Biosynthesis of Lipoprotein: Location of Nascent ApoAI and ApoB in the Rough Endoplasmic Reticulum of Chicken Hepatocytes

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Abstract. Our previous studies showed that in hepatic RER of young chickens, nascent apoAI is not associated with lipoprotein particles and only becomes part of these lipoprotein structures in the Golgi. In this study, we have used three different methodologies to determine the locations of apoAI and apoB in the RER and compared them to that of albumin. Immuno-electron microscopic examination of the RER cell fractions showed that both apoAI and apoB were associated only with the RER membrane whereas albumin was located both within the lumen and on the limiting membrane of the vesicles. To examine the possibility of membrane integration of nascent apoAI and apoB in the RER, we administered L-[³H]leucine to young chickens for 10 min, isolated RER, treated this cell fraction with buffers of varying pH, and measured the release of radioactive albumin, apoAI, and apoB. The majority of nascent apoAI (64%), nascent apoB (100%), and nascent albumin (97%) was released from RER vesicles at pH 11.2, suggesting that, like albumin, apolipoproteins are not integrated within the membrane. To determine if nascent apoproteins are exposed to the cytoplasmic surface, we administered L-[³H]leucine to young chickens and at various times isolated RER and Golgi cell fractions. Radioactive RER and Golgi cell fractions were treated with exogenous protease and the percent of nascent apoAI and apoB accessible to proteolysis was determined and compared to that of albumin. At 5, 10, and 20 min of labeling, 35–56% of nascent apoAI and 60–75% of apoB in RER were degraded, while albumin was refractive to this treatment. At all times both apolipoproteins and albumin present in Golgi cell fractions were protected from proteolysis. These biochemical and morphological findings indicate that apoAI and apoB are associated with the rough microsomal membrane and are partially exposed to the cytoplasmic surface at early stages of secretion. They may later enter the luminal side of the ER and, on entering the Golgi, form lipoprotein particles.

A POLIPOPROTEIN AI (apoAI)[1] and apolipoprotein B (apoB) are the major protein components of chicken plasma high density and very low density lipoproteins (HDL and VLDL, respectively). Both of these proteins are composed of single polypeptide chains and their structures have been well studied. However, details of their biosynthesis, assembly into lipoprotein particles, and secretion are still not clear (Chapman, 1980; Gotto et al., 1986; Hamilton, 1983). In humans and most other species, apoAI and apoB are produced mainly in liver and intestine (Gotto et al. 1986; Green and Glickman, 1981), but in avians apoAI synthesis also occurs in tissues such as breast, muscle, and kidney (Blue et al., 1982; Shackelford and Lebherz, 1983).

The sites of synthesis of the protein (Blue et al., 1982; Shackelford and Lebherz, 1983; Banerjee and Redman, 1983; Banerjee and Redman, 1984; Banerjee et al., 1985; Janero and Lane, 1983), lipid (Banerjee and Redman, 1984; Janero and Lane, 1983; Higgins and Fieldsend, 1987; Vance

and Vance, 1988), and carbohydrate (Siuta-Mangano et al., 1982) moieties of the lipoproteins have been determined. There are some studies on the nature of the nascent lipid protein complexes in the various organelles (Banerjee and Redman, 1983; Banerjee and Redman, 1984; Howell and Palade, 1982), but detailed understanding of the structure of the nascent lipoprotein particles is not available. Analysis of nascent VLDL particles, isolated by flotation from the contents of rat liver Golgi cell fractions, showed considerable variation in the size of the particles (Howell and Palade, 1982).

Our in vivo studies on HDL biosynthesis indicated that apoAI is quickly transported from the RER to the Golgi and that conjugation of lipid to protein occurs in the Golgi cell fraction where immature, not fully formed particles are assembled (Banerjee and Redman, 1983). We showed that although radioactive glycerol is quickly incorporated into lipids of the RER and Golgi apparatus, nascent lipids are mostly conjugated with apoAI in the Golgi complex and not in the RER (Banerjee and Redman, 1984). Thus, nascent apoAI and nascent lipids appear to be associated at a later step in the cellular secretory processes which occur in the

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1. *Abbreviations used in this paper:* apoAI, apolipoprotein AI; apoB, apolipoprotein B; HDL, high density lipoprotein; VLDL, very low density lipoprotein.

Golgi apparatus. In a separate study, we also measured the rate of transfer of nascent apoAI from its site of synthesis in the RER to the Golgi apparatus and showed that apoAI transfer from RER to Golgi cell fraction is very rapid and that it is difficult to measure its rate by in vivo pulse-labeling methods (Banerjee et al., 1985). This rapid movement of apoAI has later been confirmed by another group (Bamberger and Lane, 1988).

Studies with Hep G2 cells (Boström et al., 1986; Boström et al., 1988; Borén et al., 1990) and primary hepatocytes, obtained from chicken (Bamberger and Lane, 1988) and rat (Borchardt and Davis, 1987), showed that apoB movement out of the RER is slower than that of albumin, and apoB is associated with the RER membrane (Bamberger and Lane, 1988; Boström et al., 1986; Boström et al., 1988; Borén et al., 1990; Borchardt and Davis, 1987; Davis et al., 1990; Wong and Pino, 1987). Only a fraction of nascent apoB appeared to be translocated into the luminal side of the ER and secreted, while the remainder was associated with the cytoplasmic side and degraded (Davis et al., 1990). The location of nascent apoAI within the RER is not known. However, we showed that newly synthesized apoAI within the RER failed to float between densities 1.063–1.21 g/ml, whereas the apoAI present within the Golgi complex is capable of floating at a buoyant density similar to that of plasma HDL (Banerjee and Redman, 1983). This indicates that apoAI in the RER either has not been sufficiently complexed with lipids and thus cannot attain an appropriate buoyant density, or is associated with the membrane of the RER.

In this report, we examine the location of nascent apoAI and apoB within the RER by immunoelectron microscopy, by disrupting isolated cell fractions with alkaline buffers, and by treating RER vesicles with exogenous protease. The locations of apoAI and apoB are compared to that of albumin. Our observations indicate that nascent apoAI and apoB are located in the membranes of the RER and are partially exposed to the cytoplasmic surface before they complex with lipids to become part of lipoprotein particles in the Golgi apparatus.

Materials and Methods

Materials

L-[4,5-³H]leucine was obtained from Amersham Corp. (Arlington Heights, IL). Aprotinin (Trasylol) was purchased from Mobay Chemical Corp. (New York), PMSF from Calbiochem (La Jolla, CA), and 2-[N-cyclohexylamine]-ethanesulfonic acid (TPCK) and benzamidine were purchased from Sigma Chemical Co. (St. Louis, MO). All chemical reagents were of the highest quality available. Rabbit polyclonal immunoglobulin to chicken serum albumin was purchased from Cappel Laboratories (Westchester, PA) and young Leghorn chickens (5–10-d-old) were obtained from Spafas Poultry Farms (Norwich, CT).

