as the fraction of density 1.06-1.02 g/ml from the medium of monolayer cultures of rat hepatocytes by ultracentrifugation on a salt gradient (22). Rat plasma VLDL, which was obtained by modification of the method of Towbin et al. (25), was labeled with [125I] using IodoGEN iodination reagent (27). Rabbit polyclonal antibodies were prepared against rat VLDL in our laboratory by standard procedures. The protein content of samples was measured in the presence of 0.5% deoxycholate by the method of Lowry et al. (28) using bovine serum albumin as a standard. The phospholipid biosynthetic enzymes, CDP-choline:1,2-diacylglycerol cholinephosphotransferase and phosphatidylethanolamine-N-methyltransferase, were assayed as described previously (17). For preparation of the lipid microemulsion stock lipid solutions were mixed and dried at 30 °C under a stream of nitrogen. The final mixture contained 330 mmol of triolein, 35 mmol of cholesterol, 50 mmol of cholesteryl oleate, and 91 mmol of egg yolk phosphatidylcholine. The lipids were redissolved in 2 ml of diethyl ether and dried again under nitrogen. A solution of 2 ml consisting of 0.2 M NaHCO3, pH 8.3, 500 mM NaCl, 1 mM EDTA, and 0.1 mM phenyl methylsulfonyl fluoride, the suspension was shaken in a vortex mixer for 10 min and sonicated in a probe sonicator (Heat Systems-Ultrasonic Inc, Farmingdale, NY) at setting 3 for 15 min.

RESULTS AND DISCUSSION

Purity of Endoplasmic Reticulum and Golgi Membranes and Their Luminal Contents—A method has been developed for isolation and characterization of intracellular apoB-containing lipoproteins from the lumen of the endoplasmic reticulum and Golgi apparatus of rat liver. By modification (18) of the subcellular fractionation procedure of Croze and Morre (19), two endoplasmic reticulum fractions, ERI (more dense, most likely rough endoplasmic reticulum) and ERII (less dense), and Golgi vesicles were isolated. The purity of the three membrane fractions was assessed by measurement of the specific activities of three marker enzymes: NADPH:cytochrome c reductase (endoplasmic reticulum), UDP-galactose-N-acetylgalactosamine galactosyltransferase (trans-Golgi), and UDP-N-acetylgalactosaminylphosphotransferase (cis-Golgi) (Table I). According to these marker enzymes, contamination of the ERI fraction by the cis- and trans-Golgi markers was less than 5%. Contamination of the ERI fraction by the cis-Golgi marker was approximately 12.8% whereas contamination by the trans-Golgi marker was only 4.9%. Cross-contamination of Golgi membranes by the endoplasmic reticulum membrane marker enzyme, when calculated on the basis of activity in the ERII fraction, was approximately 15%. The average recovery of endoplasmic reticulum and Golgi membranes, based on comparison of marker enzyme activities in the fractions and in the homogenate, was 26.1 ± 2.9 and 13.2 ± 4.3%, respectively. Enrichment of the Golgi marker enzyme (galactosyltransferase) in the Golgi fraction relative to the homogenate was 183-fold, and enrichment of the endoplasmic reticulum marker enzyme in the ERI and ERII fractions was 15.0- to 20.3-fold, respectively.

Luminal contents containing intracellular lipoproteins were released from membrane vesicles by treatment with sodium carbonate at pH 11.3 (14). The high pH treatment did not release lipids from lipoproteins. Luminal contents were completely separated from the membranes by ultracentrifugation (14). Since apoB has been detected in microsomal membranes, as well as in luminal lipoproteins, removal of all membrane fragments from the luminal contents was essential. Lack of contamination of luminal contents by membranes was demonstrated by measurement of the activities of two membrane-bound enzymes present in both endoplasmic reticulum and Golgi membranes (17), namely CDP-choline:1,2-diacylglycerol cholinephosphotransferase and phosphatidylethanolamine-N-methyltransferase. Endoplasmic reticulum and Golgi vesicles were treated with sodium carbonate under the conditions used for release of luminal contents. The activity of cholinephosphotransferase in the endoplasmic reticulum and Golgi membranes was reduced by only 25% by the high pH treatment whereas the activity of the methyltransferase was unaffected. The specific activity of the methyltransferase and the cholinephosphotransferase in the luminal contents of the ERI, ERII, and Golgi fractions was less than 4% of that in the membranes, confirming that the luminal contents were not significantly contaminated by membrane fragments.

