# Investigation of the role of lipids in the assembly of very low density lipoproteins in rabbit hepatocytes

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**Abstract** Our aims were *i*) to determine which lipids co-localize with newly synthesized apolipoprotein (apo)B in the lumen of the rough endoplasmic reticulum (RER), and thus may play a role in the stabilization and/or translocation of this protein; and *ii*) to determine the intracellular sites of assembly of lipids into very low density lipoprotein (VLDL). In order to do this, we have developed a new method for the separation of ER-derived microsomes on self-generated gradients of iodixanol. Rabbit liver microsomes were resolved into two broad peaks, the lighter peak contained smooth vesicles and the heavier peak contained rough vesicles. Each peak was collected in a number of subfractions. A single gradient thus separates the initial events in the secretion process (RER fractions), from later events (smooth endoplasmic reticulum (SER) fractions). The microsomal fractions were separated into membranes and lumenal contents, and the mass of apoB and VLDL lipids determined by ELISA or high performance thin-layer chromatography, respectively. The biosynthetic relationships of apoB and lipids were investigated, in timed or chase experiments, by incubation of isolated rabbit hepatocytes with radiolabeled precursors of apoB or lipids, followed by isolation and analysis of the microsomal fractions. The results indicate that very small amounts of triacylglycerol, cholesterol, and cholesteryl ester co-localize with apoB into the lumen of the RER. The bulk of the VLDL lipids were in the lumen of the SER. However, some newly synthesized triacylglycerol, phospholipid, cholesterol, and cholesteryl ester were also transferred to the lumen of the RER and were chased into the SER lumen. Double-labeling experiments showed that cholesteryl ester produced from newly synthesized cholesterol (labeled with [$^3$H]mevalonate and [$^{14}$C]oleate) was almost exclusively present in the RER, while cholesteryl ester in the SER was labeled only with [$^{14}$C]oleate. Thus, distinct intracellular lipid-pools may be involved at different stages in the assembly of VLDL.—**Cartwright, I. J., J. A. Higgins, J. Wilkinson, S. Bellavia, J. S. Kendrick, and J. M. Graham.** Investigation of the role of lipids in the assembly of very low density lipoproteins in rabbit hepatocytes. *J. Lipid Res.* 1997. **38:** 531–545.

**Supplementary key words** iodixanol • self-generating gradients • apolipoprotein B • RER • SER • triacylglycerol • cholesterol • cholesteryl ester • ACAT • diacylglycerol acyltransferase

Endogenous triacylglycerol is transported from the liver in the very low density lipoproteins (VLDL). These particles consist of droplets of nonpolar lipid (triacylglycerol and cholesteryl ester) stabilized by a shell of amphipathic lipid (cholesterol and phospholipids) and protein. Apolipoprotein B (apoB) is the major protein and is essential for the secretion of VLDL. Our previous studies of the intracellular transit of apoB in rabbit and rat hepatocytes have identified three steps at which assembly and secretion of VLDL may be regulated (1–7): *i*) newly synthesized apoB is either translocated across the rough endoplasmic reticulum (RER) membrane or remains membrane bound and is subsequently degraded in the endoplasmic reticulum (ER) or Golgi membranes; *ii*) lumenal apoB is incorporated into a 'secretion-competent' light particle, or a 'secretion-incompetent' dense particle, which is degraded, without leaving the RER lumen; and *iii*) the 'secretion-competent' apoB-containing particles, which are lipid-poor compared with VLDL, move to the smooth ER (SER) and acquire the full complement of lipids before reaching the *cis*-Golgi. Similar conclusions have been reached from studies of the intracellular transit of apoB in hepatoma cell lines (8, 9). Results from other investigations are also consistent with a two-step model for assembly of VLDL (10–12), although it has also been proposed that VLDL assembly takes place in a single step co-translationally (13).

The first key step in assembly of VLDL, which determines whether apoB is secreted, is the transfer of the protein across the RER membrane. Translocation of apoB is slow and inefficient so that a large part of the newly synthesized apoB remains membrane-bound and

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; ER, endoplasmic reticulum; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; ELISA, enzyme-linked immunosorbent assay; MTP, microsomal triglyceride transfer protein.

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is accessible to antibody probes or proteases from the cytosolic side (5, 14). Recent investigations of HepG2 cells have shed considerable light on the molecular details of apoB translocation. ApoB exhibits transient translocation-arrest at a stage when the N terminal 69 kDa is on the lumenal side of the RER membrane, leaving most of the >550 kDa polypeptide on the cytosolic side (15). The arrested protein is susceptible to proteolytic digestion, so that the 85 kDa N-terminus remains on the lumenal side of the membrane and is translocated and secreted. Zhou et al. (16) have demonstrated that the cytosolic chaperone, heat shock protein-70 (hsp-70), is associated with partly translocated apoB and maintains it in a translocation-competent form. The association between apoB and hsp-70 is transient and ATP-sensitive. When hsp-70 dissociates, the membrane-bound apoB is susceptible to degradation. Provision of oleate, a lipid substrate, reduces association of apoB with hsp-70 and facilitates translocation (16). Transfer of lipid to the newly synthesized apoB, therefore, plays an important role in directing the protein to the secretory pathway. Microsomal triglyceride transfer protein (MTP), which transfers nonpolar lipids between phospholipid model membranes, is essential for apoB secretion and is believed to play a central role in this process (17–20).

Cholesterol, cholesteryl ester, triacylglycerol, and phospholipid have all been implicated in the regulation of the secretion of apoB-containing lipoproteins by hepatoma cells, hepatocytes, or whole liver (for reviews see refs. 21–24). Most of these investigations have involved manipulation of the total cellular lipid pools or the overall rate of lipid synthesis, followed by measurement of apoB secretion. However, it is clear that intracellular lipids and apoB are in several distinct pools, which differ in origin, metabolism, and/or fate (4, 6, 7, 24–28). Thus, specific lipids may be important in the transfer of apoB into the lumen of the RER, while others may be involved at other steps in the assembly of the VLDL particle. In order to determine which lipids are involved at each stage in VLDL assembly, it is necessary to examine intracellular pools in the secretory compartment.

When the liver is homogenized, the ER pinches off to form small vesicles (microsomes), trapping the lipids and proteins destined for secretion in the vesicular lumen (29). Thus, microsomes consist of a heterogeneous collection of vesicles derived from different parts of the ER. As each vesicle contains the secretory material that was present in the adjacent lumen at the time of homogenization, specific lipids involved in translocation should be in the same vesicles as newly synthesized apoB. To investigate this, we have used a new non-ionic iodinated medium, iodixanol. The inert nature of the medium and its ability to form reproducible and easily controlled self-generated gradients at relatively high centrifugal forces (unlike Percoll) have been previously reported (30). Iodixanol has been used to purify subcellular organelles (31), the endocytic compartment (32), calveolae (33), and plasma lipoproteins (34). This paper presents the first report of the use of self-generated gradients of ioxidanol to fractionate microsomes, on the basis of density, with high resolution. This approach has allowed us to identify and quantify the lipids that accompany newly synthesized apoB into the RER early in the secretory pathway, and to determine the sites, and sequence of events, of the addition of lipids to VLDL.

## METHODS

### Materials

Iodixanol (available from Nycomed as a 60% solution, Optiprep™) was purchased from Gibco. Maxidens™ was purchased from Nycomed UK. [$^{35}$S]methionine and [$^{3}$H]glycerol were from Amersham and [$^{3}$H]mevalonate, [$^{14}$C]oleoyl-CoA, and [$^{3}$H]oleate were from New England Nuclear. High performance thin-layer chromatography (HPTLC) plates were purchased from Camlab and thin-layer chromatography (TLC) plates (Merck 60F254) were purchased from BDH. Lipids and other chemicals were from Sigma, or as used previously (4–7, 25, 26, 35).

### Preparation of total microsomes from rabbit liver

Total microsomes were prepared from rabbit liver, as previously (4, 5). Briefly, livers were homogenized in 0.25 M sucrose (20% w/v) and centrifuged at 12500 rpm (12000 $g_{av}$) for 20 min in the JA-20 rotor of the Beckman J-21 centrifuge. The supernatant was centrifuged at 40000 rpm (105000 $g_{av}$) for 45 min using a Ti50 rotor in a Beckman L7 or L8 ultracentrifuge. The microsomal pellet was resuspended in 0.25 M sucrose for further fractionation. The microsomal pellet prepared in this way contains approximately 30% of the ER, determined by recovery of NADPH-cytochrome C reductase (4, 7). Previous studies demonstrated that all of the apoB in the liver is recovered in the microsomes and the Golgi fractions (4). There is no loss of apoB, when the ER disrupts to form microsomal vesicles, during homogenization.

### Preparation and incubation of rabbit hepatocytes

Rabbit hepatocytes were prepared by perfusion of rabbit livers with collagenase and characterized and incubated as described previously (6, 7, 26). Briefly, the

cells were resuspended (50% w/v) in methionine-free Eagles' minimal essential medium (MEM) which had been gassed with 95% $O_2$/5% $CO_2$. Aliquots, equivalent to 2 g of hepatocytes, in a total volume of 4 ml were incubated with [$^{35}$S]methionine (70–150 μCi), to radiolabel apoB; [$^{3}$H]glycerol (100 μCi) to radiolabel triacylglycerol and phospholipids, [$^{3}$H]mevalonate (15 μCi) to radiolabel cholesterol and cholesteryl ester, and [$^{3}$H]oleate (200 μCi), or [$^{14}$C]oleate (10 μCi), to radiolabel the acyl moiety of cholesteryl ester. Oleate (final concentration 1 mM) bound to fatty acid-free bovine serum albumin (BSA-final concentration 1%) or BSA alone were added as indicated. In some experiments the cells were incubated with the radiolabel for 30 min and isolated by centrifugation; in other experiments the hepatocytes were isolated by centrifugation, washed, and reincubated with unlabeled substrate (45 mM) for up to 120 min, to follow the transit of the radiolabeled lipid or apoB through the secretory pathway. In each set of experiments, at the end of the incubation, the hepatocyte pellets were homogenized and total microsomes were isolated as described above.

