Assembly of very low density lipoproteins in rat liver: a study of nascent particles recovered from the rough endoplasmic reticulum

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Abstract To investigate the assembly pathway for hepatic very low density lipoproteins (VLDL), nascent lipoproteins were recovered from a purified, intact rough endoplasmic reticulum (ER) fraction isolated from rat liver. Two fractions were recovered by ultracentrifugation. Particles isolated at d 1.006 g/ml were triglyceride-rich particles containing apolipoprotein (apo)B-100 or apoB-48, and apoE with very small amounts of apoA-I. Compared with VLDL recovered from the Golgi apparatus, the particles from the rough ER had less triglyceride, but more cholesteryl ester and phospholipid. The second class of particles isolated between d 1.006 and 1.210 g/ml were phospholipid-rich and contained apoB-48, apoE, and apoA-I. ApoB-100 was a minor component. Radioisotope incorporation studies utilizing [^{3}H]leucine revealed differential rates of labeling of the apoproteins in these two lipoprotein fractions. ApoB-100 and apoE followed similar patterns in both fractions with peak incorporation occurring within 15 min of isotope injection. Incorporation of [^{3}H]leucine into apoB-48 in the dense fraction peaked within 15 min of isotope administration, but peak incorporation in the d 1.006 g/ml fraction did not occur until approximately 30 min after injection. We propose that the two lipoprotein fractions recovered from the rough ER are intermediates in the assembly of VLDL by the liver. Comparison of the composition of these two particles with that of Golgi VLDL supports the sequential assembly of VLDL by the liver. Furthermore, we propose that the initial steps in the assembly of apoB-100- and apoB-48-containing lipoproteins are different with nascent apoB-100-containing particles being formed through the cotranslational association of this apoprotein with lipid while nascent apoB-48-containing VLDL are formed in the rough ER through a two-step process.—**Swift, L. L.** Assembly of very low density lipoproteins in rat liver: a study of nascent particles recovered from the rough endoplasmic reticulum. *J. Lipid Res.* 1995. **36:** 395–406.

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The intracellular events involved in the formation of very low density lipoproteins (VLDL) are poorly understood (for review see refs. 1–3). The process is initiated in the rough endoplasmic reticulum (ER) and may be driven by the translation and translocation of apolipoprotein (apo)B across the membrane into the lumen of the ER. It has been suggested that as apoB is translocated it interacts with regions in the ER where neutral lipid and phosphatidylcholine synthesis occurs, allowing the protein to interact cotranslationally with newly synthesized lipids (4). This suggestion was based on studies using cells transfected with C-terminally truncated apoB molecules which demonstrate an inverse relationship between the length of the truncated protein and the density and presumably size of the lipoprotein formed. On the other hand, the observation that a pool of apoB exists in the rough ER membrane has led to speculation that apoB-100 is cotranslationally bound to the ER membrane from which it is subsequently transferred in the initial step in VLDL formation. Pulse chase studies in HepG2 cells have supported this hypothesis, demonstrating a precursor–product relationship between membrane apoB-100 and apoB-100 in the lumen of the rough ER (5, 6).

The addition of lipid to the nascent particle has been the subject of a number of studies. Pulse-chase studies in HepG2 cells (5–7) and chicken hepatocytes (8) have suggested that apoB combines with some lipid in the ER, but additional lipid is added as the apoB-containing particles are transported through the ER and the Golgi apparatus. In this model, VLDL formation is a sequential process initiated by apoB synthesis. In contrast, Rusinol, Verkade and Vance (9) reported that the average lipid composition, density distribution, and size of apoB-containing lipoproteins in the lumina of the Golgi and heavy and light ER fractions were essentially identical and very similar to those of VLDL isolated from the media of cultured rat hepatocytes. They concluded that the site of assembly of apoB with its full complement of lipid is the ER. Finally, studies in estrogen-treated chicken hepatocytes (10) and rat liver (11, 12) suggested that apoB remains membrane-

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoprotein; ER, endoplasmic reticulum; BCA, bicinchoninic acid; ACAT, acyl-CoA:cholesterol acyltransferase; TLC, thin-layer chromatography.

bound until it reaches the Golgi apparatus, at which time it combines with lipid, forming VLDL which is then released into the lumen. In this model, the Golgi apparatus is the primary site for addition of lipid to the particle.

Studies in our laboratory have focused on understanding the assembly of VLDL by the liver. We have used the rat model and our approach has been to isolate intermediates in the assembly process from subcellular organelles and through characterization of these complexes deduce the assembly process. In this paper we report the isolation and characterization of two lipoprotein complexes recovered from hepatic rough ER fractions which we hypothesize represent early intermediates in the assembly of VLDL. The characterization of these lipoprotein fractions in combination with isotopic incorporation studies utilizing [^{3}H]leucine suggest that VLDL assembly in the rat liver is a sequential process involving the rough ER and the Golgi apparatus. In addition, our results suggest different pathways for the assembly of apoB-100-containing particles as compared to apoB-48-containing particles.

METHODS

Isolation of subcellular fractions

Male rats (Harlan Industries, Indianapolis, IN) were maintained in the Animal Care Facility under constant temperature and humidity conditions with food (Wayne Lab Blox, Allied Mills, Inc., Chicago, IL) and water ad libitum for at least 5 days prior to the experiments. The rats weighed 220–240 g at the time of the experiments. Nonfasting animals were used for all studies. Rats were anesthetized with ether and killed by exsanguination from the terminal abdominal aorta. The livers were removed immediately after exsanguination, rinsed in ice-cold water, and trimmed of excess fat and connective tissue. The method for isolating rough ER was adapted from that of Eriksson and Glaumann (13). Livers were minced finely with scalpels, and homogenized in 1.5 volumes of homogenizing media (0.1 M phosphate buffer, pH 7.3, containing 0.25 M sucrose), using a Polytron S-10 (Brinkman Instruments, Westbury, NY) at setting 0.5 for 20 sec. This method of homogenization has been used for isolating Golgi apparatus-rich fractions and is effective in rupturing hepatocytes while minimizing damage to the subcellular organelles (14). After homogenization, additional homogenizing medium was added to bring the total to 1.8 volumes. The homogenate was centrifuged at 2500 rpm for 10 min and 9500 rpm for 30 min in an SW 27 rotor (Beckman Instruments, Palo Alto, CA), after which the supernatant was decanted and adjusted to 15 mM CsCl. The supernatant was layered on discontinuous sucrose gradients consisting of 5 ml 1.975 M sucrose, 10 ml 1.375 M sucrose, and 5 ml 0.86 M sucrose with each sucrose solution containing 15 mM CsCl. The samples were centrifuged in the SW 27 rotor at 5000 rpm for 10 min, 10,000 rpm for 10 min, and 25,000 rpm for 120 min. Material banding at the 1.375/1.975 M sucrose interface was removed, diluted approximately 6-fold with ice-cold distilled water, and pelleted in the SW 27 rotor at 25,000 rpm for 30 min.