Another valid concern when studying apoB-containing lipoproteins of the Golgi lumen is that Golgi membranes prepared by most commonly used techniques are highly contaminated with endosomes which also contain apoB derived from endosomes. Minimal endosomal contamination of Golgi used in our study was...
confirmed by the following experiment. \([^{3}H]\)Leucine was injected intravenously into a rat 30 min before sacrifice which labeled endogenously synthesized apoB. Fifteen min before sacrifice, 220 \(\mu\)Ci of \([^{3}H]\)-VLDL was injected into the rat. The liver was removed and subcellular fractions were prepared. Luminal contents of the Golgi and endoplasmic reticulum fractions, ERI and ERII, were isolated and subjected to immunoaffinity chromatography using anti-human apoB-Sepharose. Apoproteins were extracted from the affinity gel, and apoB was separated by electrophoresis on a 3-15% polyacrylamide gel containing sodium dodecyl sulfate. apoB was localized on the gel by addition of apoB from rat plasma VLDL. Radioactivity in endogenously synthesized apoB \([^{3}H]\) and in endocytosed apoB \([^{125}I]\) was measured.

<table>
<thead>
<tr>
<th>Marker enzyme</th>
<th>Specific activity of marker enzyme in fraction</th>
<th>% recovery from homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPH:cytochrome c reductase</td>
<td>3.08 ± 2.1</td>
<td>26.1 ± 2.9</td>
</tr>
<tr>
<td>UDP-galactose: N-acetylglucosamine galactosyltransferase</td>
<td>13.1 ± 4.1</td>
<td>13.2 ± 4.3</td>
</tr>
<tr>
<td>UDP-N-acetylglucosamine: N-acetylglucosamine-1-phosphotransferase</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**TABLE II**

Lack of endosomal contamination of apoB isolated from endoplasmic reticulum and Golgi lumina

A rat was injected with 1 \(m\)Ci of \([^{3}H]\)Leucine 30 min before sacrifice which labeled endogenously synthesized apoB. Fifteen min before sacrifice, 220 \(\mu\)Ci of \([^{3}H]\)-VLDL was injected into the rat. The liver was removed and subcellular fractions were prepared. Luminal contents of the Golgi and endoplasmic reticulum fractions, ERI and ERII, were isolated and subjected to immunoaffinity chromatography using anti-human apoB-Sepharose. Apoproteins were extracted from the affinity gel, and apoB was separated by electrophoresis on a 3-15% polyacrylamide gel containing sodium dodecyl sulfate. apoB was localized on the gel by addition of apoB from rat plasma VLDL. Radioactivity in endogenously synthesized apoB \([^{3}H]\) and in endocytosed apoB \([^{125}I]\) was measured.

<table>
<thead>
<tr>
<th>Liver fraction</th>
<th>135I/3H ratio in apoB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.120</td>
</tr>
<tr>
<td>ERI</td>
<td>0.102</td>
</tr>
<tr>
<td>ERII</td>
<td>0.063</td>
</tr>
<tr>
<td>Golgi</td>
<td>0.028</td>
</tr>
</tbody>
</table>