### Fractionation of microsomes in self-generated iodixanol gradients

Microsomal pellets from 10 g whole liver were resuspended in 30 ml of 0.25 M sucrose; those from 2 g of hepatocytes were resuspended in 8 ml of 0.25 M sucrose. Microsomal suspensions were mixed with half their volume of 60% (w/v) iodixanol (final concentration 20%) and transferred to Optiseal tubes using a syringe. The tubes were centrifuged in the VTi65 rotor at 353000 $g_{av}$ (65000 rpm) at 4°C for 2 h. The rotor was allowed to decelerate from 2000 rpm to rest without the brake. After careful removal of the plastic sealing plug, gradients were harvested (low density end first) by upward displacement with Maxidens (using a Nycomed Gradient Unloader) in 0.5-ml fractions. Because iodixanol is inert, fractions from the gradient were analyzed directly without removal of the medium.

In some experiments, the fractions from the gradient were further separated into membrane and lumenal-content subfractions (1, 4). One ml of 0.25 M sucrose was added to each tube to dilute the iodixanol. The tubes were centrifuged at 18000 rpm (39000 $g_{av}$) in the J18.1 rotor of the J-21 centrifuge for 60 min to pellet the microsomes. The pellets were resuspended in 1.5 ml of 100 mM sodium carbonate (pH 11.0) in the same tubes, using a cone-shaped microhomogenizer, and the tubes were recentrifuged at the same speed. The supernatants (content fractions) were removed and retained. The pellets (membrane fractions) were resuspended in a small volume of 0.25 M sucrose. For assay of apoB by ELISA, it was not possible to rehomogenize the small compact membrane pellet without losing material. Therefore, to assay membrane and lumenal-content apoB, the peak fractions from the gradients were pooled, diluted with 0.25 M sucrose, and the microsomes were pelleted by centrifugation at 105000 *g* for 45 min. The microsomal pellets were then resuspended using a Potter-Elvejhem homogenizer by hand in 100 mM sodium carbonate, left on ice for 30 min, and centrifuged at 105000 *g* for 2 h to separate the pellet (membrane fraction) and supernatant (content fraction) as previously (1, 4).

### Analysis of apoB

The amount of apoB in the microsomes and the gradient fractions was determined by competitive ELISA using rabbit LDL as a standard as described previously (4). $^{35}$S-labeled apoB in the microsomes and gradient fractions was determined as previously, after separation by SDS-PAGE (6, 25).

### Analysis of lipids

The lipids of subcellular fractions were extracted as described previously (7). To determine the incorporation of radiolabeled precursors into lipid classes, the extracted lipids were dissolved in 100 μl of chloroform–methanol 1:1 (v/v) and separated on conventional TLC plates, using the solvent system heptane–diethyl ether–glacial acetic acid 60:40:2 (by volume). Lipid classes were located using iodine vapor, marked, and identified by comparison with standards. The iodine was removed completely by warming the plates. The bands were scraped from the plates, mixed with scintillation solution, and counted in a β counter. To quantify the lipids, aliquots (1–10 μl) of the lipid extracts were applied to HPTLC plates using a Desaga AS30 autospotter and separated, using heptane–diethyl ether–glacial acetic acid 60:40:2 (by volume) for the neutral lipids, and propanol–propionic acid–chloroform–water 45:30:30:15 (by volume) for the phospholipid classes. The plates were air dried, dipped in 3% cupric acetate in 8% phosphoric acid, air dried again, and heated to 140°C on a hot-plate for 30 min. A standard curve containing known amounts of phospholipid, triacylglycerol, cholesterol, and cholesteryl ester was included on each plate and used to quantitate the amount of each lipid using a Camlab CD60 densitometer (7).

### Immunoprecipitation of apoB

In some experiments the radiolabeled lipids associated with apoB in the lumenal contents were determined. Isolated hepatocytes were incubated with radiolabeled lipid precursors for 45 min. Total microsomes were prepared and separated in iodixanol gradients. The peak fractions (1–8 and 12–20) from the gradients

were pooled, diluted with 0.25 M sucrose, and the microsomes were pelleted by centrifugation at 105000 *g* for 45 min. The microsomal pellets were then resuspended using a Potter-Elvejhem homogenizer by hand in 100 mM sodium carbonate, left on ice for 30 min, and centrifuged at 105000 *g* for 2 h to separate the pellet (membrane fraction) and supernatant (content fraction). The content fractions were concentrated and aliquots were incubated with an anti-rabbit apoB monoclonal antibody (5) for 4 h at 4°C followed by anti-rat IgG coupled to Sepharose beads for 12 h at 4°C. The beads were isolated by centrifugation; the lipids were extracted from the pellet and supernatant and separated by thin-layer chromatography, as above. Preliminary experiments were performed, to determine the optimum antibody concentrations, to completely immunoprecipitate apoB. This was checked by immunoblotting.

### Determination of acyl-cholesterol:acyltransferase (ACAT) activity

ACAT was assayed essentially as described by the method of Stahlberg, Angelin, and Einarsson (36). Briefly, microsomes or microsomal subfractions from the gradients (0.1–1.0 mg protein) were incubated with [$^{14}$C]oleoyl CoA (final concentration 25 nmol, specific activity ≈ 5000–10000 dpm/nmol) in 0.2 M phosphate buffer, pH 7.4, containing 2 mM EDTA and fatty acid-free albumin (2 mg/ml) (final volume 1.0 ml). The incorporation was stopped by addition of 12 ml of chloroform–methanol 2:1 (v/v). The phases were separated by addition of 1 ml of 0.9% NaCl. The lower phase was taken to dryness and the lipids were separated on TLC plates using heptane–ethyl acetate 95:5 (by volume). The lipids were detected using iodine vapor and the cholesteryl ester band was identified by comparison with a cholesteryl oleate standard. After complete removal of the iodine, the cholesteryl ester-containing band was scraped from the plate and counted. In preliminary experiments, it was established that incorporation of [$^{14}$C]oleate into cholesteryl ester was linear for 15 min and to 2 mg of protein.

### Determination of diacylglycerol acyltransferase (DGAT) activity

DGAT activity was assayed essentially as described by Anderson et al. (37). Dioleoylglycerol (20 mg) was mixed with phosphatidylcholine (8 mg) and phosphatidylserine (8 mg) in chloroform. The solvent was removed and the lipids were suspended in 12 ml of 10 mM Tris-HCl, pH 8.0, containing 2 mM $MgCl_2$ by sonication in the MSE soniprep at maximum power. Each incubation contained 0.2 ml of the lipid suspension, 0.1 ml of buffer containing 5 mg/ml fatty acid-free albumin, 0.1 ml of [$^{14}$C]oleoyl-CoA (0.5 mM, specific activity ≈ 5000–10000 dpm/nmol) and 0.1 ml of microsomal fraction (50–200 μg protein). The reaction was stopped by addition of 10 ml of chloroform–methanol, 2:1 (v/v). The phases were separated with 1 ml of 0.5 M $CaCl_2$; the lower phase was taken to dryness and the lipids were separated by TLC using heptane–diethyl ether–glacial acetic acid 60:40:2 (by volume). The lipids were detected using iodine vapor and the triacylglycerol-containing bands were identified using triolein as a marker. After complete removal of the iodine, the triacylglycerol-containing band was scraped from the plate and counted. Preliminary experiments demonstrated that the incorporation of [$^{14}$C]oleate into triacylglycerol was linear for 30 min and to 0.25 mg of protein.

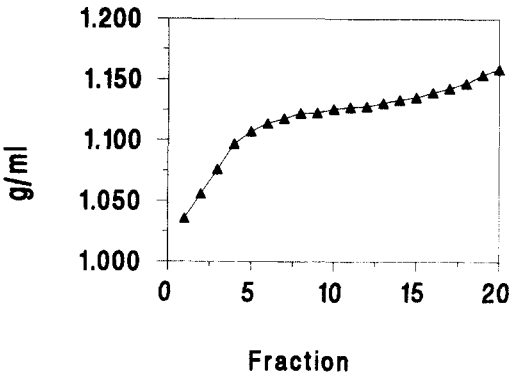


**Fig. 1.** Density of iodixanol gradient. Twenty percent iodixanol prepared by mixing 60% Optiprep with 0.25 M sucrose was centrifuged and unloaded as described in Methods. The refractive index was measured and the density was calculated according to the instructions of Nycomed, the manufacturer of Optiprep. Fraction 1 is the top and fraction 20 the bottom of the gradient.