Golgi apparatus-rich fractions were prepared by a slight modification of our previously published procedures (14). The Golgi fraction banding on the 1.2 M sucrose pad was removed, diluted 30-fold with ice-cold water, and pelleted by centrifuging at 10,000 rpm for 20 min. This modification has been shown to reduce multivesicular body contamination (15).

The final subcellular fractions were resuspended in 15 mM Tris-HCl, pH 7.4, containing 154 mM NaCl (Tris-saline) after taking a small piece of the pellet for electron microscopy. Aliquots of the suspension were frozen in liquid nitrogen for enzyme analyses, and the remainder was passed twice through the French pressure cell (American Instrument Co., Travenol Laboratories, Inc., Silver Spring, MD) under 6000 psi to release the contents. The membranes and contents were separated by centrifugation in the 40.3 rotor (Beckman Instruments) at 105,000 *g* for 45 min. The membrane pellets were suspended in Tris-saline and frozen. Lipoprotein complexes were recovered from the contents (supernatant) as described below.

Rough ER membrane and contents were also separated after treatment with 0.1 M Na_2CO_3, pH 11.2, as described by Fujiki et al. (16). The content proteins were precipitated with 10% ice-cold TCA for at least 1 h and washed one time with ethyl ether to remove TCA.

Isolation of subcellular and serum lipoproteins

Lipoproteins were isolated by ultracentrifugation using the Beckman 40.3 rotor. Two fractions were recovered from the rough ER contents. The first fraction was isolated at d 1.006 g/ml by centrifugation for 18 h at 105,000 *g* and recovered by tube slicing. The density of the infranatant was raised to 1.210 g/ml using solid KBr, and the second fraction was isolated by centrifugation for 42 h at 105,000 *g*. Both fractions were dialyzed exhaustively against 0.01% EDTA, pH 7.2, and against a final change of distilled water. Aliquots were taken for negative staining, and the remainder was lyophilized. Very low density lipoproteins were isolated from serum and from the Golgi apparatus by centrifugation at 105,000 *g* for 18 h at d 1.006 g/ml.

Chemical and enzyme analysis of subcellular fractions

Protein was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL) using bovine serum albumin as standard. NADPH cytochrome C reductase was assayed by the procedure of Masters, Williams, and Kamin (17) as modified by Wannagat, Adler, and Ockner

(18). Succinate cytochrome C reductase was measured as described by Fleischer and Fleischer (19), and galactosyl transferase by the method of Morre, Merlin, and Keenen (20). RNA content of the fractions was analyzed by the method of Schneider (21). Acyl CoA:cholesterol acyltransferase (ACAT) was analyzed by the method of Erickson and Cooper (22) with the following modifications. The assay contained 50–100 μg protein in a final volume of 200 μl, and was initiated by the addition of 5 nmol [^{14}C]oleoyl CoA (DuPont NEN, Boston, MA, 54.5 mCi/mmol) after a 5-min pre-incubation period (37°C). The reaction was stopped after 4 min by the addition of 3 ml chloroform–methanol 2:1 (v/v). [^{3}H]cholesteryl ester (DuPont NEN) was added as an internal standard, and cholesteryl esters were isolated using thin-layer chromatography (TLC), scraped into scintillation vials, and radioactivity was determined using Budget-Solve (Research Products International Corp., Mount Prospect, IL) as fluor.

Lipoprotein protein and lipid analyses

Lipoprotein protein was estimated by the BCA method with modification to eliminate lipid interference (23). Lyophilized fractions were delipidated with ethanol and ethyl ether, and individual lipid classes were separated by thin-layer chromatography on silica gel 60A thin-layer plates (Whatman K6, Fisher Scientific, Atlanta, GA) with petroleum ether–ethyl ether–acetic acid 80:20:1 (v/v/v) as the developing solvent. Lipid classes were visualized with rhodamine B. Triglycerides were scraped from the plate, eluted from the gel, and fatty acids were methylated using boron trifluoride–methanol (24). Phospholipids were methylated as described (24) without elution from the silica gel. The fatty acid methyl esters were analyzed by gas chromatography using a Hewlett-Packard 5890A gas chromatograph interfaced with an HP 3365 Chem Station and equipped with a 6 ft × 2 mm i.d. glass column packed with 10% SP 2330 on 100/120 Chromosorb W (Supelco, Bellefonte, PA). Fatty acid methyl esters were identified by comparison of retention times to those of known standards. The mass of lipid was determined by comparing the total fatty acid area of the sample to that of an internal standard (trieicosenoin and dipentadecanoylphosphatidylcholine) that was added at time of delipidation. Unesterified cholesterol and cholesteryl ester were analyzed by the method of Babson, Shapiro, and Phillips (25) without elution from the silica gel.

Individual phospholipid classes were separated by one-dimensional thin-layer chromatography on Whatman K6 plates as described by Yao and Vance (26) using chloroform–methanol–acetic acid–formic acid–H_2O 70:30:12:4:2 (v/v) as the developing solvent. The plates were developed for 1 h in the solvent system, and phospholipids were visualized using Molybdenum Blue TLC spray reagent (Alltech, Deerfield, IL). The spots were scraped from the plates, and dipentadecanoylphosphatidylcholine was added as a standard. The phospholipid fatty acids were methylated and fatty acid methyl esters were analyzed by gas chromatography. The mass of each phospholipid was calculated from the relative mass of fatty acid compared with the internal standard, considering the average molecular weight of each phospholipid class.

SDS polyacrylamide gel electrophoresis and immunoblot analyses

SDS polyacrylamide gradient (3–20%) gels were run as described by Swift et al. (27). For visualization of protein, gels were stained with 0.01% Coomassie Brilliant Blue in methanol–acetic acid–water 10:1:10 (v/v/v) and destained. For immunoblot analysis the proteins were transferred from SDS gels to nitrocellulose paper (Bio-Rad Laboratories, Hercules, CA) according to Burnette (28) using a Trans-Blot cell (Bio-Rad) and 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% (v/v) methanol as blotting buffer. Transfer was carried out at 60 volts for 16 h at 4°C. The blotted membranes were blocked using 5% nonfat dry milk in 20 mM Tris-HCl, 500 mM NaCl, pH 7.5, (TBS) and washed in TBS containing 0.05% Tween 20 (TTBS). The membranes were then incubated for 1.5 h at room temperature with rabbit antibody to rat apoB in TBS (1:500–1:800 dilution), washed with TTBS and incubated 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad, 1:3000 dilution). The membranes were washed and incubated with NBT and BCIP (Bio-Rad) for up to 30 min. Blots were screened using a Bio-Rad Imaging Densitometer, Model GE-670.