Methods

In Vivo Labeling. Leghorn chickens were maintained in a light and temperature controlled room for 24 h. They had free access to water and were fed Purina Chick Starter Chow. The birds were placed under light ether anesthesia and radiolabeled leucine was injected into the jugular vein. The birds were allowed to recover from anesthesia and at specified times were killed by decapitation.

Preparation of Cell Fractions. Livers were homogenized in 0.25 M sucrose, filtered through a layer of cheese cloth, and a postmitochondrial supernatant was removed by centrifuging the homogenate at 16,000 g for 10 min. Total membrane pellets were obtained by centrifuging the supernatant

fraction at 105,000 g for 90 min. The RER and Golgi cell fractions were further separated and characterized as described earlier (Banerjee and Redman, 1983, 1984).

High pH Washing of RER Vesicles. RER vesicles were washed with high pH buffers following the method of Paver et al. (1989). In short, aliquots (600 μl; 4 mg/ml protein) of RER vesicles were gently mixed with 7 vol of either 50 mM Hepes-KOH buffer, pH 7.5, or 25 mM CAPSO-KOH buffer, pH 9.1, containing 25 mM KCl and 0.25 M sucrose using a Pasteur pipette. The Hepes- and CAPSO-treated vesicles were layered over a cushion (2 ml) of 0.5 M sucrose containing 100 mM KCl and 50 mM Hepes-KOH, pH 7.5, and centrifuged at 105,000 g for 60 min at 4°C. The supernatant was retained for analysis of released apoAI, apoB, and albumin. The membrane pellets were solubilized with 0.5% sodium deoxycholate and 0.5% Triton X-100 and centrifuged at 105,000 g for 60 min. The detergent-soluble supernatant fraction was used to isolate apoAI, apoB, and albumin by immunoprecipitation. RER membrane vesicles were also treated with Na₂CO₃, pH 11.2, to release luminal proteins by the method of Fujiki et al. (1982).

Isolation of Radioactive ApoAI, ApoB, and Albumin from the RER. Proteins released at alkaline pH and those present in the detergent-soluble membrane fractions were supplemented with 100 μg/ml PMSF, 100 U/ml Trasylol, 1 mM benzamidine, and 1 mM TPCK. Radioactive apoAI, apoB, and albumin present in these fractions were recovered by treatment with monospecific rabbit antibodies. The antibody–antigen complexes were adsorbed to protein A-Sepharose CL-4B beads as previously described (Dixon et al., 1989). ApoAI, apoB, and albumin present in the immunoprecipitates were separated by SDS–polyacrylamide slab gel electrophoresis, detected by fluorography, and quantitated by scintillation counting and/or laser densitometric scanning of the fluorograms.

Immunoblot Analysis. Proteins released from RER cell fractions with alkaline buffers were precipitated with 5% ice-cold TCA for 18 h, solubilized in electrophoresis sample buffer, separated by SDS-PAGE, and transferred to Immobilon-P membrane in 25 mM Tris, 192 mM glycine, 0.05% SDS, and 20% methanol at 60 V for 8 h (Towbin et al., 1979). The blotted membranes were blocked in PBS containing 2% BSA for 1 h at room temperature. Blots were washed briefly with distilled water and incubated at 4°C overnight with rabbit antibody to chicken albumin in PBS, 1% BSA, 0.1% Triton X-100, and 0.02% SDS. Blots were washed (5 × 10 min) in the same buffer and incubated with ¹²⁵I-labeled goat anti–rabbit IgG at 1:1,000 dilution for 60 min. Blots were again washed as described, dried, and exposed to Kodak XAR-5 film.

Protease Protection Assay. The proteolysis conditions were essentially as described by Walter and Blobel (1983). In short, samples were placed on ice and supplemented with CaCl₂ to a final concentration of 2 mM. Proteinase K was added to a final concentration of 50 μg/ml and digestions were performed for 30 min on ice. After digestion PMSF was added to a concentration of 3 mM, and the samples were left on ice for another 5 min. The membrane vesicles were sedimented through a 0.25 M sucrose solution to remove the protease. The pellets were dissolved in NET buffer containing proteolytic inhibitors, 0.5% sodium deoxycholate and 0.5% Triton X-100 and recentrifuged at 105,000 g for 1 h. The supernatant fraction was used to isolate apoAI, apoB, and albumin by immunoprecipitation and analyzed by SDS-PAGE and fluorography. ApoAI was first immunoprecipitated and the supernatant fraction was then used to serially immunoprecipitate albumin, followed by apoB.

Electron Microscopy. The procedures for fixation and embedding of RER cell fractions were as described before (Banerjee and Redman, 1983). For immunolocalization, the RER vesicles were fixed in suspension with cold phosphate-buffered para-formaldehyde, glutaraldehyde, and picric acid solution containing MgCl₂ (3% para-formaldehyde, 0.1% glutaraldehyde, 0.1% picric acid, 5 mM MgCl₂, and 50 mM sodium phosphate buffer, pH 6.5) for 1 h at 0°C. With the exception of tannic acid the fixative was as described by Aris and Blobel (1988). The fixed RER vesicles were diluted and centrifuged in an Eppendorf microfuge. The pellets were washed with the same buffer and quenched with 50 mM NH₄Cl for 1 h. The minced pellets were dehydrated with 5-min steps in 70% ethanol, infiltrated in 1:1 mixture of 70% ethanol and LR White for 15 min using a shaker, followed by three changes of pure LR White, 30 min each. The samples were embedded in fresh LR White using gelatin capsules and polymerized at 52°C for 24 h. Ultrathin sections on coated nickel grids were equilibrated for 10 min in PBS, blocked with normal goat serum diluted 1:30 in PBS for 20 min, and incubated for 1 h at 37°C with affinity-purified primary rabbit antibody. After three 5-min washes with PBS containing 0.05% Tween 20 (PBS/Tween 20), the sections were incubated with 10-nm colloidal gold conjugated with goat anti–rabbit IgG at 1:40 dilution with PBS/Tween 20 for 1 h at room temperature, washed as above, and rinsed with water before

staining with uranyl acetate and lead citrate. The sections were examined with a Philips EM 410 electron microscope.

For examination of liver sections, 15-d-old chicken embryos were used. The embryos were decapitated and their livers were flushed with 0.9% NaCl until blanched and then fixed by perfusion with cold 4% para-formaldehyde, 0.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2 until livers hardened. After perfusion, livers were removed, cut into small pieces and fixed an additional 30–90 min on ice. The pieces were dehydrated in graded dimethylformamide (DMF), infiltrated, and embedded in Lowicryl K4M according to the procedure of Altman et al. (1984). Incubations with primary antibody and gold-labeled secondary antibody were as described above except the sections were incubated overnight at 4°C with primary antibody. Lowicryl seemed to be the best embedding media for the whole liver while LR White gave the best result for the isolated RER vesicles. In both cases nonimmune rabbit serum was used as negative control.