## RESULTS

### Fractionation of microsomes in self-generating gradients of iodixanol

On centrifugation, 20% iodixanol formed a gradient, which was steep at the top between fractions 1 and 5, and more or less linear for the remainder, between fractions 6–20 (**Fig. 1**). The microsomes distributed on the gradient in two visible broad peaks, one of which was towards the top of the gradient and one towards the bottom. The distribution of phospholipid, a membrane marker, and cytochrome c reductase, a marker for the endoplasmic reticulum, showed a similar distribution, with two broad peaks between fractions 1 and 7 and between fractions 14 and 19 (**Fig. 2**). Protein distributed in the same two peaks (Fig. 2) and also showed a high concentration in fraction 20, which contained

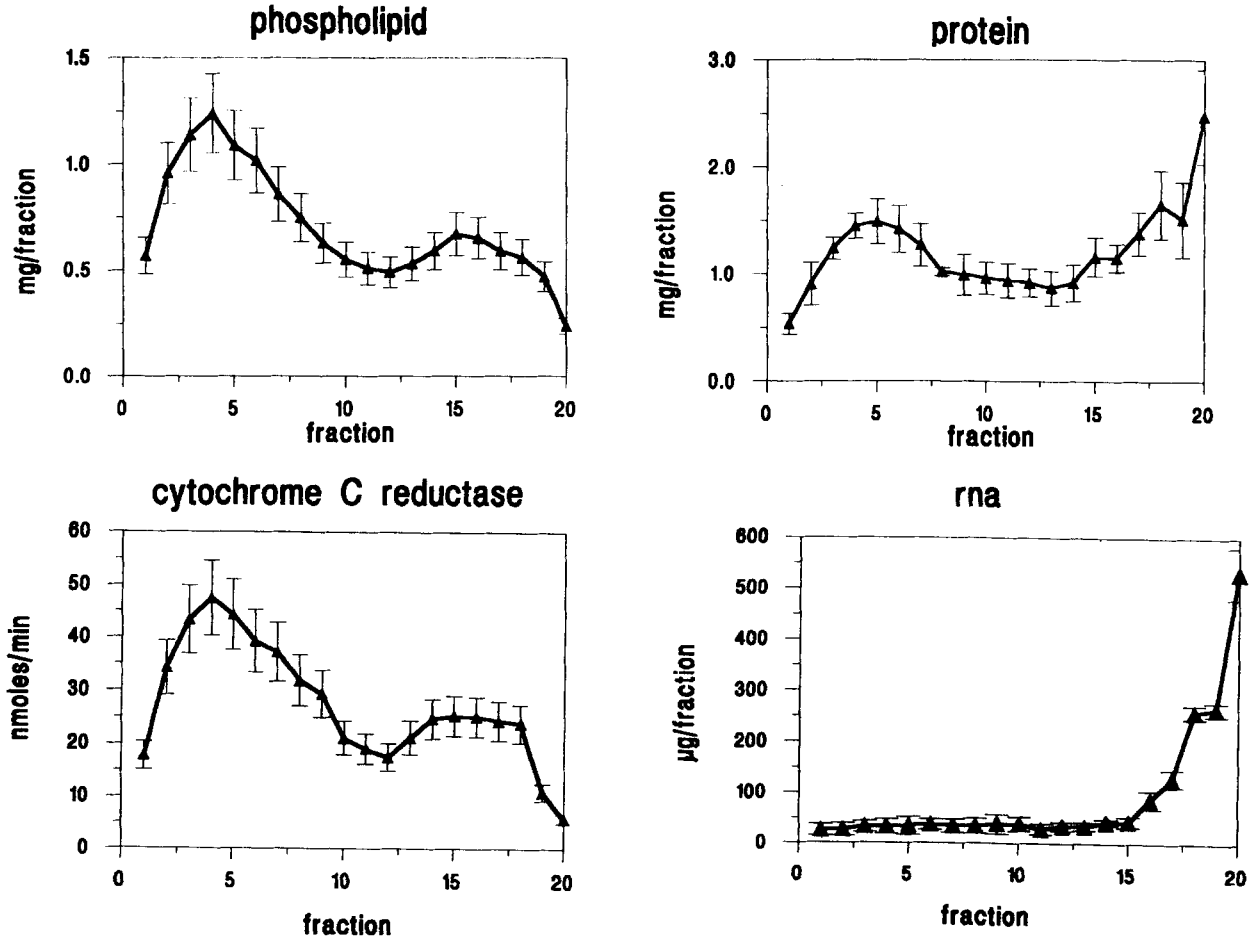


**Fig. 2.** Distribution of membrane components in iodixanol gradients. Microsomes were separated on 20% iodixanol gradients as described in Methods. The protein, phospholipid, and RNA were determined and the activity of NADPH cytochrome C reductase was assayed. The results plotted are the mean of four determinations on separate preparations ± standard deviation. Each component was measured in the original microsomal suspension. Recoveries of each component from the gradient was >90%.

approximately 50% soluble protein, and which did not pellet with the microsomes (presumably the cytosolic proteins, which were trapped in the initial microsomal pellet). The concentration of RNA was low, except in fractions 16–20, with the highest concentration in fraction 20 (Fig. 2). There was no detectable UDP-galactose-galactosyltransferase activity in any of the fractions or the original total microsomes, indicating very low contamination with Golgi membrane vesicles (data not shown). This differs from our previous findings in which total rabbit microsomes contained detectable galactosyltransferase activity (4). In the latter case, however, activity in total microsomes was very low and only detected in concentrated microsomal suspensions. In the present experiments, the microsomal fractions were diluted for preparation of iodixanol gradients. Overall, these results indicate the rough (heavy peak) and smooth microsomes (light peak) separate on iodixanol and that, in each peak, the microsomal vesicles were spread in the gradient yielding a number of subfractions that differ in density. In the heavy peak there was a heterogeneous distribution, such that the vesicles with the greatest number of bound ribosomes were at the bottom of the gradient.

### Distribution of VLDL components in microsomal fractions separated in iodixanol gradients

ApoB, cholesterol, cholesteryl ester, and triacylglycerol all exhibited peaks at the top of the gradient similar to, but sharper than, the light peak of smooth microsomes (**Fig. 3**). ApoB was also at high levels at the bottom of the gradient in fractions 18–20, coincident with the most dense rough microsome fractions, and there was a small peak of cholesteryl ester between fractions 14 and 15. In the peak fractions at the top of the gradient, a large fraction of each lipid was in the lumen of

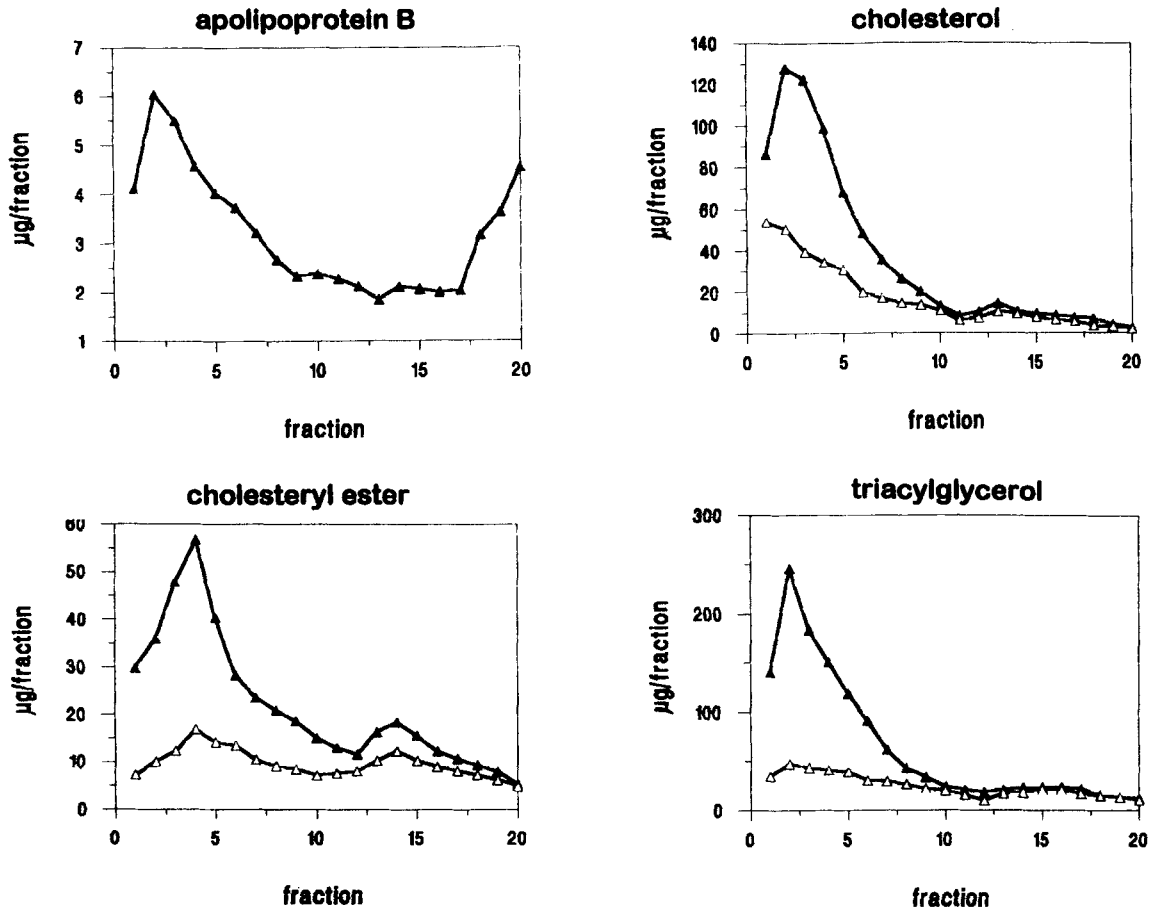


**Fig. 3.** Distribution of VLDL components in microsomal subfractions separated in iodixanol gradients. Microsomes were separated on 20% iodixanol gradients, as described in Methods. Parallel samples were separated into membrane and lumenal content fractions and the lipids were extracted and determined by HPTLC, as described in Methods. ApoB in total fractions was determined by ELISA, as described in Methods. ApoB in pooled membrane fractions was also determined and the results are given in the text; solid triangles, total microsomes; open triangles, membrane fractions. The results plotted are the mean of three determinations. Each component was measured in the original microsomal suspension. Recovery of apoB was between 85 and 90% and recoveries of the lipid components were >80%.

the microsomal vesicles. The cholesterol content of the membranes increased steadily from the bottom of the gradient to the top. As described in Methods, it was not possible to analyze apoB in individual fractions and instead pooled fractions were analyzed. However, most of the apoB was lumenal with 88.24 ± 1.36%, 86.92 ± 2.60%, and 95.75 ± 0.24 (n = 3) in the lumen of pooled fractions 1–8, fractions 9–16, and fractions 15–20, respectively.