Electron microscopy

Small pieces of the final rough ER pellets were fixed in 2% glutaraldehyde in phosphate buffer (pH 7.2) overnight. The samples were rinsed in 7.5% phosphate-buffered sucrose and post-fixed in Millonig's osmium tetroxide. They were stained en bloc with aqueous uranyl acetate, dehydrated, and embedded in Araldite. Thin sections were doubly stained with uranyl acetate and lead citrate. Lipoprotein fractions were negatively stained on carbon-stabilized Formvar-coated grids (Electron Microscopy Sciences, Ft. Washington, PA) using aqueous 2% phosphotungstic acid, pH 6.5. Grids were viewed in a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) and various fields were selected and photographed. Lipoprotein diameters were determined by measuring all spherical lipoprotein particles in a given field.

In vivo labeling studies

Rats were anesthetized with ether, and 100 μCi L-[4,5 ^{3}H]leucine (ICN Biomedicals, Inc., Costa Mesa, CA, 110 Ci/mmol) in 154 mM NaCl, 10 mM phosphate buffer (pH 7.4) was injected into the jugular bulb. After injec-

tion the rats were placed in a warm box and allowed to recover. The anesthetized rats were killed by exsanguination at 10, 15, 30, or 45 min after injection. The liver was removed, and nascent particles were recovered from the subcellular fractions as described above. The labeled apoproteins were separated on SDS-PAGE, stained, destained, and sliced into 2-mm slices. The gel segments were prepared for scintillation counting using NCS tissue

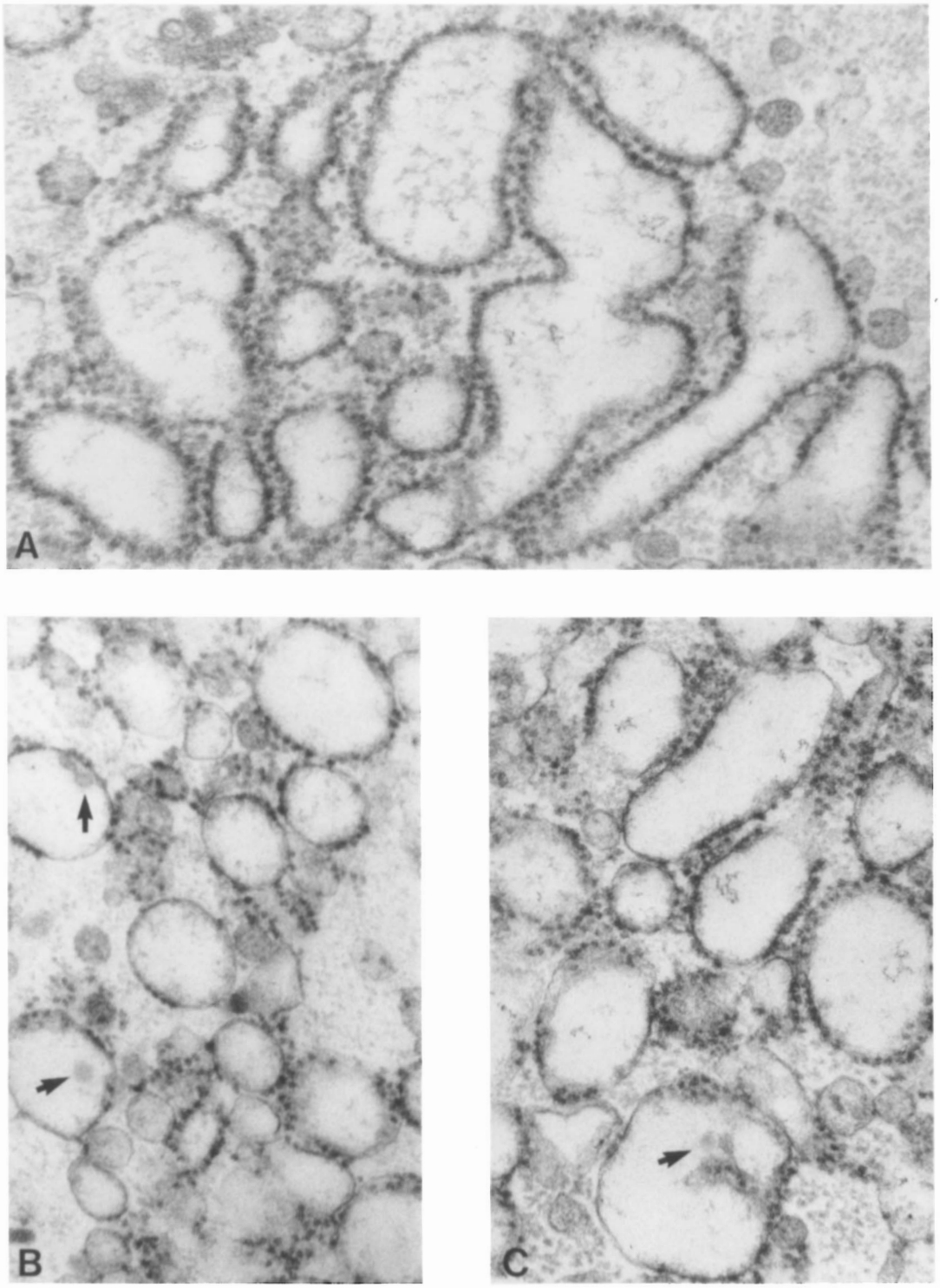

Fig. 1. Electron micrographs of rough ER fractions from rat liver. Ribosome-studded membranes appeared in large sheets (A) which may represent dilated cisternae. Vesicular structures were also noted (B, C). Osmiophilic particles (arrows) with diameters of 300–475 Å were noted generally closely associated with the membranes. Uranyl acetate and lead citrate. ×62,750.