Results

### Immunocytochemical Localization of ApoAI, ApoB, and Albumin in RER Vesicles

Immunoelectron microscopy, using monospecific polyclonal rabbit antibodies to chicken apoAI and apoB, was used to ex-amine the location of apoAI and apoB in liver sections and in isolated RER vesicles. In whole liver sections antibodies to apoAI and apoB and to serum albumin, used as a control, decorated the RER area (Fig. 1, a–c). The gold particles appeared on both the cytoplasmic and the luminal side of the RER membrane. Anti-apoAI and anti-apoB binding occurred to a lesser extent than antialbumin binding (compare Fig. 1, a and b with c). With nonimmune rabbit serum only background levels of gold labeling were detected (Fig. 1 d).

For a more detailed examination the RER cell fraction was prepared from chicken livers and processed for immunoelectron microscopy. Electron micrographs revealed that gold particles, representing apoAI and apoB, were mainly associated with the membrane of RER cell fractions and very few particles appeared to be in the lumen of the vesicles (Fig. 2, a and b). In contrast, gold particles representing albumin were distributed in both the lumen and the membrane (Fig. 2 c). As shown in Table I nearly all of apoAI (99%) and apoB (90%) were associated with the membrane. In the case of albumin, the gold particles were distributed between the mem-
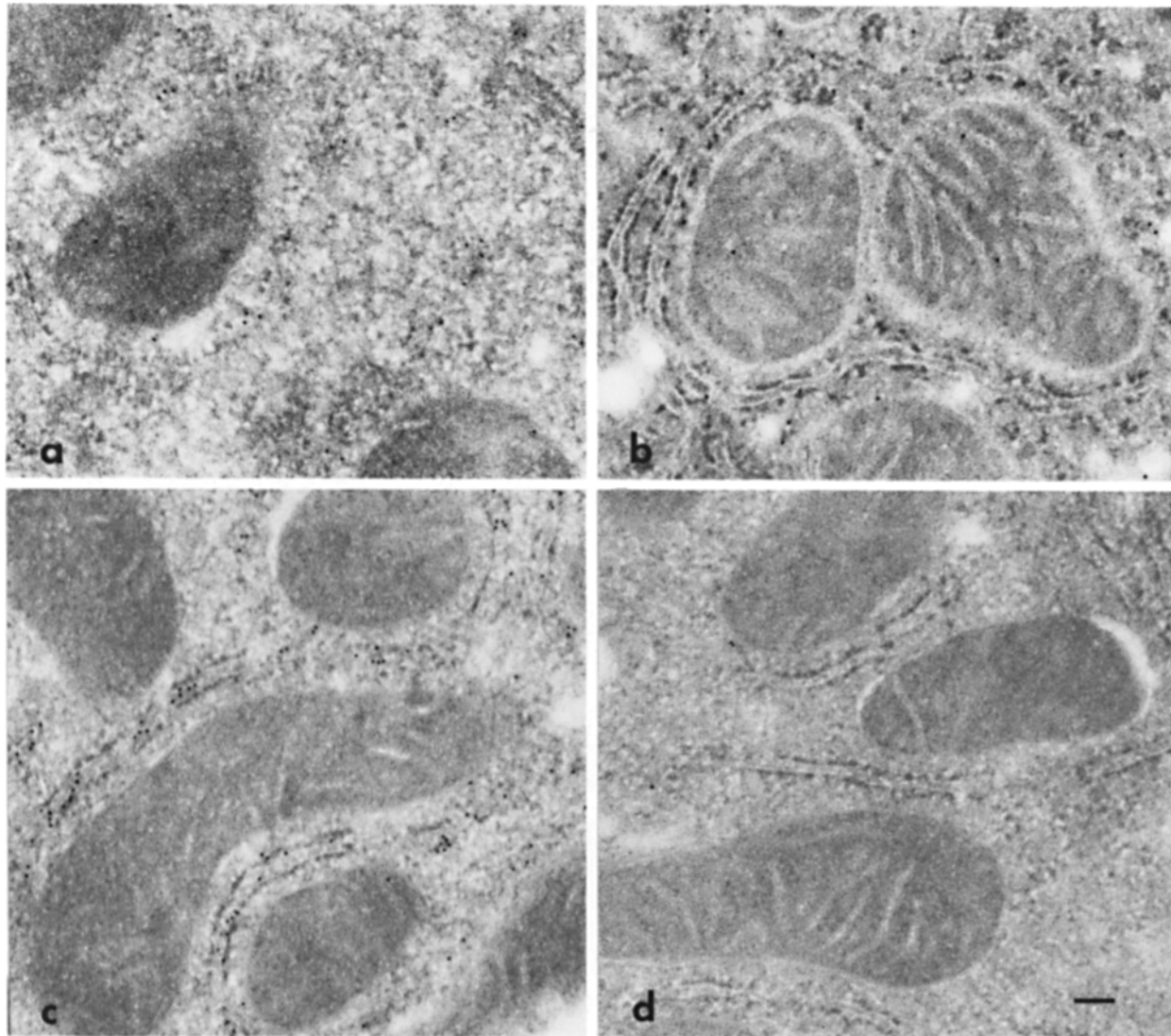
Figure 1. Embryonic chick hepatocytes. The embryonic livers were prepared for immunoelectron microscopy as described in Materials and Methods. (a) ApoA-I; (b) apoB; (c) albumin; and (d) nonimmune serum. Bar, 100 nm.
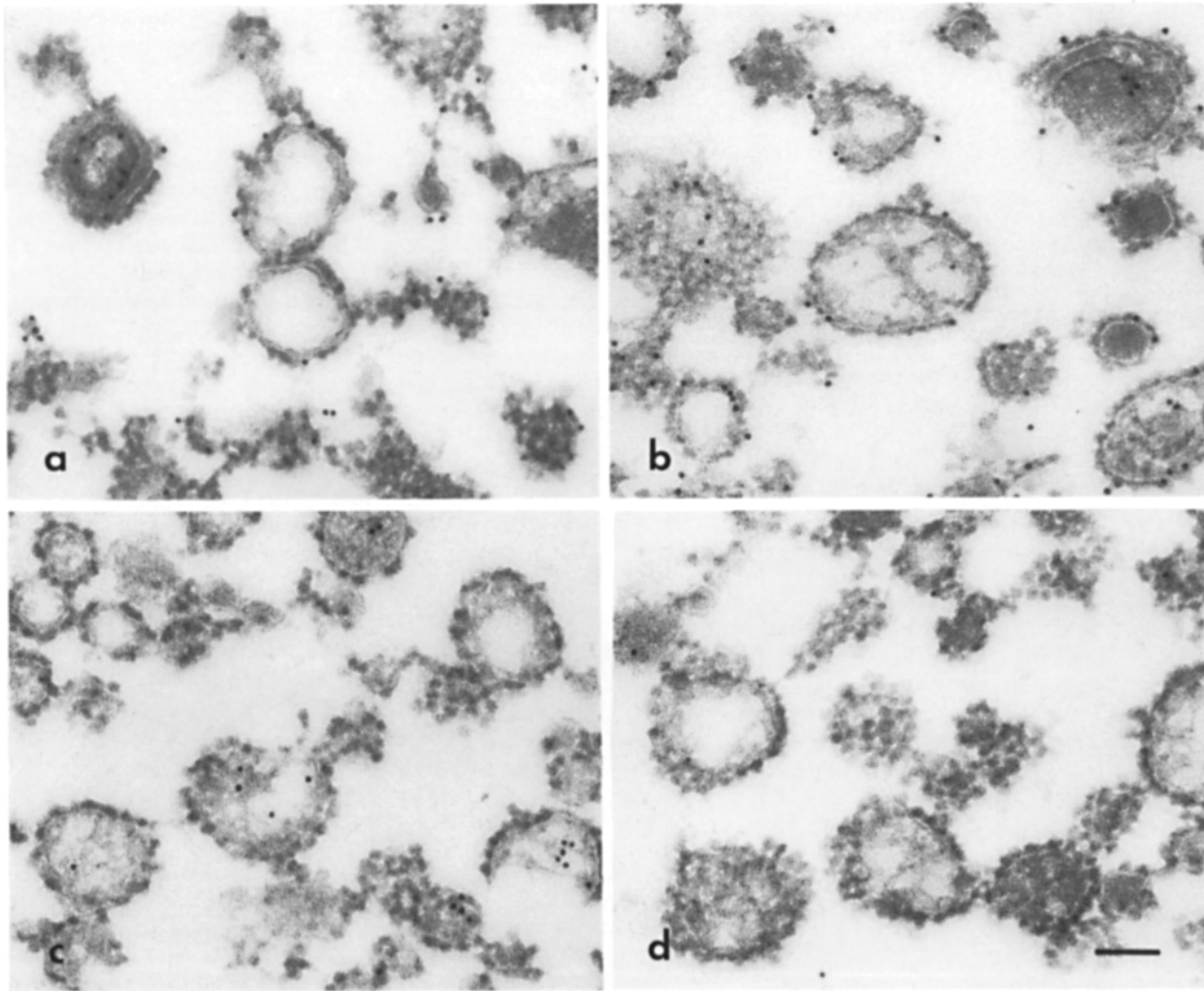
Figure 2. Immunoelectron microscopic localization of apoA-I, apoB, and albumin in isolated RER vesicles. The RER vesicles, isolated from young chicken liver, were fixed and the membranes were pelleted and prepared for EM as described in Materials and Methods. The vesicles are mostly lined with ribosomes. (*a*) ApoA-I; (*b*) apoB; (*c*) albumin; and (*d*) nonimmune serum. Bar, 100 nm.