The average composition of the lumenal contents of fractions 1–6 was 12.39 ± 3.34 µg of cholesterol, 26.79 ± 5.77 µg of triacylglycerol, and 6.68 ± 1.76 µg of cholesteryl ester for each µg of apoB (**Fig. 4**). In contrast, the lumenal contents of fractions 17–20 contained very small amounts of triacylglycerol, cholesterol, and cholesteryl ester (Fig. 4). Apart from cholesterol, which increased in the membrane fractions from the bottom to the top of the gradient, all membrane fractions had a similar lipid composition with 34.50 ± 8.19 µg of triacylglycerol and 14.42 ± 2.91 µg of cholesteryl ester per mg of phospholipid (Fig. 4).

### Distribution of newly synthesized VLDL components on iodixanol gradients

To determine the distribution of newly synthesized VLDL components in the microsomal subfractions, hepatocytes were incubated with [$^{35}$S]methionine to label apoB, [$^{3}$H]mevalonate to label cholesterol and cholesteryl ester, or [$^{3}$H]glycerol to label triacylglycerol and phospholipid. Incubations were performed for 30 min, which has previously been shown to incorporate sufficient radiolabel into VLDL components, and were carried out in the absence and presence of added oleate, which in previous studies has been shown to stimulate apoB secretion (7).

The distribution of newly synthesized VLDL compo-

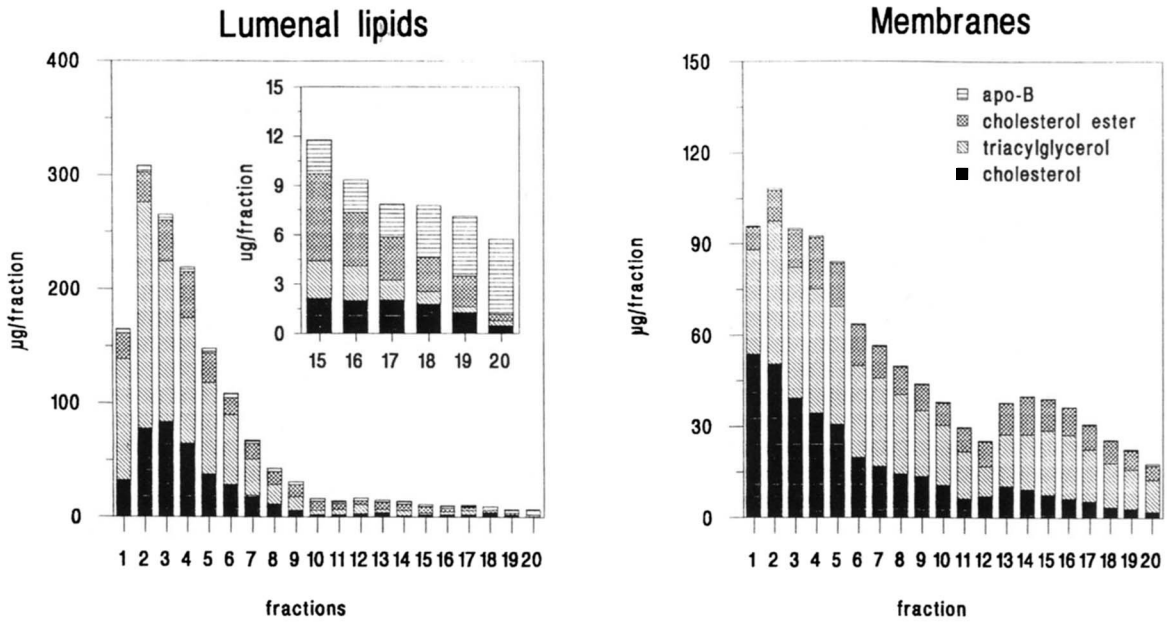


**Fig. 4.** Distribution of apoB and lipids in the lumenal and membrane fractions. Microsomes were separated on 20% iodixanol gradients, and further separated into membranes and lumenal contents, and the lipids and apoB were determined by HPTLC and ELISA, respectively. The proportion of apoB in the membranes and contents was calculated from the results for pooled fractions (see text), and the lipid and apoB composition plotted. The lumenal contents in fractions 15–20 are shown in expanded scale in the inset graph.

nents on iodixanol gradients differed from the distribution of the mass of these components (**Fig. 5**). Labeled apoB was in two peaks. However, the highest radioactivity was in the rough microsomal fractions, 17–20, with a smaller peak in the smooth microsomal fractions. Labeled cholesterol was also in two peaks, one coincident with the smooth microsomes and the mass of cholesterol, and a peak in the dense rough microsome. Labeled cholesteryl ester was at the highest concentration in the dense rough microsomes, in fractions 18–20, coincident with newly synthesized apoB, with very little radiolabel in the smooth microsome peak. Radiolabeled triacylglycerol and phospholipid were mainly in the light smooth microsome-containing peak, with smaller amounts in the dense rough microsomes, in fractions 18–20. The major effect of addition of oleate to the incubation medium was to increase incorporation of [$^3$H]glycerol into triacylglycerol in the light peak, with little effect on the synthesis of triacylglycerol in the heavy peak. There was also a small increase in the incorporation of the radiolabeled precursor into apoB and cholesterol in the light peak; and into cholesteryl ester in the heavy peak. The microsomal fractions taken from iodixanol gradients were also separated into membrane and lumenal subfractions. The patterns of incorporation of the radiolabeled precursors into these subfractions were similar to those found for the total microsomal fractions: >80% of the radiolabeled lipids were in the membrane fraction (data not shown). Thus, the radiolabeled lipids did not show the same distribution as the mass of lipids, most of which were in the lumen of the light fractions.

The calculated specific activities of apoB, triacylglycerol, cholesteryl ester, and cholesterol were all higher in the RER than the SER (compare Figs. 3 and 5). This was particularly striking for cholesteryl ester, newly synthesized from [$^3$H]mevalonate, which was almost completely restricted to the RER, although the mass of cholesteryl ester was highest in the lumen of the SER. However, when the fatty acyl moiety was labeled using [$^3$H]oleate, a different pattern of incorporation was found. Cholesteryl ester labeled with oleate was found in two peaks, one in the smooth microsomes and one in the rough microsomes. This was verified by a double-labeled experiment in which hepatocytes were incubated simultaneously with [$^3$H]mevalonate and [$^{14}$C]oleate and the microsomes were subfractionated and the lipids were analyzed (**Fig. 6**). The ratio of $^{14}$C/$^3$H in the smooth microsomes peaks (fractions 1–3) was 6.85 ± 0.28 while that in the rough microsomal peaks (fractions 18–20) was 1.39 ± 0.35 (n = 4). The ratios were similar in both membrane and lumenal contents. Thus, cholesterol synthesized from mevalonate in the rough microsomes appears to be selectively channelled into the esterification pathway, while cholesteryl ester in the smooth microsomes is formed from cholesterol synthesized before incubation of the hepatocytes with [$^3$H]mevalonate.

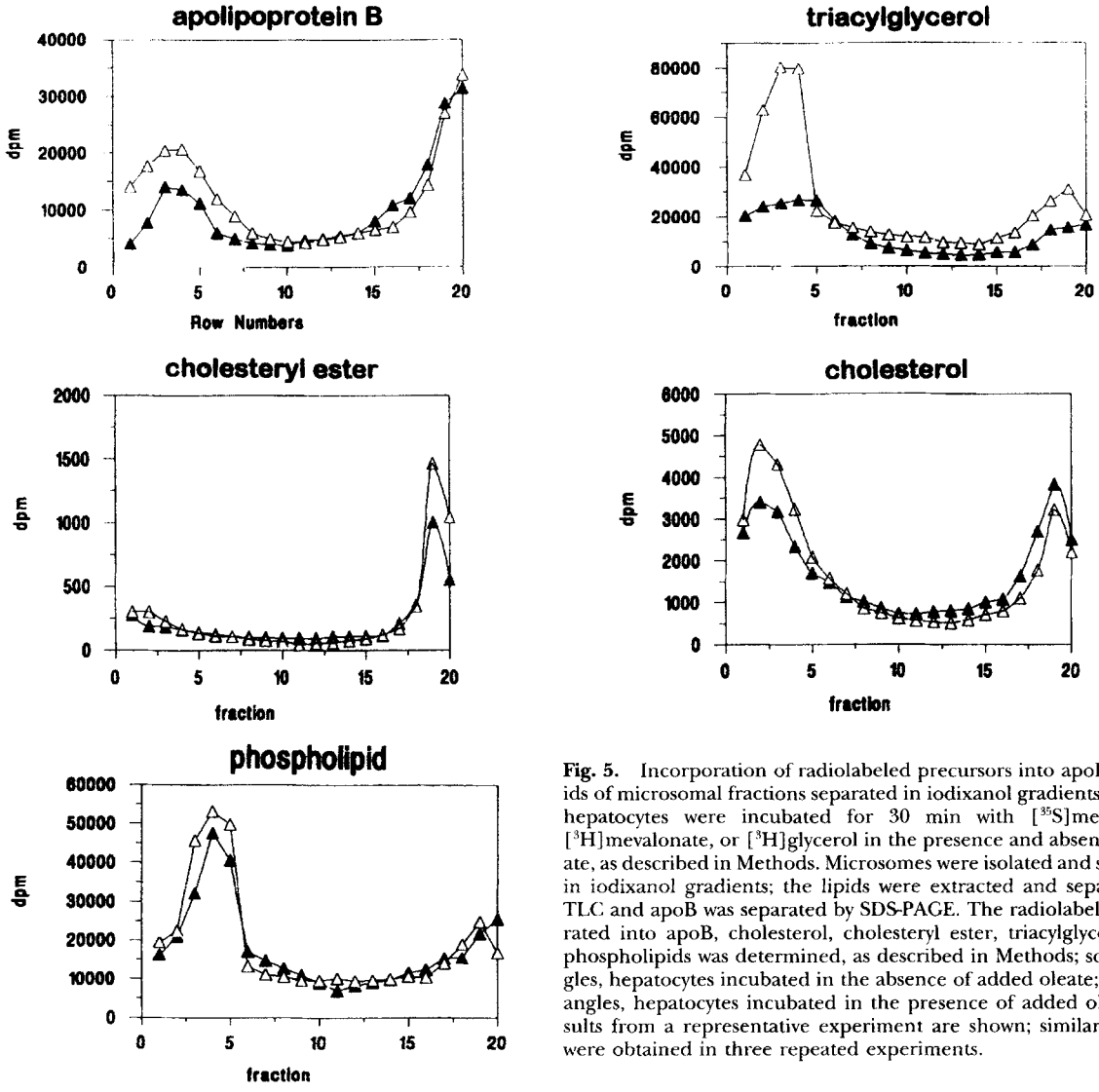


**Fig. 5.** Incorporation of radiolabeled precursors into apoB and lipids of microsomal fractions separated in iodixanol gradients. Isolated hepatocytes were incubated for 30 min with [$^{35}$S]methionine, [$^{3}$H]mevalonate, or [$^{3}$H]glycerol in the presence and absence of oleate, as described in Methods. Microsomes were isolated and separated in iodixanol gradients; the lipids were extracted and separated by TLC and apoB was separated by SDS-PAGE. The radiolabel incorporated into apoB, cholesterol, cholesteryl ester, triacylglycerol, and phospholipids was determined, as described in Methods; solid triangles, hepatocytes incubated in the absence of added oleate; open triangles, hepatocytes incubated in the presence of added oleate. Results from a representative experiment are shown; similar patterns were obtained in three repeated experiments.