TABLE 1. Enzyme and chemical analyses of subcellular fractions from rat liver

Fraction	NADPH-Cytochrome C Reductase	Galactosyl Transferase	ACAT	RNA	Cholesterol / Phospholipid
	μmol/min/mg protein	*nmol/h/mg protein*	*pmol/min/mg protein*	*μg/mg protein*	
Homogenate	$2.44 \pm 0.19 \times 10^{-2}$	5.60 ± 0.83	112.6 ± 20.7	0.165 ± 0.29	
Rough ER	$12.24 \pm 2.06 \times 10^{-2}$ (5.0)	7.94 ± 4.44 (1.4)	1056.4 ± 158.9 (9.4)	0.839 ± 0.081 (5.1)	0.044 ± 0.008
Golgi apparatus		420.8 ± 159.0 (75.1)			0.183 ± 0.043

Values represent mean ± SD of at least four rough ER preparations and three Golgi preparations. Numbers in parentheses represent fold enrichment over homogenate.

solubilizer (Amersham, Arlington Heights, IL). Samples were counted in a Beckman LS 6800 scintillation counter.

Statistics

Data were analyzed by Student's *t*-test.

RESULTS

Morphology of isolated rough ER fractions

Rough ER fractions isolated from rat liver consisted of several membrane structures. Ribosome-studded membranes appeared in large sheets as well as in vesicles (**Fig. 1**). Nearly all membranes had associated ribosomes, although some were more heavily studded than others. Some of the large sheets appeared to be individual intact cisternae of the rough ER complex. The large cisternae and vesicles contained amorphous material. In some vesicles spherical particles (300–475 Å) were noted, and in general these particles appeared in close apposition with the membrane. The Golgi fractions displayed characteristic morphology which we have described previously, namely plates with associated anastomosing tubular network. Lipoprotein-filled vesicles were prevalent.

Enzyme and chemical analyses of rough ER

Table 1 presents the results of the enzymatic characterization of the rough ER preparations. The fractions were enriched 5-fold in NADPH cytochrome C reductase, 5-fold in RNA, and 8- to 10-fold in ACAT compared with homogenate. We were unable to detect any succinate cytochrome C reductase activity, and galactosyl transferase activity was similar to that of the homogenate. The ratio of unesterified cholesterol to phospholipid in the Golgi membranes was significantly larger than for the rough ER membranes, as has been previously reported (29).

Composition of lipoprotein complexes recovered from rough ER

The contents of the rough ER were fractionated by ultracentrifugation into two fractions: d < 1.006 g/ml and d 1.006–1.210 g/ml. The lipid compositions of these two fractions are given in **Table 2.** The fraction isolated at d 1.006 g/ml was triglyceride-rich; however, compared with VLDL isolated from Golgi fractions, this fraction had a decreased content of triglyceride and an increased content of cholesteryl ester and phospholipid. The second class of lipoproteins had little core material (cholesteryl ester and triglyceride), but was rich in phospholipid. Compared with the complexes isolated at d 1.006 g/ml, they were also enriched in unesterified cholesterol. The cholesterol/phospholipid ratios of the three fractions were significantly different, decreasing from the more dense rough ER fraction to the Golgi VLDL. By SDS polyacrylamide gel electrophoresis, the d 1.006 g/ml fraction from the rough ER contained apoB-100, apoB-48, and apoE (**Fig. 2**). By scanning densitometry the ratio of apoB-100/apoB-48 was 0.11 ± 0.04 (n = 6), suggesting

TABLE 2. Lipid composition of nascent lipoproteins recovered from rough ER and Golgi

	Rough ER (8)		Golgi (3)
	d 1.006–1.210 g/ml	d 1.006 g/ml	d 1.006 g/ml
		weight %	
Cholesteryl esters	12.75 ± 4.57	11.51 ± 2.22	4.40 ± 1.31
Triglycerides	9.64 ± 2.78	57.51 ± 5.28	70.10 ± 4.48
Unesterified cholesterol	15.36 ± 4.54	2.66 ± 1.47	0.93 ± 0.45
Phospholipid	62.26 ± 5.72	28.33 ± 2.52	24.60 ± 5.25
$\frac{\text{Cholesterol}}{\text{Phospholipid}}$ ratio	0.258 ± 0.084	0.089 ± 0.044	0.040 ± 0.021

Data represent mean ± SD. Number of determinations are shown in parentheses.

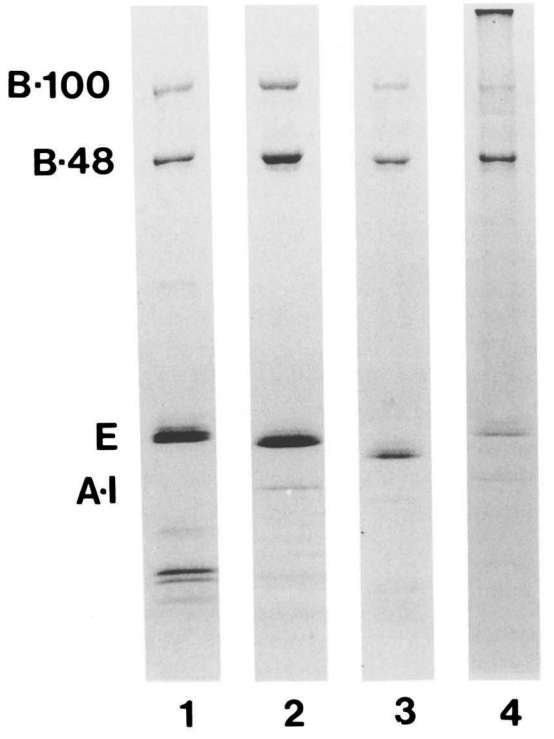

Fig. 2. SDS polyacrylamide gel electrophoretograms of serum VLDL (1), Golgi VLDL (2), d 1.006 g/ml (3), and d 1.006–1.210 g/ml (4) fractions from rough ER. Proteins were separated on 3–20% continuous acrylamide gradient gels and stained with Coomassie Blue.

that apoB-48 is clearly the more prominent B protein in this fraction. Fast migrating low molecular weight protein bands were consistently observed, but the identity of these bands is unknown. The d 1.006–1.210 g/ml fraction contained apoB-48 and only small amounts of apoB-100. In addition, the fraction contained apoE and apoA-I. Based on staining intensity, apoE and A-I were present in almost equal amounts. It is important to note that apoA-I was far more abundant in this fraction that in the d 1.006 g/ml fraction. Other unidentified Coomassie-staining bands were also noted in this fraction.

Figure 3 presents the particle size distribution of these two classes of lipoprotein particles. The d 1.006 g/ml particles averaged 338 Å in diameter (338 ± 91 Å; n = 1251) with particles sizes ranging from 146 to 780 Å. Consistent with their density and composition, the d 1.006–1.210 g/ml particles were smaller particles, ranging from 65 to 419 Å and averaging 170 Å (170 ± 65 Å; n = 536) in diameter.