brane (58%) and lumen (42%). Gold particles at an average distance of 7.0 ± 5.2 nm on either side of the middle of the visible membrane were considered membrane associated while those that were further inside from the membrane were considered luminal. Quantitation was obtained by counting 300–400 RER vesicles.

Interestingly, 96% of the RER vesicles contained apoAI and apoB while only 41% had albumin. This is unexpected because liver makes more albumin than apoAI or apoB (see Fig. 1 c). Since albumin is a soluble luminal protein, it is possible that some of the RER vesicles lost albumin during cell fractionation. ApoAI and apoB, which appear to be membrane-associated, are less likely to be released during rupture of the RER before fractionation. These immunoelectron microscopic results suggest that unlike albumin, apoAI and apoB are associated with the RER membrane.

## Treatment of RER Cell Fractions with Alkaline Buffers

The immunocytochemical findings raise the possibility that upon completion of synthesis apoAI and apoB might be integrated within the RER membrane rather than being released, as albumin, in the luminal compartment. Treat-

ment of cell fractions with alkaline buffers is a commonly used method for separating peripheral and luminal proteins from membrane-integrated proteins. This method is often used to open sealed rat hepatic RER vesicles (Fujiki et al., 1982). The effectiveness of this technique for releasing albu-

Table I. Distribution of Gold Particles over RER

	Antisera used		
	Apo AI	Apo B	Albumin
Relative frequency	95.8%	95.7%	41.3%
Total gold count	641	753	100
Number of RER profiles	288	345	385
% of gold particles			
Membrane associated	99.0%	89.8%	58.2%
Within the lumen	1.0%	10.2%	41.8%

Immunolabeling was performed as described in Materials and Methods. Relative frequency represents the percentage of gold-labeled vesicles. Gold particle was counted as membrane associated when particle occured within an average distance of 7.0 ± 5.2 nm on either side of the middle of the visible membrane. All other gold particles were counted as either in lumen or nonspecific. Particles obtained by nonimmune serum were subtracted from all samples.
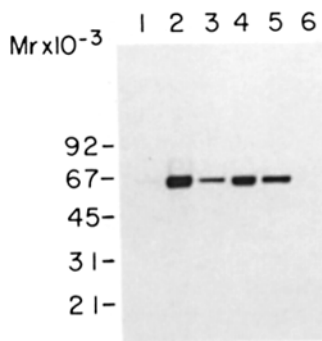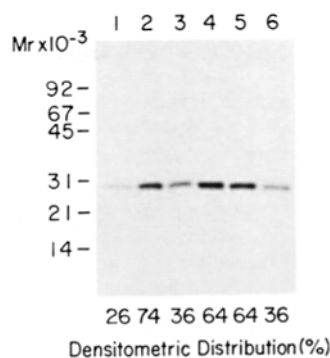
Figure 3. pH-dependent release of endogenous albumin. Chicken liver RER membranes were washed once with buffers of varying pH and the supernatant and pellet fractions were resolved by 8% SDS-PAGE, immunoblotted on to nitrocellulose, and probed with goat anti–rabbit IgG as described in Materials and Methods. (Lanes 1 and 2) pH 7.5; (lanes 3 and 4) pH 9.1; (lanes 5 and 6) pH 11.2. Lanes 1, 3, and 5 represent released supernatant fractions and lanes 2, 4, and 6 are protein retained in the membrane pellets.