**FIG. 1.** Recovery of apoB from the Golgi luminal contents after immunoaffinity chromatography. Panel A, known amounts of \([^{3}H]\)leucine-labeled apoB-containing lipoproteins were diluted with sodium bicarbonate, pH 8.3, and incubated with Sepharose 4B (1 ml affinity matrix) covalently linked to sheep antibody prepared against human apoB. Up to 9 \(\mu\)g of apoB was incubated with 1 ml of affinity gel. The bound proteins were extracted from the beads and electrophoresed on a polyacrylamide gel. The bands containing apoproteins B were cut from the gel, and radioactivity was counted. Panel B, a rat was injected with 1 \(m\)Ci of \([^{3}H]\)Leucine into the portal vein. After 40 min the liver was excised, and the Golgi luminal contents were obtained. A fixed amount of \([^{3}H]\)leucine-labeled Golgi luminal contents (1 mg of luminal protein) was subjected to immunoaffinity chromatography in the presence of various amounts of unlabeled rat plasma VLDL. The bound proteins were extracted from the affinity gel, and apoproteins B were isolated by polyacrylamide gel electrophoresis. Radioactivity in recovered apoB was determined as a function of the amount of unlabeled apoB present. For the sample incubated in the absence of unlabeled VLDL 53,283 disintegrations/minute were present in apoproteins B-48 and B-100 combined.
The lipids were extracted from the affinity gel, and the mass of phospholipids, were incubated with Sepharose 508 nmol of total lipids of which the mol glycerols/cholesterol/cholesteryl circles) or absence (closed circles) of a lipid microemulsion containing to antibody directed against human apoB in the presence amounts of triacylglycerols, cholesterol plus cholesteryl esters, and phospholipids. As shown in Fig. 2 (closed circles), recovery of VLDL lipids after immunoaffinity chromatography was greater than 90% for triacylglycerols (panel A) and greater than 80% for cholesterol plus cholesteryl esters (panel B) and phospholipids (panel C). Since the luminal contents of the endoplasmic reticulum and Golgi contained some lipids that were not bound to apoB it was important to ascertain that lipids not associated with apoB did not bind to the anti-apoB-Sepharose affinity gel. Therefore, samples of rat plasma VLDL were incubated with the affinity matrix in the presence or absence of a lipid microemulsion (508 nmol of total lipids) containing no apoB. The mol % lipid composition of the microemulsion was triacylglycerol/cholesterol/cholesteryl esters/phospholipids, 65:7:10:18. Neither the linearity of the assay nor the quantitative recovery of apoB-associated lipids was compromised by the presence of the lipid microemulsion (Fig. 2) (open circles).

Amount of ApoB-associated Lipids in the Lumina of the Endoplasmic Reticulum and Golgi—For elucidation of the intracellular site(s) of assembly of apoB with each of its lipid components, first, the moles of each lipid moiety associated with a unit mass of apoB (apoproteins B-48 and B-100 combined) in the two endoplasmic reticulum and Golgi fractions was determined. The total amounts of triacylglycerols, phospholipids, and cholesterol plus cholesteryl esters/microgram of apoB (Fig. 3) were similar in all three luminal fractions. Triacylglycerol contributed 74-83%, phospholipids 10-16%, and cholesterol plus cholesteryl esters 7-11% of the total lipid associated with apoB. The molar ratio of cholesterol/cholesteryl esters in Golgi lipoproteins was 1.43 ± 0.41, which was similar to that in the endoplasmic reticulum fractions. The nanomoles of each lipid component/microgram of apoB was also measured in VLDL secreted by cultured rat hepatocytes and in rat plasma VLDL (Fig. 3). The amount of triacylglycerols/microgram of apoB was slightly less in the two samples of secreted VLDL than in the intracellular apoB-containing lipoproteins. The reason for this apparent variation in composition is not clear but may be due to lipolysis of triacylglycerols in the culture medium or the circulation. These data demonstrate that the amount of each lipid associated with one apoB molecule is approximately the same in the luminal contents of the endoplasmic reticulum and Golgi and in VLDL secreted by cultured rat hepatocytes and plasma VLDL. The amount of luminal apoB, and its associated lipids, per milligram of intact organelle protein was, however, 7-10-fold higher in the Golgi than in either endoplasmic reticulum fraction ('Table III').