The fatty acid composition of phospholipids from the two fractions, serum VLDL, and rough ER membranes is presented in **Table 3.** The fatty acid composition of the serum VLDL phospholipids was quite different from that of the rough ER d 1.006 g/ml fraction containing more C16:0 and C18:2, and less C18:0, C20:4, and C22:6. The fatty acid compositions of the rough ER fractions differed slightly with the d 1.006 g/ml fraction containing less C16:0 and more C22:6 than the d 1.006–1.210 g/ml fraction. The fatty acid composition of rough ER membranes and the d 1.006–1.210 g/ml fraction differed in that the latter fraction contained more C16:0, but less C18:2, C20:4, and C22:6 than the membranes.

The phospholipid composition of the rough ER membranes and content fractions along with the phospholipid composition of serum VLDL is shown in **Table 4.** The phospholipid compositions of the two content fractions were similar. There were minor differences between the rough ER membranes and the d 1.006–1.210 g/ml fraction with the membranes containing more phosphatidylinositol and less sphingomyelin than the d 1.006–1.210 g/ml fraction. The phospholipid composition of serum VLDL differed markedly from that of the rough ER membranes and content fractions. Serum VLDL have less phos-

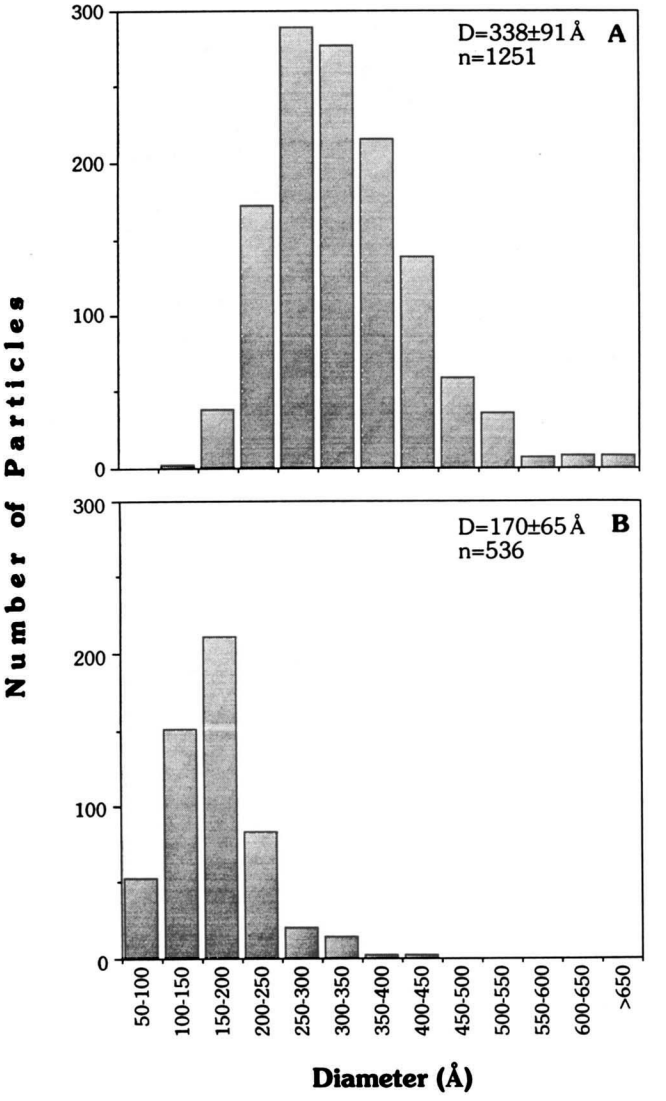

Fig. 3. Diameters of lipoprotein particles recovered from rough ER. A: d 1.006 g/ml; B: d 1.006–1.210 g/ml. D, diameter (mean ± SD); n, number of particles measured.

TABLE 3. Fatty acid composition of phospholipids and triglycerides

Fatty Acid	Membranes	d 1.006–1.210 g/ml	d 1.006 g/ml	Serum VLDL
14:0	0.23 ± 0.07	0.54 ± 0.14	0.79 ± 0.29	0.52 ± 0.19
16:0	18.24 ± 0.91[a]	22.25 ± 1.04[b]	19.85 ± 0.69[a,b]	22.62 ± 1.31[b]
16:1	1.00 ± 0.16	0.73 ± 0.42	0.86 ± 0.28	0.62 ± 0.43
18:0	20.37 ± 1.12	21.42 ± 1.62[c]	22.42 ± 2.91[a]	18.86 ± 1.96[b,d]
18:1	7.55 ± 0.44[c]	8.13 ± 0.86[a,b]	9.31 ± 0.52[a,d]	8.90 ± 0.63[b]
18:2	15.50 ± 0.82	13.28 ± 1.34	14.11 ± 1.73	24.32 ± 2.18
20:3n-6	0.23 ± 0.17	0.06 ± 0.13	N.D.	0.12 ± 0.16
20:3n-9	1.40 ± 0.17	1.66 ± 0.25	1.32 ± 0.18	1.49 ± 0.22
20:4n-6	23.06 ± 1.28[a]	20.07 ± 1.40[a,b]	21.16 ± 1.70	14.55 ± 1.74[b]
20:5n-3	1.23 ± 0.22	0.66 ± 0.60	0.78 ± 0.55	0.46 ± 0.47
22:4	0.55 ± 0.40	1.72 ± 0.50	N.D.	1.26 ± 0.49
22:5n-3	1.91 ± 0.27	1.88 ± 0.32	1.63 ± 0.23	1.23 ± 0.28
22:6n-3	8.28 ± 0.69	6.58 ± 0.58	7.45 ± 0.61	4.38 ± 0.62
	(n = 7)	(n = 5)	(n = 7)	(n = 7)

N.D., not detected.
[a,b]$P < 0.01$; [c,d]$P < 0.05$.

phatidylethanolamine and phosphatidylserine and more phosphatidylcholine than the rough ER fractions. In addition, like the rough ER d 1.006–1.210 g/ml fraction, serum VLDL have more sphingomyelin than rough ER membranes.

The rough ER membranes and luminal proteins were separated after sodium carbonate treatment, and the proteins were separated by SDS-PAGE. After transfer from the gel to nitrocellulose and immunolocalization using antibody to apoB, the blots were scanned and the relative amounts of apoB-100 and apoB-48 in the membrane and contents were assessed. **Table 5** presents the results of these studies. Approximately 80% of apoB-100 was found in the contents with the remainder localized in the membrane. In contrast, approximately 60% of the total apoB-48 was found in the contents with 40% remaining with the membrane.