Figure 4. pH-dependent release of apoAI from the RER membrane. Young chickens received L-[^{3}H]leucine (2.5 mCi/100 g body weight) by i.v. injection for 10 min and radioactive rough microsomes were prepared from their livers. The isolated radiolabeled RER vesicles were treated with buffers of varying pH and separated into soluble supernatant and membrane fractions. Radioactive apoA-I was immunoprecipitated from both the supernatant and from detergent-solubilized membrane pellets, analyzed by 12% SDS-PAGE and fluorography as described in Materials and Methods. The position of molecular weight markers is indicated at left and relative densitometric distribution of major band at bottom. Lanes 1, 3, and 5 represent supernatants and lanes 2, 4, and 6 represent pellets. (Lanes 1 and 2) pH 7.5; (lanes 3 and 4) pH 9.1; (lanes 5 and 6) pH 11.2.

min, a content protein, from chicken hepatic RER cell fractions was first established. The isolated RER vesicles were washed with alkaline buffers of varying pH and the release of endogenous albumin and nascent pulse-labeled albumin were measured. The results are shown in Fig. 3. Very little endogenous albumin was released upon treatment of RER vesicles with pH 7.5 buffer; at pH 9.1 ~30% was detected in the supernatant fraction whereas at pH 11.2, 95% was released. Similar results were obtained when albumin was radiolabeled by intravenous injection of L-[^{3}H]leucine for 10 min (Table II), indicating that, at the time period used for radiolabeling, most of the nascent proteins have been released from polysomes and have entered into the lumen of the RER cell fraction. These results confirm that, as noted in rat liver microsomes, high pH washing of chicken liver microsomes also releases endogenous and nascent soluble luminal proteins.

To examine the possibility that in absence of lipids nascent apolipoproteins might be integrated in the RER membrane and thus fractionate differently from albumin, radiolabeled RER cell fractions were treated with buffers of varying pH and separated into soluble supernatant and membrane fractions by centrifugation. The occurrence of nascent apoAI and apoB in the supernatant fraction and in solubilized membrane fractions was determined by immunoprecipitation. The results are given in Figs. 4 and 5. Treatment at either pH 7.5 or 9.1 only released a small fraction of apoAI (Fig. 4, lanes 1 and 3). The majority (64-70%) of nascent apoAI

was still associated with the membrane fraction (Fig. 4, lanes 2 and 4). When RER vesicles were treated with sodium carbonate pH 11.2, 36% of nascent apoAI remained with the membrane (Fig. 4, lane 6), while 64% was released into the supernatant (Fig. 4, lane 5). A different pattern was obtained with apoB. At pH 7.5 and 9.1 there was no detectable release of nascent apoB from the RER membrane (Fig. 5, lanes 2 and 4), but nearly all of the apoB was released from RER membrane at pH 11.2 (Fig. 5, lane 5). The higher bands, observed in Fig. 5 (lanes 2 and 4) are aggregated apoB which failed to enter the gel. These results indicate that albumin, apoAI, and apoB are released from RER cell fractions on treatment with alkaline buffers. In general, albumin and apoB are more readily released at alkaline pH than apoAI, suggesting that apoAI has greater affinity to the membrane. However, the large majority of all the apolipoproteins are released at pH 11.2 which would not occur if they were fully integrated into the membrane.

Protease Protection of Nascent ApoAI, ApoB, and Albumin in Isolated RER and Golgi Cell Fractions

Treatment of membrane vesicles with exogenous protease

Table II. Distribution of Newly Synthesized Protein and Nascent Albumin between Soluble and Membrane Fractions Obtained from Various pH-treated RER

	Radioactivity			
	Total		Albumin	
	Supernatant	Pellet	Supernatant	Pellet
	cpm ± SD	cpm ± SD	cpm ± SD	cpm ± SD
Hepes, pH 7.5	17,219 ± 1,725 (15%)	97,364 ± 9,779 (85%)	1,978 ± 418 (11%)	15,333 ± 332 (86%)
Capso, pH 9.1	37,881 ± 2,839 (31%)	83,160 ± 8,861 (69%)	6,820 ± 1,643 (31%)	15,080 ± 1,279 (69%)
Carbonate, pH 11.2	86,550 ± 6,638 (95%)	4,825 ± 2,786 (5%)	13,053 ± 1,329 (95%)	600 ± 461 (4%)

Radioactive microsomes were prepared as described in Materials and Methods. Aliquots containing 2.4 mg membrane protein were treated for 30 min at 20°C. Values of total proteins were obtained by TCA precipitation, values for albumin obtained by immunoprecipitation.
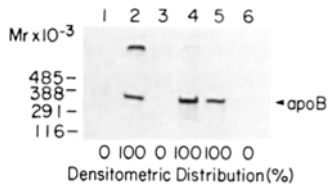
Figure 5. pH-dependent release of apoB from RER. The effect of pH on the release of apoB from isolated RER fractions was determined. Conditions were essentially as described in the legend of Fig. 4 for apoAI except that the analysis of radioactive apoB was by a 3–15% gradient SDS polyacrylamide gel. The position of apoB is indicated by arrowhead. Relative densitometric distribution of apoB band at bottom. (Lanes *1* and *2*) pH 7.5; (lanes *3* and *4*) pH 9.1; and (lanes *5* and *6*) pH 11.2. Lanes 1, 3, and 5 represent supernatants and lanes 2, 4, and 6 represent pellets.

under certain defined conditions only degrades surface-exposed proteins (Walter and Blobel, 1983). To determine whether the nascent apoproteins are exposed to the cytoplasmic surface, the isolated RER and Golgi cell fractions, labeled in vivo for various times, were incubated with exogenous protease and the percent of nascent proteins protected from degradation was measured. The results are shown in Fig. 6 (*A–C*). At all time points the majority of nascent albumin was protected from degradation in both RER and Golgi cell fractions (Fig. 6 *A*, lanes *1, 2, 4,* and *5*). When RER or Golgi vesicles were incubated with both Triton X-100 and

proteinase K, the albumin radioactivity completely disappeared (Fig. 6 *A*, lanes *3* and *6*). The complete protection of nascent albumin in intact vesicles from exogenous protease digestion shows the usefulness of the technique in determining whether nascent proteins are located within isolated membrane vesicles derived from the RER and Golgi.

The same protease-treated organelles were then used to determine the location of nascent apoAI and apoB. At the earliest time of in vivo labeling (5 min) 45% of nascent apoAI present in RER was protected from protease digestion. At 10 and 20 min of labeling there was a gradual increase, up to 65%, of nascent apoAI protection from protease digestion (Fig. 6 *B*, lanes *1* and *2*). This indicates that at these time intervals ∼35–55% of nascent apoAI is exposed to the cytoplasmic side of the RER vesicles and suggests that with increasing time there is transfer of apoAI from the cytoplasmic surface.