Density Gradient Ultracentrifugation of Luminal Contents from the Endoplasmic Reticulum and Golgi—The composition of the apoB-containing entities in the lumina of the endoplasmic reticulum and Golgi suggests that fully formed, apoB-containing lipoproteins exist in the endoplasmic reticulum as well as in the Golgi. Direct evidence supporting this hypothesis was obtained from two additional experiments. First, the luminal contents from each of the three organelles were centrifuged on a salt gradient, and six fractions of densities ranging from 1.25 to 1.02 g/ml were collected from the gradients. The apoproteins in each density fraction were analyzed by polyacrylamide gel electrophoresis and immunoblotting. As shown in Fig. 4, in the luminal of the endoplasmic reticulum (ERI and ERII) and Golgi fractions the majority of apoB-100 was present in the fraction of density 1.02 g/ml. Small amounts of apoproteins B and E were also found in the pellet at the bottom of the centrifuge tube. In contrast to apoB-100, apoB-48 from the lumina of the three organelles was distributed throughout the density gradient in fractions of densities from 1.25 to 1.02 g/ml. Therefore, although approximately half of the apoB-48 was present in particles of density less than 1.02 g/ml (i.e. in the LDL/VLDL density range), significant amounts of apoB-48 were also present in particles of higher density (i.e. in the LDL/high density lipoprotein density range). However, the pattern of distribution of apoproteins B in the density gradient was the same regardless of whether the sample of luminal contents was obtained from the ERI, ERII, or Golgi. The same pattern of density distribution of apoB-48 and apoB-100 from lipoproteins secreted by cultured rat hepatocytes has been observed by us and

Fig. 2. Recovery of apoB-associated lipids after immunoaffinity chromatography. Rat plasma VLDL, containing known amounts of triacylglycerols, cholesterol plus cholesteryl esters, and phospholipids, were incubated with Sepharose 4B covalently linked to antibody directed against human apoB in the presence (open circles) or absence (closed circles) of a lipid microemulsion containing 508 nmol of total lipids of which the mol % composition was triacylglycerols/cholesterol/cholesteryl esters/phospholipids, 65:7:10:18. The lipids were extracted from the affinity gel, and the mass of triacylglycerols (panel A), phospholipids (panel B), and cholesterol plus cholesteryl esters (panel C) bound to the beads was determined.

Fig. 3. The amount of apoB-associated lipids in the lumen of the endoplasmic reticulum and Golgi and in plasma VLDL and VLDL secreted by cultured rat hepatocytes. The amount of apoB in the luminal contents of three rat liver subcellular fractions (ERI, ERII, and Golgi) was quantitated by an enzyme-linked immunoassay. Aliquots from the lumina of two endoplasmic reticulum ERI and ERII fractions, as well as Golgi, were incubated with anti-human apoB-Sepharose. Lipids were extracted from the affinity beads and the mass of triacylglycerols (solid bars), cholesterol plus cholesteryl esters (open bars), and phospholipids (hatched bars) was measured. Plasma VLDL (PLAS) (isolated by sequential ultracentrifugation) and VLDL from hepatocyte medium (MED) (isolated by ultracentrifugation on a single salt gradient) were treated similarly. The data are expressed as nanomoles of each lipid/microgram of apoB in the luminal contents. Values represent means ± S.D. from 8 to 10 independent subcellular fractionations.
ApoB-containing lipoproteins were isolated from the luminal contents of two endoplasmic reticulum fractions (ERI and ERII) and Golgi vesicles from rat liver. The amount of apoB, as well as the amount of each lipid moiety, was determined per milligram of vesicle protein.

In a second experiment, the lipoproteins in the top fraction from ERI, ERII, and Golgi fractions (22.3, 18.8, and 6.0 mg of vesicle protein, respectively) were ultracentrifuged on a salt gradient, and the nanomoles of each lipid component/microgram of density lipoprotein.