Radioisotope incorporation studies

Radioisotope incorporation studies were carried out to investigate the relationship between the two fractions recovered from the rough ER. In these studies [^{3}H]leucine was injected via the jugular bulb, and the animals were killed at 10, 15, 30, and 45 min after injection. The rough ER fractions were isolated, the apoproteins were separated by SDS-PAGE, and radioactivity in the apoproteins was determined by liquid scintillation techniques. Total counts recovered with the apoproteins of both fractions (d 1.006 g/ml and d 1.006–1.210 g/ml) peaked by 15 min at 9600 dpms. At 10, 15, and 30 min postinjection, over 80% of the radioactivity was recovered with the d 1.006 g/ml fraction. At the 45-min time point, at which point total incorporation was approximately 2200 dpms, 65% of the radioactivity was recovered with the d 1.006 g/ml fraction, and 35% with the d 1.006–1.210 g/ml fraction.

The incorporation of [^{3}H]leucine into the apoproteins of the d 1.006 g/ml and d 1.006–1.210 g/ml fractions is shown in **Fig. 4.** Peak incorporation into apoB-48 in the d 1.006 g/ml fraction was approximately 31% higher than in apoB-100 (1239 vs. 946 dpms) and 75% higher in the d 1.006–1.210 g/ml fraction (748 vs. 427 dpms). Peak incorporation into apoE occurred by 15 min at approximately 5800 dpms in the d 1.006 g/ml fraction and approximately 750 dpms in the d 1.006–1.210 g/ml fraction.

In the d 1.006 g/ml fraction the labeling patterns of apoB-100 and apoE were similar, each reaching peak incorporation by 15 min and declining in parallel (Fig. 4A).

TABLE 4. Phospholipid composition of rough ER membranes, content fractions, and serum VLDL

	Membranes	d 1.006–1.210 g/ml	d 1.006 g/ml	Serum VLDL
Phosphatidylethanolamine	23.91 ± 1.57[a]	27.40 ± 3.33[a]	23.11 ± 3.00[a]	4.81 ± 2.08[b]
Phosphatidylserine	3.26 ± 0.61[a]	4.81 ± 1.93[a]	2.71 ± 1.32[c]	0.91 ± 0.40[b]
Phosphatidylinositol	12.91 ± 0.36[a]	6.26 ± 3.44[b]	7.85 ± 4.26	8.07 ± 6.06
Phosphatidylcholine	57.13 ± 2.28[a]	52.13 ± 6.43[a]	52.13 ± 6.43[a]	74.04 ± 5.12[b]
Sphingomyelin	2.81 ± 1.00[a]	9.14 ± 3.65[b]	6.58 ± 4.71	8.76 ± 1.82[b]
Lysophosphatidylcholine	N.D.	N.D.	N.D.	3.43 ± 4.01

N.D., Not determined.
[a,b]$P < 0.01$; [b,c]$P < 0.05$.

TABLE 5. Distribution of apoB-100 and apoB-48 between membrane and content fractions of rough ER

	ApoB-100	ApoB-48
Membrane	21.9 ± 10.6	41.7 ± 8.2
Contents	78.1 ± 10.6	58.3 ± 8.2

Values represent mean ± SD of four measurements for apoB-100 and five measurements for apoB-48.

Least squares analyses of the data excluding the 10-min time point suggest that apoE and apoB-100 move out of the rough ER at approximately the same rate of 2.98 and 2.82% per min, respectively. The incorporation of [^{3}H]leucine into apoB-48 lagged that of apoB-100 and apoE, attaining its maximum by 30 min after isotope injection. While there is only one time point after the peak for apoB-48, the movement of apoB-48 out of the rough ER seems to parallel that of apoB-100 and apoE.

The patterns of incorporation into the apoproteins of the d 1.006–1.210 g/ml fraction were slightly different (Fig. 4B). As in the d 1.006 g/ml fraction, peak isotope incorporation into apoB-100 and apoE occurred within 15 min of isotope injection; however, unlike the d 1.006 g/ml fraction, peak isotope incorporation into apoB-48 occurred within 15 min of isotope injection.

Because of the difference in the patterns of isotope incorporation into apoB-48 in the two fractions, we expressed the data as percent activity recovered in the individual apoproteins in the two fractions. The data are shown in **Fig. 5.** Approximately 70% of the total radioactivity recovered with apoB-100 in the contents was recovered in the d 1.006 g/ml fraction at all time points (Fig. 5A). Approximately 80% of the total activity recovered with apoE was found in the d 1.006 g/ml fraction (Fig. 5C). In neither apoB-100 nor apoE was there any suggestion of movement of these apoproteins from the d 1.006–1.210 g/ml fraction to the d 1.006 g/ml fraction. In contrast, for apoB-48 at the earliest time point there was more activity in the d 1.006–1.210 g/ml fraction than in the d 1.006 g/ml fraction, but this changed with time, suggesting that the label appearing in the d 1.006–1.210 g/ml fraction moved to the d 1.006 g/ml fraction (Fig. 5B). It should be noted that by scanning densitometry we estimated similar amounts of apoB-48 in the d 1.006 g/ml and d 1.006–1.210 g/ml fractions.

We attempted to determine radioactivity in apoB-100 and apoB-48 in the membranes of the rough ER in a manner similar to that used for determination of activity in the content apoproteins. Notwithstanding the fact that approximately 20% of the total apoB-100 in the rough ER fraction and 40% of the apoB-48 were found in the membrane (Table 5), we were unable by scintillation counting techniques to detect any radioactivity in either B protein in the membrane.

DISCUSSION

It was the aim of these studies to investigate VLDL assembly in the liver by isolating and characterizing intermediates in the assembly pathway. Previous studies in our laboratory have focused on the intermediates recovered from the Golgi apparatus (14, 30). In this study we recovered nascent lipoprotein complexes from hepatic rough ER fractions that were shown to retain morphologic integrity as judged by electron microscopy (Fig. 1) and were not contaminated by other organelles as judged by marker enzyme analyses (Table 1).