ApoB presented a more complex distribution within RER vesicles. After a short pulse (5 and 10 min with radioactive leucine), two major bands and many minor bands were obtained by immunoprecipitation (Fig. 6 *C*, lane *1*). Only 33–40% of the major radioactive band was protected from protease digestion (Fig. 6 *C*, lane *2*). At 20 min, a period which is sufficient for apoB synthesis to be completed, only 25% of the main radioactive product immunoprecipitated
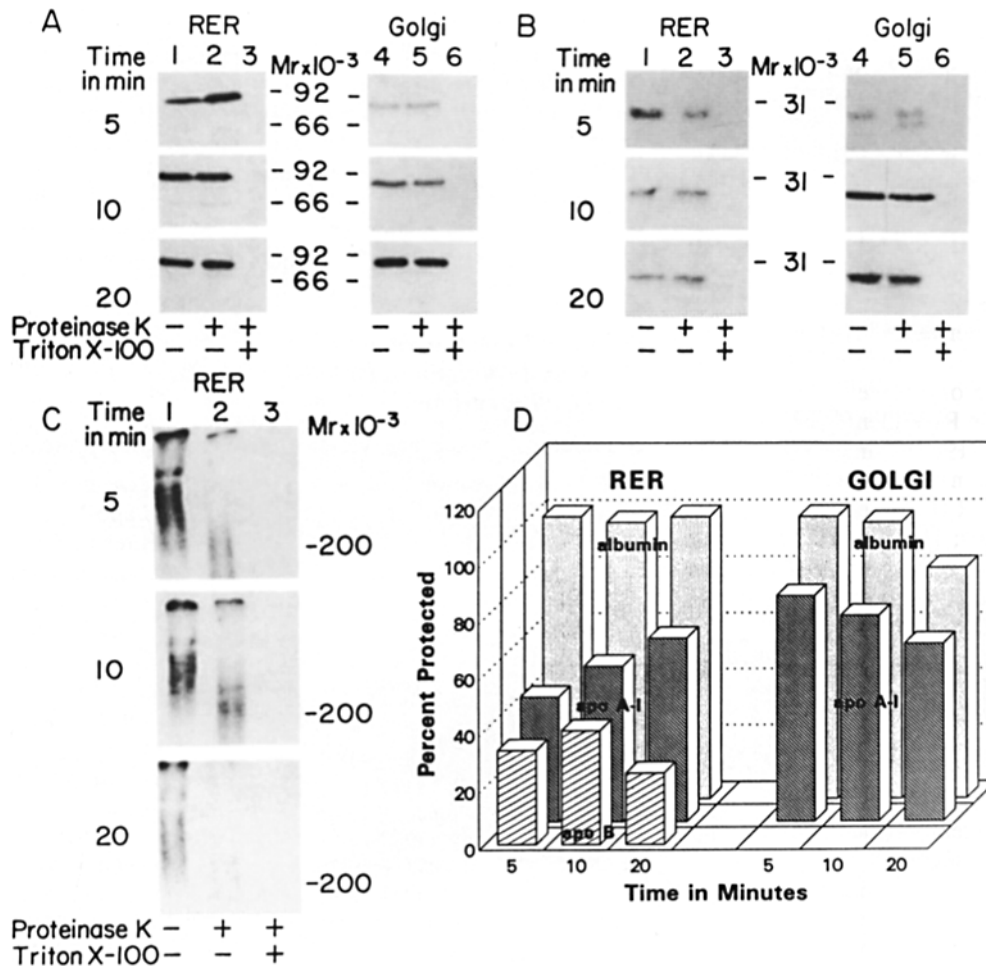
Figure 6. Effect of exogenous protease treatment on nascent albumin, apoAI, and apoB present in RER and Golgi cell fractions. Chickens were injected intravenously with L-[³H]leucine (2.5 mCi/100 g body weight). At 5, 10, and 20 min, livers were removed and radioactive rough microsomes and Golgi cell fractions were prepared (Banerjee and Redman, 1984). Aliquots of cell fractions were incubated with proteinase K and membranes were separated from the soluble fraction by centrifugation. The pellets were solubilized with detergent and radioactive albumin (*A*), apoAI (*B*), and apoB (*C*) present in solubilized fractions were recovered by immunoprecipitation and analyzed by SDS-PAGE and fluorography. For details see Materials and Methods. (Lanes *1* and *4*) Control fractions incubated in buffer alone; (lanes *2* and *5*) cell fractions incubated with proteinase K (50 μg/ml); (lanes *3* and *6*) cell fractions incubated with proteinase K (50 μg/ml) and Triton X-100 (1%). (Lanes *1–3*) RER; (lanes *4–6*) Golgi cell fraction. (*D*) Densitometric quantitation of the results given in *A–C*.

with anti-apoB remained inaccessible to the exogenous protease. This indicates that the majority (75%) is still exposed to the cytoplasmic surface. Since it has been reported that the synthesis of a full apoB molecule (~350 kD) requires ~14 min (Bamberger and Lane, 1988), it is possible that the smaller radioactive polypeptides present in the untreated sample at 5 and 10 min of labeling (Fig. 6 C, lane 1) are incomplete polypeptides still attached to the polysomes and accessible to exogenous protease (Fig. 6 C, lane 2).

In Golgi cell fractions, nascent apoAI was mostly resistant to exogenous protease digestion. The percent of protected protein decreased from 80 to 63% as the time increased (Fig. 6 B, lanes 4 and 5). It should be noted, however, that in these vesicles there was also a slight decrease in the amount of nascent albumin protected from exogenous protease (Fig. 6 D). As a control when Golgi vesicles were incubated in the presence of Triton X-100, apoAI was completely degraded (Fig. 6 B, lane 6).

It was difficult to get sufficient radioactive apoB from isolated Golgi fractions to perform protease protection experiments. The reason for this is not clear. Since a significant amount of nascent apoB degrades in the RER (Davis et al., 1990; Sato et al., 1990), it is possible that very little apoB accumulates in the Golgi. Borén et al. (1990) also failed to find any apoB in the Golgi cell fractions of Hep G2 cells. A summary of the protection of apoAI, apoB, and albumin on incubation of RER and Golgi cell fractions with proteinase K is presented in Fig. 6 D.

Discussion

Important issues in the study of lipoprotein biosynthesis are to determine the intracellular location where apoproteins and lipids combine to form lipoprotein particles and the mechanisms by which this is accomplished. Our earlier in vivo studies showed that the signal sequence is cleaved from apoAI polypeptide and pro-apoAI is the only form of apoAI present in the RER cell fractions. At this early step of secretion it contains very little lipid. Most of the lipids complex to nascent apoAI at a later stage of the secretory process, when apoAI enters the Golgi (Banerjee and Redman, 1983, 1984, 1985).

It is thought that most secretory proteins must pass through an aqueous channel of the RER membrane to enter the lumen (Gilmore and Blobel, 1985). Since both apoAI and apoB are lipid binding proteins and appear not to be complexed to nascent lipids in RER (Banerjee and Redman, 1983, 1984), the question remains: How do they maintain their solubility during translocation? An answer to this question is provided by the results presented in this study which demonstrate that most apoAI and apoB present in the RER are associated with the membrane. This finding is an important step in understanding how lipoproteins, VLDL and HDL, are formed. Our results are in agreement with recent findings that apoB is associated with the ER membrane in Hep G2 cells (Boström et al., 1986, 1988; Borén et al., 1990) and in rat (Davis et al., 1990) and chicken hepatocytes (Bamberger and Lane, 1988). Taken together these results suggest that in contrast to soluble secretory proteins which directly enter the lumen of the RER, or to membrane proteins which become fully integrated into the membrane; apolipoproteins have a modified translocation process. Per-

haps apolipoproteins interact transiently with RER membrane lipids before entering the lumen of secretory vesicles. Later they assemble with a different set of lipids, which are destined to be secreted.