To determine whether newly synthesized lumenal lipids were associated with lumenal apoB, hepatocytes were incubated with radiolabeled lipid precursors in the presence of oleate. The microsomal fraction was isolated and separated in iodixanol gradients. Fractions 1–8 (SER) and 12–20 (RER) were pooled and the lumenal contents were isolated, concentrated, and apoB immunoprecipitated, as described in Methods. In two separate determinations, 38–39% of the phospholipid, 52–53% of the triacylglycerol, 57–58% of the cholesterol, and 44–45% of the cholesteryl ester radioactivity in the RER lumen immunoprecipitated with apoB; 83–84% of the phospholipid, 78–80% of the triacylglycerol, 57–58% of the cholesterol, and 44–46% of the cholesteryl ester radioactivity in the SER lumen immunoprecipitated with apoB. Thus, only about half of the newly synthesized lipids in the lumen of the RER and approximately 80% of the triacylglycerol and phospholipid in the lumen of the SER are associated with apoB. These observations raise the possibility that lipids may be associated with proteins, in addition to apoB, in the RER lumen, and that part of cholesterol and cholesteryl ester in the SER lumen may be in a separate pool from triacylglycerol and phospholipid. These questions are under further investigation.

### Transfer of radiolabeled apoB and lipid between intracellular pools

In the iodixanol gradient, the denser fractions contain the RER-derived vesicles and the lighter fractions contain the SER-derived vesicles. Thus, in a single gradient the early RER events in VLDL assembly are separated from the later events in the SER. As the RER is the site of protein synthesis it is reasonable to conclude

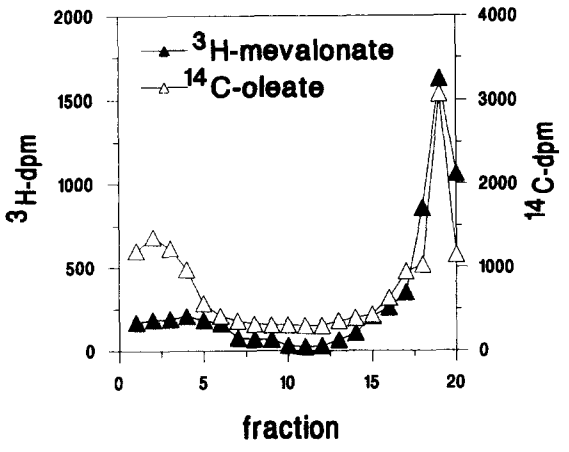


**Fig. 6.** Incorporation of [$^3$H]mevalonate and [$^{14}$C]oleate into cholesterol ester of microsomal fractions. Isolated hepatocytes were incubated for 30 min with [$^3$H]mevalonate and [$^{14}$C]oleate. Microsomes were isolated and separated in iodixanol gradients; the fractions were separated into membranes and lumenal contents, and the lipids were extracted and separated by TLC, as described in Methods. The incorporation of the two labeled precursors into the cholesteryl ester was determined using the double-label program in the Hewlett-Packard scintillation counter. The data plotted are the mean of three determinations.

that apoB in the SER has been transferred from the RER either as a lumenal or a membrane constituent. However, VLDL lipids are synthesized in both RER and SER and the lipid in the RER may not necessarily be transferred to the SER with apoB. To determine the relationship between the lipids and apoB in the RER and the SER, a series of experiments was performed in which the hepatocytes were preincubated with the appropriate radiolabeled precursor, isolated by centrifugation, washed, and resuspended in the presence of unlabeled precursor for a range of times, before isolation and fractionation of the microsomes. This is not strictly a pulse-chase experiment, as it is not possible to label one pool of lipid or apoB and follow its fate. There is significant incorporation of radiolabeled precursors into all lipid and apoB pools within a few minutes incubation (7). However, this protocol does allow us to determine whether radiolabeled apoB or lipid in the RER is transferred to the SER. Four fractions were taken from the gradients. Fraction 1 contains the light peak of smooth microsomes and fraction 4 the heavy peak of rough microsomes with fractions 2 and 3 intermediate.

During the "chase" period the radiolabeled apoB, triacylglycerol, phospholipid, and cholesterol decreased in membrane and lumenal pools in fraction 4 and increased in fraction 1 (**Fig. 7A, B, and C**). Therefore, these VLDL components synthesized in the RER are transferred to the SER. However, there was little transfer of the radiolabeled apoB, triacylglycerol, or phospholipid from the membrane pools to the lumenal pools. Radiolabeled cholesteryl ester decreased in fraction 4 and in this case there appeared to be some transfer of the newly synthesized lipid from the lumen and membrane of the RER to the lumen of the SER.

### Distribution of ACAT and DGAT activity in the iodixanol gradient

The activity of ACAT, the enzyme responsible for synthesis of cholesteryl ester, was distributed in two peaks corresponding to the dense rough microsome peak and the light smooth microsome peak (**Fig. 8**). The specific activities of the enzyme (activity/mg membrane protein) were similar in all of the microsome fractions. The capacity of the microsomal subfractions to synthesize cholesteryl ester, therefore, is broadly distributed. The activity of DGAT, the enzyme that catalyzes the final step in the synthesis of triacylglycerol, was also broadly distributed and exhibited a peak coincident with the smooth microsome peak and a small peak coincident with the dense RER fractions (Fig. 8). DGAT activity is, therefore, present in all fractions that contain newly synthesized triacylglycerol, although its specific activity (activity/mg membrane protein) is somewhat higher in the SER fractions.

## DISCUSSION

The aim of this investigation was to determine which lipids move into the lumen of the RER, and hence may play a role in the stabilization of apoB or its translocation across the ER membrane. A second aim was to determine the site and sequence of transfer of the VLDL lipids into the lumen of the ER. Because we wish to extrapolate our findings to human liver, we have opted to use adult rabbit hepatocytes for our studies. Rabbit hepatocytes, like human liver, secrete apoB-100-containing VLDL-sized particles. Rat hepatocytes and the rat hepatoma cell line, McArdle RH7777, secrete both apoB-48 and apoB-100, which differ in their intracellular transit and their assembly into lipoproteins (7, 38). The human hepatoma cell line, HepG2, which has been extremely useful in studies of the translocation of apoB-100, secretes HDL/LDL-sized particles and also exhibits significant differences in lipid metabolism from normal adult hepatocytes (39, 40). It has been suggested that these cells lack the second step in the assembly of VLDL. This step may be an important determinant of the size of VLDL particles, which in turn affects their atherogenicity (41).

Iodixanol is a relatively new centrifugation medium and has been used in a number of separation methods (30–34). This is the first study in which this medium