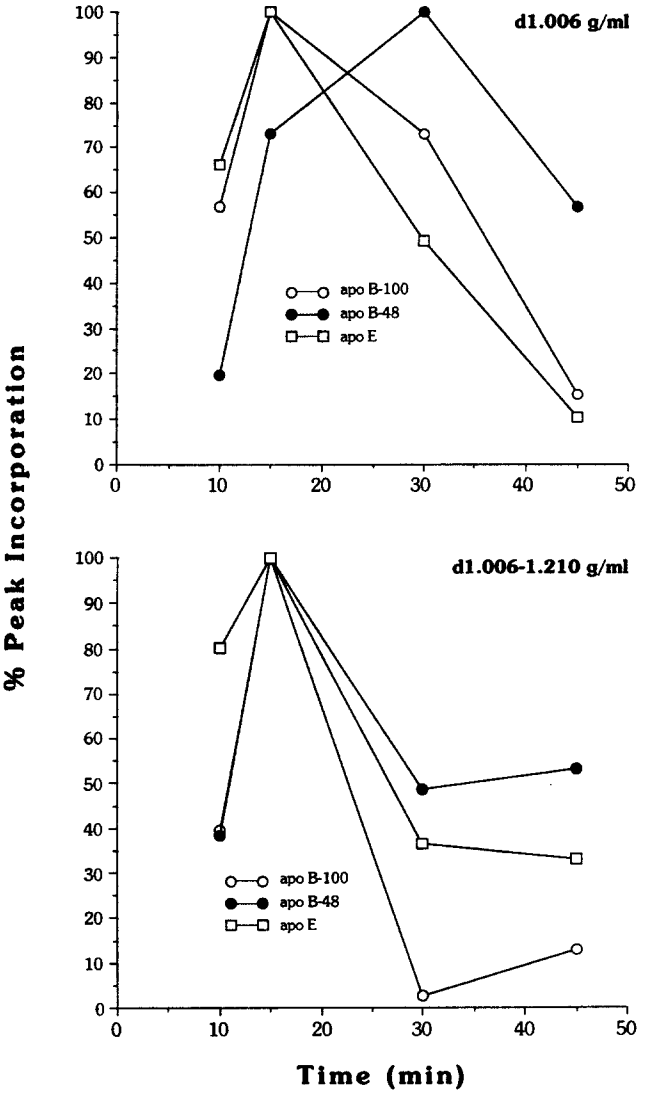

Fig. 4. Time course of [^{3}H]leucine incorporation into apoB-100 (—○—), apoB-48 (—●—), and apoE (—□—) of d 1.006 g/ml fraction (top) and d 1.006–1.210 g/ml fraction (bottom) recovered from hepatic rough ER. The lipoprotein fractions were isolated, apoproteins were separated by SDS polyacrylamide gel electrophoresis, and the radioactivity in individual apolipoprotein was determined by liquid scintillation techniques. Each point represents the average of two separate experiments.

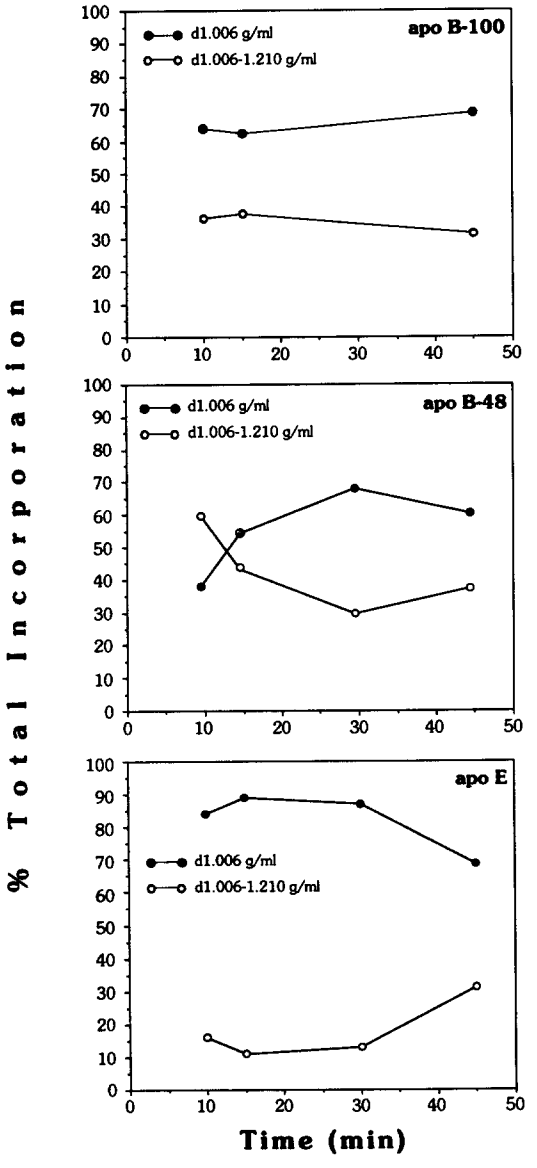

Fig. 5. Distribution of radioactivity between the d 1.006 g/ml fraction and d 1.006–1.210 g/ml fraction for apoB-100, apoB-48, and apoE. The lipoprotein fractions were isolated from the rough ER, apoproteins were separated by SDS polyacrylamide gel electrophoresis, and the radioactivity in each apolipoprotein was determined by liquid scintillation techniques. The total DPMs in each apoprotein were determined, and the percent recovered in each fraction was calculated. Each point represents the average of two separate experiments.

Two lipoprotein fractions were isolated from the contents of the rough ER by preparative ultracentrifugation. While the d 1.006 g/ml particles were triglyceride-rich, they were smaller than either Golgi VLDL or serum VLDL, containing proportionately less core material. If this particle is a precursor to Golgi VLDL and eventually to serum VLDL as we believe it to be, additional lipid, specifically triglyceride and phospholipid, must be added either as the particle is transported from the rough ER to the Golgi or perhaps in the Golgi apparatus as suggested by Bamberger and Lane (10). Compared with Golgi VLDL we estimate that the d 1.006 g/ml fraction has approximately 50% of its total triglyceride complement and perhaps 60–70% of its total phospholipid. While we have no experimental data concerning the subsequent addition of lipid to the d 1.006 g/ml particle, it is interesting to note that the phospholipid composition of the d 1.006 g/ml fraction can be changed to one very similar to that of Golgi VLDL, as reported by Hamilton and Fielding (31), simply by adding phosphatidylcholine. It is also important to note that the fatty acid composition of the phospholipids in the d 1.006 g/ml fraction was quite different from that of serum VLDL, consistent with our suggestion that a specific phospholipid class (e.g., phosphatidylcholine) might be added in the maturation of the particle.