The release of nascent apoAI and apoB from RER fractions by high pH is of interest since others had indicated that apoB is integrated in the ER membranes in Hep G2 cells and in rat hepatocytes (Boström et al., 1986, 1988; Borén et al., 1990; Davis et al., 1990). Our studies indicate that apoAI and apoB are membrane associated. However, they are not fully integrated in the membrane since they are released by treatment at pH 11.2. Conventional integral membrane proteins are retained in the bilayer when vesicles are treated at pH 11.2 (Fujiki et al., 1982).

ApoB release by high pH washing contrasts with results obtained from Hep G2 cells (Boström et al., 1986) and rat primary hepatocytes in culture (Davis et al., 1990). These discrepancies may reflect different amounts of nascent apoB present in hepatocytes in culture as compared to that present in intact chicken livers. Alternatively, the different results may represent procedural differences as indicated by recent in vitro studies in which apoB-15 was completely extractable from the membrane (Chuck et al., 1990) while apoB-17 was only partially released (Pease et al., 1991). Chuck et al. (1990) studied the in vitro translation of an NH_2-terminal portion of apoB-100 (termed apoB-15) which contains the signal sequences and several hydrophobic domains and represents ~15% of the total polypeptide. Their studies demonstrated that apoB-15 spans the lipid bilayer without being permanently integrated in it. At early stages of translation apo B-15 was in the microsomal membrane and was isolated as a short protease–protected transmembrane polypeptide. With increasing translation the short protease–protected domain became progressively larger and fully protected chains accumulated (Chuck et al., 1990). Using apoB-17 and a similar in vitro system, Pease et al. (1991) failed to confirm the above finding, and based on their alkaline carbonate and saponin disruption results suggested that apoB-17 is cotranslationally inserted into the inner leaflet of the ER.

Both the high pH washing experiments and immunocytochemistry indicated that nascent apoproteins are located on the membranes of the RER. However, these techniques did not allow us to determine whether or not nascent apoAI and apoB are exposed to the outer or luminal surfaces. Because of this protease digestion experiments were undertaken. In the protease digestion studies, at early times (between 5, 10, and 20 min), portions of radioactive apoAI and apoB, present on the RER, are accessible to exogenous protease. Only 44–65% of nascent apoAI and 25–40% of nascent apoB are protected. In contrast, in the same RER vesicles, nascent albumin is nearly all protected. This is a strong indication that some apoAI and apoB are exposed to the outer surface. Kinetic studies, from 5 to 20 min, show a gradual increase in the amount of apoAI that is protected, suggesting that, with time, nascent apoAI moves from the outer surface to a more protected environment. This shift was not discerned with apoB (Fig. 6 D).

In the Golgi cell fraction a different situation occurs. At the earliest time points (5–10 min) 73–80% of nascent apoAI is protected, while 98–100% of nascent albumin is protected. Unexpectedly, at 20 min of pulse labeling, only 63% of nascent apoAI was protected. However, it is noteworthy

that at this time point only 82% of our control, nascent albumin, was protected, suggesting that the Golgi preparations in this experiment were permeable to the protease. Or perhaps less likely, that just before exocytosis, all secretory proteins are somewhat accessible to exogenous protease.

The results of our protease protection studies with apoAI contrast with the results of in vitro experiments (Stoffel et al., 1981). We find that a portion of nascent apoAI in chicken liver RER vesicles is digested, indicating that it is exposed to the cytoplasmic surface of the ER membrane. Stoffel et al. (1981) reported that apoAI, produced by translating apoAI mRNA in vitro in the presence of dog pancreatic microsomes, was completely protected. The reason for this difference is not clear. One possibility is that dog pancreatic microsomes behave differently in translocating nascent apoAI compared to chicken liver microsomes. For example, there is evidence that dog pancreatic microsomes, but not rat liver microsomes (Paver et al., 1989), release a resident luminal protein, protein disulfide isomerase, upon mild alkaline treatment. Another possibility is that the in vitro system lacks factors present in liver, which are necessary for holding apolipoproteins in the RER membrane.

The presence of some nascent apoAI and apoB on the cytoplasmic surface of the RER vesicles may be attributed to a redistribution of radioactive apoproteins released from membrane compartments during cell fractionation. This is unlikely since there is unequal distribution of radioactive apoAI and apoB on the surface of RER and Golgi vesicles. Also, the kinetic studies which indicate greater protection of apoAI with increasing in vivo labeling time do not favor nonspecific binding to the membrane surface.

Recently Davis et al. (1990) showed that in rat hepatocytes in culture only a fraction of de novo synthesized apoB is secreted and essentially all apoB that is associated with the cytoplasmic side of the ER membrane is degraded. The fates of nascent apoAI and apoB which are associated with the cytoplasmic side of the chicken liver RER vesicles are not known. It is possible that they are also degraded and never enter the secretory path. However, this will be difficult to verify by in vivo studies. The increased protection of apoAI to proteinase K treatment with increased in vivo labeling time (5-20 min) suggests that apoAI enters the RER membrane.

The observations that both apoAI and apoB are associated with the ER membrane seems to support an earlier suggestion that the assembly of lipoproteins starts within the bilayer of the ER membrane (Olofsson et al., 1987; Norum et al., 1983). This hypothesis assumes that neutral lipids present in the lipid core of the particle are deposited between the lipid leaflets of the ER and that apoproteins interact with these deposits within the membrane (Scow and Blanchett-Mackie, 1985; Hashimoto and Fogelman, 1980). Our earlier studies suggested that lipid particles rich in triglycerides are formed very early in the secretory process in the RER, but at that stage these lipid particles do not contain much apoAI (Banerjee and Redman, 1984).

The intracellular events that lead to the formation of core triglycerides and cholesterol ester surrounded by a layer of apoproteins, phospholipids, and cholesterol are still not understood. Yet, it appears that before lipoprotein formation, nascent apolipoproteins are associated with the lipid bilayer of the RER cell fraction and at the earliest stage a portion

of these apolipoproteins are exposed to the cytoplasmic surface. How nascent apolipoproteins are translocated to the luminal surface, and whether this process is involved in lipoprotein formation, remains to be determined.

References

Altman, L. G., B. G. Schneider, and D. S. Papermaster. 1984. Rapid embedding of tissues in Lowicryl K4M for immunoelectron microscopy. *J. Histochem. Cytochem.* 32:1217-1223.