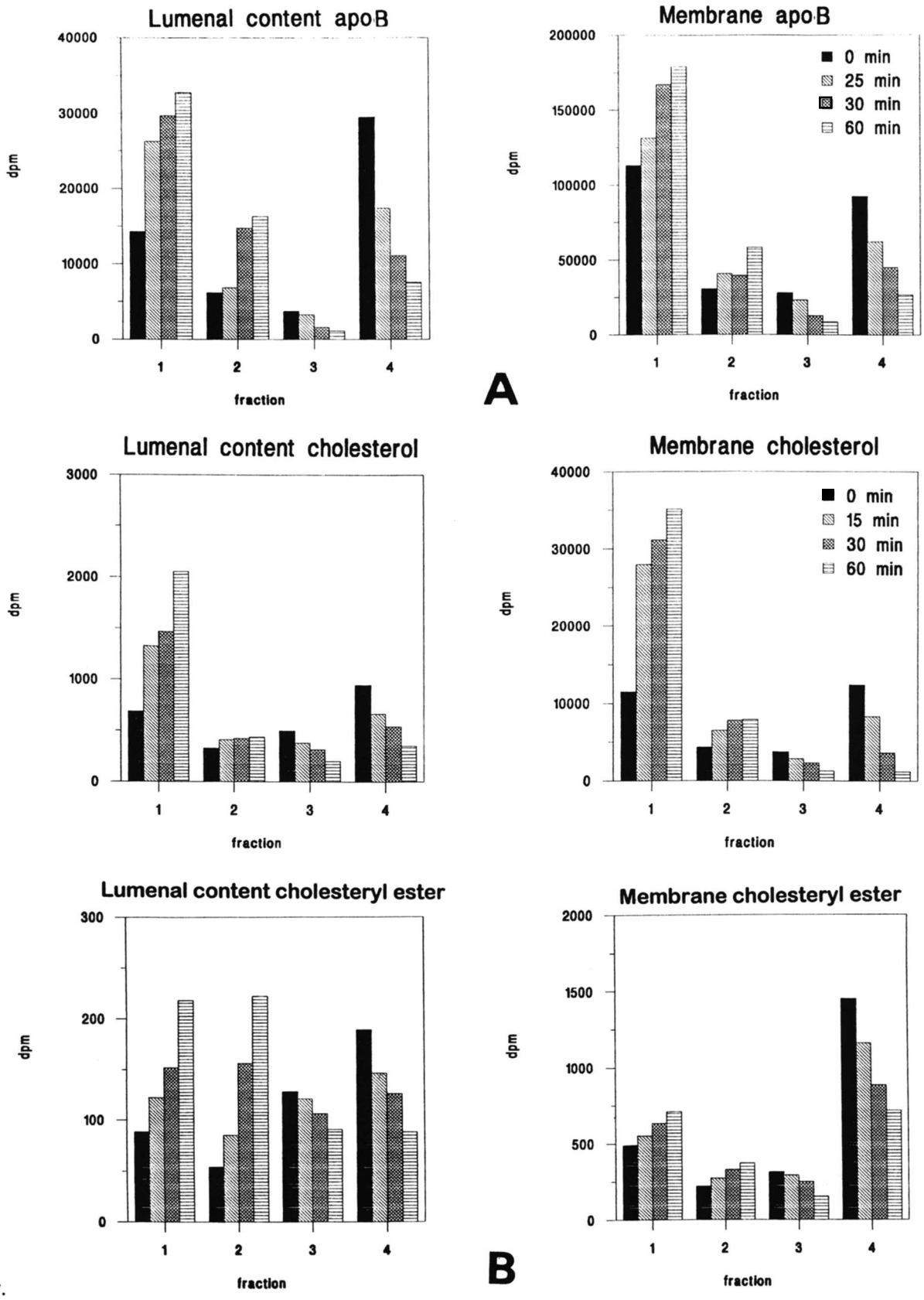


Fig. 7.

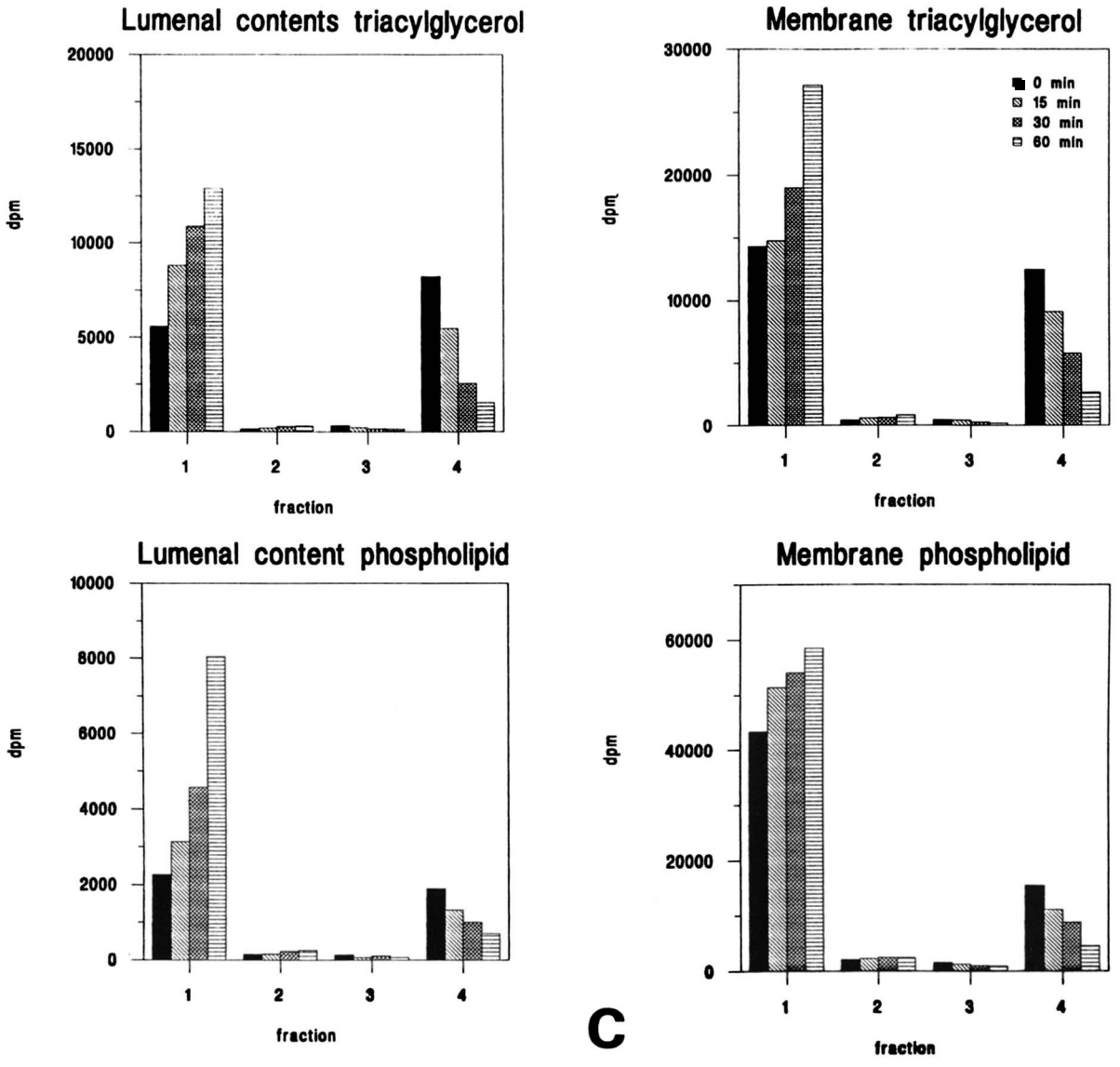


**Fig. 7.** Fate of radiolabeled VLDL components in "chase" incubation. Isolated hepatocytes were incubated for 30 min with [$^{35}$S]methionine, [$^{3}$H]mevalonate, or [$^{3}$H]glycerol in the absence of oleate as described in Methods. The cells were isolated by centrifugation, and reincubated with an excess of the unlabeled substrate in the presence of oleate for 0, 30, 60, and 120 min. At each time point, microsomes were isolated and separated in iodixanol gradients, as described in Methods. Four equal fractions (1–4) were taken corresponding to 1–5, 5–10, 11–15, and 16–20 on the gradients illustrated in Figs. 1–6. The fractions were separated into membranes and lumenal contents; the lipids were extracted and separated by TLC and apoB was separated by SDS-PAGE. The radiolabel incorporated into apoB, cholesterol, cholesteryl ester, triacylglycerol, and phospholipids was determined as described in Methods. A: $^{35}$S-labeled apoB; B: $^{3}$H-labeled cholesterol and cholesteryl ester; and C: $^{3}$H-labeled triacylglycerol and total phospholipid.

has been used to subfractionate microsomal vesicles. Based on analysis of protein, phospholipid, RNA, and NADPH cytochrome C reductase, we conclude that ER-derived microsomes separate into two broad peaks, the heavy peak is enriched in RER vesicles and the light peak with SER vesicles. Within these broad peaks, the microsomal vesicles separate into subfractions on the basis of small differences in density. The distribution of RNA suggests that the heavy peak is heterogenous with the denser fractions containing the highest concentration of ribosomes. Although there are no established biochemical or morphological markers to differentiate and identify regions of the SER, it is likely that the vesicles also have a heterogeneous distribution in the light peak. For the purpose of the present study we wished to subfractionate microsomal vesicles, in order to determine which lipids are in the ER lumen early and late in the secretory pathway. Iodixanol gradients allowed us

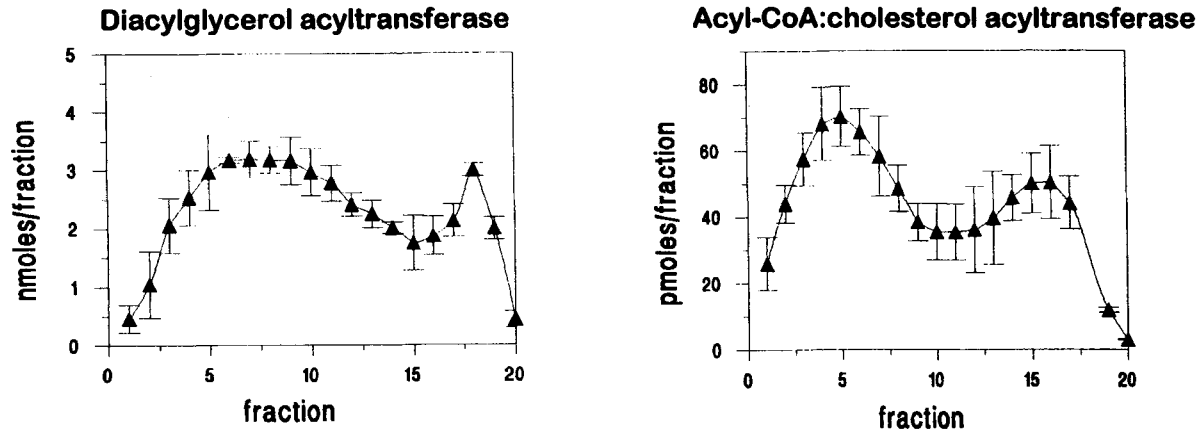


**Fig. 8.** ACAT and DAGT activity of microsomal fractions separated in iodixanol gradients. Liver microsomes were separated on 20% iodixanol gradients and the ACAT and DAGT activities were determined as described in Methods. The results plotted are the mean of results from three separate preparations assayed in duplicate ± standard deviation.

to achieve this aim although the separation is essentially operational. A further advantage of the method is that it presents a "snap-shot" of the secretory pathway in the ER. Thus, the early events in secretion, in the RER, are separated from the later events, in the SER, in a single gradient.