The particles recovered between the densities 1.006 and 1.210 g/ml were deficient in core material, containing primarily surface material. The diameter of these particles (170 Å) was consistent with the lipid composition. The cholesterol/phospholipid ratio in this fraction was higher than in the membrane (0.258 ± 0.084 vs. 0.044 ± 0.008) suggesting that membrane contamination is minimal. In addition, while we do see some unidentified bands in this fraction by SDS-PAGE that could be membrane proteins, the primary proteins are apoB-48, apoE, and apoA-I. The composition of this particle suggests that it might be an early precursor to VLDL. It has been suggested that apoB might associate with lipid as it is translated and translocated across the membrane of the rough ER (4). It has also been suggested that membrane-associated apoB might be 'ejected' from the membrane along with lipids into the lipoprotein assembly pathway (5, 6). The primary lipids involved in these initial assembly steps might be expected to be phospholipid (perhaps membrane phospholipid) as well as cholesterol, some of which may be esterified by ACAT, which we and others (32, 33) have shown is concentrated in the rough ER. Thus the initial particle formed might be rich in phospholipid and contain both esterified and unesterified cholesterol, like the particles that we retrieve from the rough ER at d 1.006–1.210 g/ml. The addition of triglyceride to this particle, perhaps at the smooth surfaced ends of the rough ER, as suggested by Alexander, Hamilton, and Havel (34), could convert it to the particle isolated at d 1.006 g/ml. In this regard it is interesting to note that the addition of triglyceride to the d 1.006–1.210 g/ml particle changes the lipid composition to one similar to that of the particles that we isolated at d 1.006 g/ml.

The isotope incorporation studies provided additional insight into the assembly pathway and the relationship between the particles isolated at d 1.006 g/ml and those isolated at d 1.006–1.210 g/ml. The labeling patterns of apoB-100 and apoE in the d 1.006 g/ml fraction were similar, each attaining peak isotope incorporation by 15 min after injection. This peak in isotope incorporation for

apoB-100 in the rough ER was similar to that found by Boström and coworkers for apoB-100 in rough ER d 1.006 g/ml fraction in HepG2 cells (5), and, as noted by these workers, is consistent with an estimated translation time of 14 min (i.e., approximately 6 amino acids/sec). The decay of activity after the peak was approximately linear, and assuming the influx of apoB and apoE into the rough ER was negligible during this period, we determined the efflux to be approximately 3% per min, slightly slower than the rate of movement of apoB-100 from the rough ER to Golgi in HepG2 cells (5). In contrast, apoB-48 did not attain peak incorporation until approximately 30 min after isotope injection, but appeared to move out of the rough ER at a rate similar to that of apoB-100 and apoE. The labeling patterns in the d 1.006–1.210 g/ml fraction were slightly different with all three apoproteins attaining peak activity within 15 min of isotope injection.

The distribution of radioactivity in the individual apoproteins between the two fractions was quite different. For apoB-100 and apoE most of the radioactivity (70–80%) was found in the d 1.006 g/ml fraction at all time points, while there was a clear time-dependent shift in the distribution of radioactivity of apoB-48 from the denser fraction to the d 1.006 g/ml fraction.

In interpreting these data, it must be kept in mind that lipoprotein assembly in rat liver is complicated by the fact that the liver synthesizes both apoB-100 and apoB-48. Therefore, assuming only one copy of apoB (either apoB-100 or apoB-48) per lipoprotein particle as has been suggested (35), the rat liver then produces two VLDL particles—those containing apoB-100 and those containing apoB-48. From our compositional and isotopic labeling data we propose the following pathways for lipoprotein assembly.

We propose that there are different pathways for the assembly of apoB-100-containing VLDL compared with apoB-48-containing VLDL. We propose that apoB-100 particles recovered in the d 1.006 g/ml fraction are formed by a co-translational association of lipid with apoB-100 similar to the model proposed by Borén et al. (4). Therefore apoB-100 would form the triglyceride-rich particle during translocation to the lumen of the endoplasmic reticulum. This would explain why apoB-100 is found primarily with triglyceride-rich particles with only very small amounts in the d 1.006–1.210 g/ml fraction and also why there is no indication from the isotopic incorporation study that labeled apoB-100 on particles recovered in the dense fraction moves to the d 1.006 g/ml fraction.

We propose a two-step process for the assembly of apoB-48-containing, triglyceride-rich particles. We propose that apoB-48 associates first primarily with phospholipid and unesterified cholesterol forming the particle recovered in the d 1.006–1.210 g/ml fraction with subsequent conversion of this particle to a triglyceride-rich particle via addition of triglyceride, perhaps by the activity of the microsomal triglyceride transfer protein (36). Because of this additional step in assembly compared with apoB-100-containing particles, the peak labeling of apoB-48 in the d 1.006 g/ml fraction is delayed compared to that of apoB-100. After addition of triglyceride to the apoB-48-containing particle it appears to move to the Golgi at the same rate as do apoB-100-containing particles. Why should there be a two-step process in the assembly of apoB-48-containing particles? While we do not know the answer to this question, it may be related to the relative sizes of apoB-100 and apoB-48. The studies of Spring et al. (37) demonstrated a direct relationship between apoB size and lipoprotein core circumference. Yao et al. (38), utilizing a rat hepatoma cell line stably transfected with a variety of human truncated apoB cDNA constructs, examined the relationship between the size of apoB and the formation and secretion of apoB-containing lipoproteins. They reported that the size of apoB governs the lipid content of these lipoproteins as well as the kinetics of secretion. Borén et al. (4) also noted an inverse relationship between the size of C-terminally truncated apoB polypeptides and the density of the lipoprotein assembled in HepG2 cells. We would suggest that apoB-48, because of its size, is able to form smaller particles than is possible with apoB-100. These particles, which we isolated at d 1.006–1.210 g/ml, may have different fates, with some being converted to larger triglyceride-rich particles, a process which delays the movement of apoB-48 through the secretory pathway. In contrast, the size of apoB-100 precludes its association with small particles, and therefore it enters the lipoprotein assembly pathway only with larger triglyceride-rich particles.

We propose that not all of the dense apoB-48-containing particles (d 1.006–1.210 g/ml) are converted to triglyceride-rich particles. This is based on the distribution of radioactivity in apoB-48 between d 1.006 and d 1.006–1.210 g/ml particles. It appears that approximately 30% of these particles is not converted. They may be degraded, or they may be secreted as apoB-containing HDL such as the particles originally described by Fainaru and coworkers (39) and subsequently observed by others (40).

Finally, while our data demonstrate clearly that both apoB-100 and apoB-48 are associated with the rough ER membrane, we have been unable to find any evidence from our isotopic incorporation studies that these pools are utilized in lipoprotein assembly under normal metabolic conditions. Perhaps under normal metabolic conditions these pools are destined for degradation; however, in periods of stress or increased hepatic lipogenesis, these pools could conceivably be used in lipoprotein assembly.

In summary, our studies have provided additional insight into lipoprotein assembly by the liver. We have recovered two lipoprotein fractions from the rough ER that we postulate are intermediates in the assembly process.

Comparison of the compositions of these two particles with that of Golgi VLDL support the sequential assembly of VLDL by the liver. Isotope incorporation experiments suggest that the actual assembly process for apoB-100- and apoB-48-containing lipoproteins may be different. Future experiments will examine the biochemical differences between these particles, as well as explore the differences in assembly.

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