Aris, J. P., and G. Blobel. 1988. Identification and characterization of a yeast nucleolar protein that is similar to a rat liver nucleolar protein. *J. Cell Biol.* 107:17-31.

Bamberger, M. J., and M. D. Lane. 1988. Assembly of very low density lipoprotein in the hepatocyte. *J. Biol. Chem.* 263:11868-11878.

Banerjee, D., and C. M. Redman. 1983. Biosynthesis of high density lipoprotein by chicken liver: nature of nascent intracellular high density lipoprotein. *J. Cell Biol.* 96:651-660.

Banerjee, D., and C. M. Redman. 1984. Biosynthesis of high density lipoprotein by chicken liver: conjugation of nascent lipids with apoprotein AI. *J. Cell Biol.* 99:1917-1926.

Banerjee, D., T. K. Mukherjee, and C. M. Redman. 1985. Biosynthesis of high density lipoprotein by chicken liver:intracellular transport and proteolytic processing of nascent apolipoprotein AI. *J. Cell Biol.* 101:1219-1226.

Blue, M. L., P. Ostapchuk, J. S. Gordon, and D. L. Williams. 1982. Synthesis of apolipoprotein AI by peripheral tissues of the rooster. *J. Biol. Chem.* 257:11151-11159.

Borchardt, R. A., and R. A. Davis. 1987. Intrahepatic assembly of very low density lipoproteins. *J. Biol. Chem.* 262:16394-16402.

Borén, J., M. Wettesten, A. Sjöberg, T. Thorlin, G. Bondjers, O. Wiklund, and S.-O. Olofsson. 1990. The assembly and secretion of apo B-100 containing lipoproteins in Hep G2 cells. *J. Biol. Chem.* 265:10556-10564.

Boström, K., M. Wettesten, J. Borén, G. Bondjers, O. Wiklund, and S.-O. Olofsson. 1986. Pulse-chase studies of the synthesis and intracellular transport of apolipoprotein B-100 in Hep G2 cells. *J. Biol. Chem.* 261:13800-13806.

Boström, K., J. Borén, M. Wettesten, A. Sjöberg, G. Bondjers, O. Wiklund, P. Carlsson, and S.-O. Olofsson. 1988. Studies on the assembly of apo B-100-containing lipoproteins in Hep G2 cells. *J. Biol. Chem.* 263:4434-4442.

Chapman, M. J. 1980. Animal lipoproteins: chemistry, structure and comparative aspects. *J. Lipid Res.* 21:789-853.

Chuck, S. L., Z. Yao, D. Blackhart, B. J. McCarthy, and V. R. Lingappa. 1990. New variation on the translocation of proteins during early biogenesis of apolipoprotein B. *Nature (Lond.).* 346:382-385.

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.* 265:10005-10011.

Dixon, J. L., R. Battini, S. Ferrari, C. M. Redman, and D. Banerjee. 1989. Expression and secretion of chicken apolipoprotein AI in transfected COS cells. *Biochim. Biophys. Acta.* 1009:47-53.

Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* 93:97-102.

Gilmore, R., and G. Blobel. 1985. Translocation of secretory proteins across the microsomal membrane occurs through an environment accessible to aqueous perturbants. *Cell.* 42:497-505.

Gotto, A. M. Jr., H. J. Pownall, and R. J. Havel. 1986. Introduction to the plasma lipoproteins. *Methods Enzymol.* 128:3-41.

Green, P. H., and R. M. Glickman. 1981. Intestinal lipoprotein metabolism. *J. Lipid Res.* 22:1153-1173.

Hamilton, R. L. 1983. Hepatic secretion of nascent plasma lipoproteins. *In* Plasma protein secretion by the liver. H. Glauman, T. Peters Jr., and C. Redman, editors. Academic Press, Ltd., London. 357-374.

Hashimoto, S., and A. M. Fogelman. 1980. Smooth microsomes: a trap for cholesteryl ester formed in hepatic microsomes. *J. Biol. Chem.* 255:8678-8684.

Higgins, J. A., and J. K. Fieldsend. 1987. Phosphatidyl-choline synthesis for incorporation into membranes or for secretion as plasma lipoproteins by Golgi membranes of rat liver. *J. Lipid Res.* 28:268-278.

Howell, K., and G. Palade. 1982. Heterogeneity of lipoprotein particles in hepatic Golgi fractions. *J. Cell Biol.* 92:833-845.

Janero, D. R., and M. D. Lane. 1983. Sequential assembly of very low density lipoproteins, apolipoproteins, triacylglycerol and phosphoglycerides by the

intact liver cell. *J. Biol. Chem.* 258:14496-14504.

Norum, K. R., T. Berg, P. Helgerud, and C. A. Drevon. 1983. Transport of cholesterol. *Physiol. Rev.* 63:1343-1419.

Olofsson, S.-O., G. Bjursell, K. Boström, P. Carlsson, J. Elovson, A. A. Potter, M. A. Reuben, and G. Bondjers. 1987. Apolipoprotein B: structure, biosynthesis and role in the lipoprotein assembly process. *Atherosclerosis.* 68:1-17.

Paver, J. L., H. C. Hawkins, and R. B. Freedman. 1989. Preparation and characterization of dog pancreas microsomal membranes specifically depleted of protein disulphid-isomerase. *Biochem. J.* 257:657-663.

Pease, R. J., G. B. Harrison, and J. Scott. 1991. Cotranslocational insertion of apolipoprotein B into the inner leaflet of the endoplasmic reticulum. *Nature (Lond.).* 353:448-450.

Sato, R., T. Imanaka, A. Takatsuki, and T. Takano. 1990. Degradation of newly synthesized apolipoprotein B-100 in a pre-Golgi compartment. *J. Biol. Chem.* 265:11880-11884.

Scow, R. O., and E. J. Blanchett-Mackie. 1985. Why fatty acids flow in cell membranes. *Prog. Lipid Res.* 24:197-241.

Shackelford, J. E., and H. G. Lebherz. 1983. Synthesis and secretion of apolipoprotein AI by chick breast muscle. *J. Biol. Chem.* 258:7175-7180.

Siuta-Mangano, P., S. C. Howard, W. J. Lennarz, and M. D. Lane. 1982. Synthesis, processing and secretion of apolipoprotein B by the chick liver cell. *J. Biol. Chem.* 257:4292-4300.

Stoffel, W., G. Blobel, and P. Walter. 1981. Synthesis *in vitro* and translocation of apolipoprotein AI across microsomal membranes. *Eur. J. Biochem.* 120: 519-522.

Towbin, T. C., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.

Vance, J. E., and D. E. Vance. 1988. Does rat liver Golgi have the capacity to synthesize phospholipids for lipoprotein secretion? *J. Biol. Chem.* 263: 5898-5909.

Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* 96:84-93.

Wong, L., and R. M. Pino. 1987. Biogenesis of very low density lipoproteins in rat liver. *Eur. J. Biochem.* 164:357-367.