Incorporation of apoB into VLDL may occur in two ways: *i*) complete VLDL particles are formed in a single step in the ER lumen, or *ii*) lipid-poor apoB/VLDL precursors are formed and these acquire the full complement of lipid later in the secretory pathway. The results from the present study support the latter model. The bulk of the VLDL lipid is transferred into the lumen of the SER and this is stimulated by oleate, which stimulates secretion. Approximately 80% of the newly synthesized triacylglycerol and phospholipid in the SER lumen is associated with immunoprecipitated lumenal apoB. In the RER lumen only small amounts of cholesterol, cholesteryl ester, and triacylglycerol are found and only about half of the newly synthesized lumenal lipids is associated with immunoprecipitated apoB. The alternative model, i.e., that complete VLDL are formed in one step, is not supported by our observations. Complete assembly does not take place in the RER lumen, as the mass of lipid is very low relative to the mass of apoB. The evidence is also against a model in which apoB moves to the SER, as a membrane component, and is post-translationally incorporated into VLDL in a single step. Although post-translational translocation of apoB across the ER membrane may occur, this takes place close to the site of synthesis (8, 26, 40). In addition, the results reported here and previous studies of rat and rabbit heptaocytes have shown that newly synthesized apoB in the RER membrane is not chased into the SER lumen (6, 7).

Our observations do not implicate any specific class of lipid in the translocation of newly synthesized apoB. Newly synthesized triacylglycerol, phospholipid, cholesterol, and cholesteryl ester were all transferred into the lumen of the RER, and all of these lipids moved to the SER in chase experiments. With the exception of a small fraction of the cholesteryl ester, radiolabeled lipids and radiolabeled apoB in the RER membrane did not move into the lumen during the chase experiments. This observation suggests that, like apoB, lipids in the RER are committed either to secretion or membrane retention during or shortly after synthesis.

Although there is no evidence that a single lipid class is involved in translocation of apoB, the labeling patterns indicate that lipids at different sites in the secretory pathway are not in equilibrium. Although the mass of each lipid was greatest in the SER lumen, the specific activities of the lipids in the RER membrane and lumen were greater. A striking observation was the difference in the origin of cholesteryl ester in the RER and SER. Thus, cholesteryl ester synthesized from radiolabeled mevalonate is largely restricted to the RER, while cholesteryl ester synthesized from radiolabeled oleate is in both the RER and SER. Cholesterol is synthesized in both RER and SER, and ACAT activity is present in these fractions. Newly synthesized cholesterol in the RER is directed to esterification and it is possible, although at present speculative, that this pool of lipid is involved in apoB translocation, while in the SER, preexisting cholesterol is esterified and added, with triacylglycerol, to the maturing VLDL particle.

A large number of studies have been carried out to investigate the roles played by cholesterol, cholesteryl ester, and triacylglycerol in regulating secretion of apoB-containing lipoproteins. Addition of oleate to the incubation medium of HepG2 cells (42–44) or adult hepatocytes (6, 7) stimulates secretion of apoB. How-

ever, this may be a result of effects on apoB transit and degradation, rather than a direct effect on cholesteryl ester or triacylglycerol (26, 42, 43). Studies of HepG2 cells have suggested that cholesterol/cholesteryl ester loading stimulates secretion of apoB (45–47). Consistent with this, inhibition of HMG-CoA reductase or ACAT lowers secretion of apoB (45, 48). In contrast, other studies have shown that the size of the triacylglycerol pool in hepatoma cells correlates with apoB secretion, rather than that of cholesteryl ester (44, 49, 50), and that inhibition of cholesterol or cholesteryl ester synthesis has no effect on apoB secretion (44, 50). It has been suggested that these discrepancies are due to the fact that the preexisting intracellular pool of cholesterol or cholesteryl ester is regulatory, and that acute inhibition, of either cholesterol or cholesteryl ester synthesis, does not modulate apoB secretion (47). Studies of adult rabbit, rat, or human hepatocytes, perfused rat livers, and of humans, African green monkeys, chickens, rats, and pigs have also implicated cholesterol or cholesteryl ester synthesis in the regulation of VLDL secretion (51–61). In hamster and hamster hepatocytes, inhibition of fatty acid synthesis inhibits VLDL secretion, indicating that triacylglycerol synthesis is important (61). All of these, apparently conflicting, conclusions can be reconciled by taking into account the fact that, in all studies, total cellular lipids were pooled. This would mask differences due to lipid pools at different sites in the assembly process. Our observations indicate that translocation of apoB into the secretory pathway involves small pools of lipid synthesized in the RER, while assembly of the completed VLDL particles involves lipids in the SER. These pools may be differentially modified by the various strategies adopted to alter triacylglycerol, cholesterol, or cholesteryl ester.

In summary, our results are consistent with a two-step assembly of VLDL. In first step, apoB moves into the RER lumen accompanied by small amounts of cholesteryl ester, synthesized from mevalonate and oleate, and cholesterol and triacylglycerol. The second step in the SER results in addition of triacylglycerol, cholesterol, and cholesteryl ester and is stimulated by oleate.

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## REFERENCES

1. Higgins, J. A., and J. L. Hutson. 1984. The roles of Golgi and endoplasmic reticulum in the synthesis and assembly of lipoprotein lipids in rat hepatocytes. *J. Lipid Res.* **25:** 1295–1305.
2. Higgins J. A., and J. K. Fieldsend. 1987. Phosphatidylcholine synthesis for incorporation into membranes or for secretion as plasma lipoproteins by Golgi membranes of rat liver. *J. Lipid Res.* **28:** 268–278.
3. Higgins, J. A. 1988. Evidence that during very low density lipoprotein assembly in rat hepatocytes most of the triacylglycerol and phospholipids are packaged with apolipoprotein B in the Golgi complex. *FEBS Lett.* **232:** 405–408.
4. Wilkinson, J., J. A. Higgins, P. H. E. Groot, E. Gherardi, and D. E. Bowyer. 1992. Determination of the intracellular distribution and pool sizes of apolipoprotein B in rabbit liver. *Biochem. J.* **288:** 413–419.
5. Wilkinson, J., J. A. Higgins, P. H. E. Groot, E. Gherardi, and D. E. Bowyer, 1993. Topography of apolipoprotein B in subcellular fractions of rabbit liver probed with a panel of monoclonal antibodies. *J. Lipid Res.* **34:** 815–825.
6. Cartwright, I. J., A-M. Hebbachi, and J. A. Higgins. 1993. Transit and sorting apolipoprotein B within the endoplasmic reticulum and Golgi compartments of isolated hepatocytes from normal and orotic acid fed rats. *J. Biol. Chem.* **268:** 20937–20952.
7. Cartwright, I. J., and J. A. Higgins. 1995. Intracellular events in the assembly of very low density lipoprotein lipids with apolipoprotein B in isolated rabbit hepatocytes. *Biochem. J.* **310:** 897–907.
8. Boren, J., S. Rustaeus, M. Wettensten, M. Andersson, A. Wiklund, and S-O. Olofsson. 1993. Influence of triacylglycerol-biosynthetic rate on the assembly of apo-B100 containing lipoproteins in Hep-G2 cells. *Arterioscler. Thromb.* **13:** 1743–1754.
9. Boren, J., S. Rustaeus, and S-O. Olofsson. 1994. Studies on the assembly of apolipoprotein B-100 and B-48 containing very low density lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **269:** 25879–25888.
10. Spring, D. J., L. W. Chen-Lin, J. E. Chatterton, J. Elvoson, and V. N. Schumaker. 1992. Lipoprotein assembly: apo-B size determines lipoprotein core circumference. *J. Biol. Chem.* **267:** 14839–14845.
11. Elvoson, J., G. T. Bell, M. H. Doolittle, and M. Phillips. 1992. A two-step model for very low density lipoprotein assembly: characterisation of intermediate lipoprotein particles. *Circulation.* **86 (suppl)** 1–692.
12. Swift, L. L. 1995. Assembly of very low density lipoproteins in rat liver: a study of nascent particles recovered from the rough endoplasmic reticulum. *J. Lipid Res.* **35:** 395–406.
13. Rusinol, A. E., E. Y. W. Chan, and J. E. Vance. 1993. Assembly of rat hepatic very low density lipoproteins in the endoplasmic reticulum. *J. Biol. Chem.* **268:** 25168–25175.
14. Davis, R. A., R. N. Thrift, C. C. Wu, and K. E. Howell. 1990. Apolipoprotein B is both integrated into and translocated across the endoplasmic reticulum membrane. *J. Biol. Chem.* **26:** 10006–10011.
15. Du, E. Z., J. Kurth, S-L. Wang, P. Humiston, and R. A. Davis. 1994. Proteolysis coupled secretion of the N terminus of apolipoprotein-B. *J. Biol. Chem.* **269:** 24169–24176.
16. Zhou, M., X. Wu, L-S. Huang, and H. N. Ginsberg. 1995. Apolipoprotein B-100, an inefficiently translocated secretory protein is bound to the cytosolic chaperone, heat shock protein 70. *J. Biol. Chem.* **270:** 25220–25224.
17. Hamilton, R. L., and R. J. Havel. 1993. Is microsomal triacylglycerol transport protein the missing link in apolipoproteinemia? *Hepatology.* **18:** 460–463.
18. Sharpe, D., L. Blindermam, K. A. Combs, B. Kienzie, B. Ricci, K. Wagner-Smith, C. M. Gil, C. W. Turck, M. E. Bouma, D. J. Rader, L. P. Aggerbeck, R. E. Gregg, D. A. Gordon, and J. R. Wetterau. 1993. Cloning and gene de-