Apo E was also distributed throughout all fractions collected from the density gradient but were clearly visible in the fraction of density 1.02 g/ml from all three luminal fractions. The fraction of density 1.02 g/ml was collected from all three luminal contents and examined by negative staining electron microscopy. The fraction from the ERI and ERII contents was concentrated by ultrafiltration on a polyether sulfone membrane prior to examination by electron microscopy. The size bar represents 100 nm. The size distribution of the particles, as determined by measurement of 100 particles in each sample, is shown in the right-hand panel. ERI, solid line; ERII, small dashes; Golgi, alternating long and short dashes.

ApoB-containing Lipoproteins in the Lumen of the Endoplasmic Reticulum Are Not the Result of Contamination from Golgi Lipoproteins—Since the concentration of luminal apoB and lipids/milligram of total organelle protein was 7–10-fold higher in the Golgi than in the endoplasmic reticulum (Table III), the possibility existed that the apoB-containing lipoproteins isolated from the endoplasmic reticulum fractions were derived from the small (approximately 5%) contamination of the endoplasmic reticulum by Golgi vesicles (Table I). This possibility was eliminated by examination of the sensitivity of the luminal apoproteins to endoglycosidase H. Apoprotein B in plasma is a glycoprotein containing multiple N-glycosylated sites (33–35). The initial events in the process of N-glycosylation of proteins occur in the endoplasmic reticulum where a "high mannose" intermediate is cotranslationally formed (36). Endoglycosidase H cleaves the β-(1→4) linkage between 2 N-acetylglucosamine residues of the high mannose structure (36). Sensitivity of a glycoprotein to this glycosidase indicates the presence of the high mannose oligosaccharide structure. Upon entering the Golgi, glycoproteins are processed so that their N-linked oligosaccharide structures are

---

2 A. Rusinol and J. E. Vance, unpublished observations.
and Golgi was examined. Luminal contents of the ERI, ERII, and Golgi vesicles were fractionated by ultracentrifugation on a salt gradient. The fraction of density less than 1.02 g/ml from each organelle was incubated in the presence or absence of endoglycosidase H (Endo H). The proteins were subjected to polyacrylamide gel electrophoresis, then transferred to a polyvinylidene difluoride membrane. The membrane was probed first with concanavalin A linked to horseradish peroxidase (Con A), then with anti-VLDL. The immunoreactive species were visualized with enhanced chemiluminescence reagents.

The proteins were subjected to polyacrylamide gel electrophoresis, and are no longer sensitive to endoglycosidase H. In contrast, when the fractions of density less than 1.02 g/ml from the organelle or vesicle were incubated with endoglycosidase H, apoB-48 and apoB-100 were found to have lost their ability to react with the lectin. As expected, in the Golgi fractions the intensities of the protein bands that bound to concanavalin A were unaffected by endoglycosidase H treatment. If the luminal apoproteins isolated from the two endoplasmic reticulum fractions had been derived from the Golgi, the putative "endoplasmic reticulum" apoproteins would have been insensitive to endoglycosidase H. Instead, the sensitivity of the apoproteins of the two endoplasmic reticulum fractions to endoglycosidase H eliminates the possibility that the luminal lipoproteins isolated from ERI and ERII originated from contamination by Golgi vesicles.

The aim of this study was to determine the intracellular site(s) of assembly of apoB with lipids during formation of hepatic VLDL. Our experiments demonstrate that the average lipid composition, density distribution, and size of the apoB-containing lipoproteins in the lumina of the Golgi and the heavy and light endoplasmic reticulum fractions were essentially identical. Moreover, these properties of the intracellular apoB-containing lipoproteins were very similar to those of VLDL isolated from the medium of cultured rat hepatocytes and from rat plasma.