fects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia. *Nature.* **365:** 65–69.
19. Gordon, D. A., J. R. Wetterau, and R. E. Gregg. 1995. Mircrosomal triglyceride transfer protein: a protein complex equired for the assembly of lipoprotein particles. *Trends Cell Biol.* **5:** 317–321.
20. Jamil, H., J. E. Dickson, C. Ching-Hsuen, M. W. Lago, J. K. Rinhhaert, S. A. Biller, R. E. Gregg, and J. R. Wetterau. 1995. Microsomal triglyceride transfer protein: specificity of lipid binding and transport. *J. Biol. Chem.* **270:** 6549–6554.
21. Dixon, J. L., and H. N. Ginsberg. 1993. Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins; information obtained from cultured liver cells. *J. Lipid Res.* **34:** 167–179.
22. Sniderman, A. D., and K. Cianflone. 1993. Substrate delivery as a determinant of hepatic apoB secretion. *Arterioscler. Thromb.* **13:** 629–636.
23. Gibbons, G. W., and D. Wiggins. 1995. The enzymology of hepatic very low density lipoproteins assembly. *Biochem. Soc. Trans.* **23:** 495–500.
24. Thompson, G., R. P. Naoumova, and G. F. Watts. 1996. Role of cholesterol in regulating apolipoprotein B secretion by the liver. *J. Lipid Res.* **27:** 439–447.
25. Cartwright, I. J., and J. A. Higgins. 1992. Quantification of apolipoprotein B-48 and B-100 in rat liver endoplasmic reticulum and Golgi. *Biochem. J.* **285:** 153–159.
26. Cartwright, I. J., and J. A. Higgins. 1996. Intracellular degradation in the regulation of secretion of apolipoprotein B-100 by rabbit hepatocytes. *Biochem. J.* **314:** 377–384.
27. Suckling, K. E., and E. F. Stange. 1985. Role of acyl CoA-cholesterol acyltransferase in cellular cholesterol metabolism. *J. Lipid Res.* **26:** 647–671.
28. Dietchy, J. M., S. D. Turley, and D. K. Spady. 1993. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species including humans. *J. Lipid Res.* **34:** 1637–1659.
29. Palade, G. E., and P. Siekevitz. 1956. Pancreatic microsomes: an integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* **2:** 671–680.
30. Ford, T., J. M. Graham, and D. Rickwood. 1994. Iodixanol: a nonionic isoosmotic centrifugation medium for the formation of self-generated gradients. *Anal. Biochem.* **220:** 360–366.
31. Ford. T., J. M. Graham, and D. Rickwood. 1994. The preparation of subcellular organelles from mouse liver in self-generated gradients of Iodixanol. *Anal. Biochem.* **220:** 367–373.
32. Graham, J. M., and D. Billington. 1996. Iodixanol—a new density gradient medium for the dissection of the endosomal compartment. *Z. Gastroenterol.* **34:** 76–78.
33. Smart, E. J., Y-S. Ying, C. Mineo, and R. G. W. Anderson. 1995. A detergent-free method for purifying calveolae membranes from tissue culture cells. *Proc. Natl. Acad. Sci. USA.* **92:** 10104–10108.
34. Graham, J. M., J. A. Higgins, T. Gillot, T. Taylor, J. Wilkinson, T. Ford, and D. Billington. 1996. A novel method for the rapid separation of plasma lipoproteins using self-generated gradients of Iodixanol. *Atherosclerosis.* **124:** 125–135.
35. Higgins, J. A. 1984. The transverse distribution of phospholipids in the membranes of Golgi subfractions of rat liver. *Biochem. J.* **219:** 261–272.
36. Stahlberg, D., B. Angelin, and K. Einarsson. 1989. Effects of treatment with clofibrate, bezafibrate and ciprofibrate on the metabolism of cholesterol in rat liver microsomes. *J. Lipid Res.* **30:** 953–958.
37. Anderson, M., M. Wettesten, J. Boren, A. Magnusson, A. Sjoberg, S. Rusteaus, and S-O. Olofsson. 1994. Purification of diacylglycerol:acyltransferase from rat liver to near homogeneity. *J. Lipid Res.* **35:** 535–545.
38. Rustaeus, S., K. Lindberg, J. Boren, and S-O. Olofsson. 1995. Brefeldin A reversibly inhibits the assembly of apoB-containing lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **270:** 28879–28886.
39. Gibbons, G. F. 1994. A comparison of in vitro models to study hepatic lipid and lipoprotein metabolism. *Current Opin. Lipidol.* **5:** 191–198.
40. Gibbons, G. F., R. Khurana, A. Odwell, and M. C. L. Seelender. 1994. Lipid balance in HepG2 cells: active synthesis and impaired mobilization. *J. Lipid Res.* **35:** 1801–1808.
41. Packard, C. J. 1996. Plasma lipid and lipoprotein metabolism in the 1990s—what we know and what we need to know. *In* Lipids: Current Perspectives. Vol. I. Lipids and Lipoproteins. D. J. Betteridge, editor. Martin Dunitz, London, UK. 1–20.
42. Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* **266:** 5080–5086.
43. White, A. L., D. L. Graham, J. LeGros, R. J. Pease, and J. Scott. 1992. Oleate-mediated stimulation of apolipoprotein B secretion from rat hepatoma cells. *J. Biol. Chem.* **267:** 15657–15664.
44. Wu, X., N. Sakata, E. Lui, and H. N. Ginsberg. 1994. Evidence for a lack of regulation of assembly and secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by cholesteryl ester. *J. Biol. Chem.* **266:** 12375–12382.
45. Dashti, N., 1992. The effect of low density lipoproteins cholesterol and 25-hydroxycholesterol on apolipoprotein B gene expression in HepG2 cells. *J. Biol. Chem.* **267:** 7160–7169.
46. Cianflone, K. M., Z. Yasruel, M. A. Rodrieguez, D. Vas, and A. D. Sniderman. 1990. Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge. *J. Lipid Res.* **31:** 2045–2055.
47. Kohen-Avramoglu, R., K. M. Cianflone, and A. D. Sniderman. 1995. The role of the neutral lipid accessible pool in the regulation of secretion of apoB-100 lipoprotein particles by HepG2 cells. *J. Lipid Res.* **36:** 2513–2528.
48. Musanti, R., L. Giorgini, P-P. Lovisolo, A. Pirillo, A. Chiari, and G. Ghiselli. 1996. Inhibition of acylCoA-cholesterol acyltransferase decreases apolipoprotein B-100-containing lipoprotein secretion from HepG2 cells. *J. Lipid Res.* **37:** 1–14.
49. Furiukawa, S., and T. Hirano. 1993. Rapid stimulation of apolipoprotein B secretion by oleate is not associated with cholesteryl ester biosynthesis in HepG2 cells. *Biochim. Biophys. Acta.* **1170:** 32–37.
50. Sato, R., T. Imanara, and T. Tanko. 1990. The effect of the HMG-CoA reductase inhibitor CS 514 on the synthesis and secretion of apolipoprotein B and A-I by the human hepatoma HepG2. *Biochim. Biophys. Acta.* **1042:** 36–41.
51. Kosykh, V. A., A. P. Surguchov, E. A. Podres, D. K. Novikov, and Y. Sudarickoc. 1988. VLDL apoprotein secretion and apoB mRNA levels in primary culture of cholesterol-loaded rabbit hepatocytes. *FEBS Lett.* **232:** 103–106.
52. Kosykh, V. A., S. N. Preobrazhensky, L. V. Fuki, O. E. Zai-

kina, V. P. Tsibulksy, V. S. Repin, and V. N. Sirnov. 1985. Cholesterol can stimulate secretion of apolipoprotein B by cultured human hepatocytes. *Biochim. Biophys. Acta.* **836:** 385–389.
53. Tanaka, M., H. Jingami, H. Otani, M. Cho, Y. Ueda, H. Arai, Y. Nagano, T. Doi, M. Yokode, and T. Kita. 1993. Regulation of apolipoprotein B production and secretion in response to the change of intracellular cholesterol ester contents in rabbit hepatocytes. *J. Biol. Chem.* **268:** 12713–12718.
54. Huff, M. W., D. E. Telford, J. Barrett, T. Bilheimer, and P. J. Gillies. 1994. Inhibition of hepatic ACAT decreases apoB secretion in miniature pigs fed a cholesterol-free diet. *Arterioscler. Thromb.* **14:** 1498–1508.
55. Khan, B. V., T. V. Fungwe, H. G. Wilcox, and M. Heimsberg. 1990. Cholesterol is required for the secretion of very low density lipoproteins—in vivo studies. *Biochim. Biophys. Acta.* **1044:** 297–304.
56. Fungwe, T. V., L. Cagen, H. G. Wilcox, and M. Heimsberg. 1992. Regulation of hepatic secretion of very low density lipoproteins by dietary cholesterol. *J. Lipid Res.* **33:** 179–191.
57. Craig, W. Y., R. Nutik, and A. D. Cooper. 1988. Regulation of apolipoprotein synthesis and secretion in the human hepatoma HepG2. *J. Biol. Chem.* **263:** 13869–13890.
58. Arad, Y., R. Ramakrishnan, and H. N. Ginsberg. 1990. Lovastatin therapy reduces low density lipoprotein apoB levels in subjects with combined hyperlipidemia by reducing production of apoB-containing lipoproteins: implications for the pathophysiology of apoB production. *J. Lipid Res.* **30:** 567–582.
59. Tarugi, P., S. Nicolini, G. Ballarini, L. Marchi, C. Duvigneau, P. Tartoni, and S. Calandra. 1996. Synthesis and secretion of B-100 and A-I apolipoproteins in response to changes of intracellular cholesteryl ester content in chick liver. *J. Lipid Res.* **37:** 493–507.
60. Carr, T. P., R. L. Hamilton, and L. L. Rudel. 1995. ACAT inhibitors decrease secretion of cholesteryl esters and apolipoprotein B by perfused livers of African green monkeys. *J. Lipid Res.* **36:** 25–30.
61. Arbeeny, C. M., D. S. Meyers, K. F. Bergquist, and R. E. Gregg. 1992. Inhibition of fatty acid synthesis decreases very low density lipoprotein secretion in the hamster. *J. Lipid Res.* **33:** 843–851.