Our observations complement the recent report of Boren et al. (9) who concluded that in the human hepatoma cell line, HepG2, apoB-100 is cotranslationally associated with lipid during translocation of apoB across the endoplasmic reticulum membrane into the lumen. In addition, our data support the suggestion of Borchardt and Davis (8) that in rat hepatocytes apoB is assembled with its lipids in the endoplasmic reticulum. Our data are not, however, in accord with the conclusion of Higgins and co-workers (11, 12) or Bamberg and Lane (10) that the majority of triacylglycerols and phospholipids are assembled into VLDL particles in the Golgi rather than in the endoplasmic reticulum. The explanation for the different conclusions regarding the intracellular site of hepatic VLDL assembly is not clear. The use of different experimental models (e.g., rat hepatocytes (8), rat liver (11, 12), HepG2 cells (9, 14–16), and estrogen-treated chicken hepatocytes (10, 13)) may be one explanation for the apparent discrepancies. Another explanation may be that in most of the previous studies cultured cells were used, and the results depended upon isolation of pure subcellular fractions. The results of studies involving subcellular fractionation of rat liver are less likely to be ambiguous because this procedure generates much more material and more pure fractions than from cultured cells.

Our experiments demonstrate clearly that the amount of each lipid component/microgram of apoB is constant throughout the secretory pathway. The data show that VLDL/LDL-like, apoB-containing lipoprotein particles are present in the lumen of the endoplasmic reticulum. There was no general increase in the average size of the apoB-containing particles, nor an increase in their lipid content, nor a change in their density distribution when the particles reached the Golgi. Therefore, we conclude that the site of assembly of apoB with its full complement of lipids is the endoplasmic reticulum. From the data in this study, we cannot compare the relative lipid compositions of intracellular apoB-100- versus apoB-48-containing lipoproteins, except to note that the former particles were nearly all present in the VLDL/LDL density range and therefore were presumably, on the average, more "lipid-rich" than the latter particles, which were distributed throughout the VLDL/LDL/high density lipoprotein density range. Our results do not preclude the possibility that as VLDL progress through the secretory pathway (i.e. from endoplasmic reticulum to Golgi to secretory vesicles to secretion) there is an exchange, but not a net acquisition, of phospholipids and possibly some triacylglycerols between the fully formed lipoprotein particles and the membrane of the organelle or vesicle. Such an exchange has been implicated by the report of Howell and Palade (37) and is also compatible with the pulse-chase study of Janero and Lane (13) showing that radiolabeled phospholipids were added to VLDL of chicken hepatocytes in both the endoplasmic reticulum and the Golgi.

**Fig. 6.** Apoproteins isolated from the luminal contents of the endoplasmic reticulum are sensitive to treatment with endoglycosidase H. Luminal contents from ERI, ERII, and Golgi fractions were subjected to ultracentrifugation, and the fractions of density less than 1.02 g/ml were incubated in the presence or absence of endoglycosidase H (Endo H). The proteins were subjected to polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The proteins on the membrane were subsequently probed with concanavalin A (a lectin with high affinity for mannose residues) linked to horseradish peroxidase. The membranes were reprobed with antibody directed against rat plasma VLDL. As shown in Fig. 6, the amounts of apoB-48 and apoB-100 from the ERI, ERII, and Golgi fractions that reacted with the anti-VLDL antibody were the same in samples incubated in the presence or absence of endoglycosidase H. When the membrane was probed with concanavalin A linked to horseradish peroxidase apoproteins B-100 and B-48 were visible in the luminal contents of the ERI, ERII, and Golgi fractions that had not been treated with endoglycosidase H. In contrast, when the fractions of density less than 1.02 g/ml from the luminal contents of the ERI and ERII were incubated with endoglycosidase H, apoB-48 and apoB-100 were found to have lost their ability to react with the lectin. As expected, in the Golgi fractions the intensities of the protein bands that bound to concanavalin A were unaffected by endoglycosidase H treatment. If the luminal apoproteins isolated from the two endoplasmic reticulum fractions had been derived from the Golgi, the putative "endoplasmic reticulum" apoproteins would have been insensitive to endoglycosidase H. Instead, the sensitivity of the apoproteins of the two endoplasmic reticulum fractions to endoglycosidase H eliminates the possibility that the luminal lipoproteins isolated from ERI and ERII originated from contamination by Golgi vesicles.
REFERENCES