Isolation and properties of nascent lipoproteins from highly purified rat hepatocytic Golgi fractions

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Abstract Two procedures were used to isolate hepatocytic Golgi fractions from rat liver. One procedure yields a light Golgi fraction (GF_{1+2}) and the other "intact" stacks of cisternae. Triglyceride fatty acids in nascent very low density lipoproteins (VLDL) were labeled by injection of [^{3}H]palmitate intravenously, and radiolabeled lipoproteins were injected as markers of potentially contaminating endosomes. GF_{1+2} fractions were en riched manyfold in the endosomal markers, indicative of substantial endosomal contamination, whereas intact Golgi fractions from the same livers were about 7% as contaminated. By electron microscopy, GF_{1+2} fractions contained mainly multivesicular bodies (MVBs), together with some Golgi-derived secretory vesicles. The small endosomal contamination of intact Golgi fractions was further reduced by a simple modification of the procedure, which removed most entrained endosomes. The surface constituents of Golgi VLDL (d < 1.010 g/ml) released from these highly purified intact Golgi fractions differed from those of plasma VLDL. Golgi VLDL contained fivefold less unesterified cholesterol than plasma VLDL, but twofold more phospholipids. Golgi VLDL and plasma VLDL contained similar amounts of cholesteryl esters and triglycerides. The protein content of Golgi VLDL was substantially lower than that of plasma VLDL. ApoB-100 and apoB-48 were similarly represented, but nascent VLDL contained less of the C apolipoproteins. ApoA-I was present mainly as the proprotein in Golgi VLDL, but was virtually lacking in plasma VLDL. ApoE comprised about 22% of the protein mass of Golgi VLDL as well as plasma VLDL; the distribution of apoE isoforms was also similar. Apolipoproteins E and pro A-I released from ruptured Golgi cisternae were largely bound to the Golgi VLDL or were associated with Golgi membranes. Particles resembling low density lipoproteins (LDL) and high density lipoproteins (HDL) were not seen by electron microscopy in contents of intact Golgi fractions. These observations indicate that nascent Golgi VLDL are the primary particulate precursors of rat plasma lipoproteins of hepatocytic origin, and suggest that particles with the density of plasma HDL and LDL do not exist within the secretory pathway of normal hepatocytes. Thus, the results of this research on the properties of nascent plasma lipoprotein precursors contained within uncontaminated hepatocytic Golgi frac ions differ substantially from previous published work. —**Hamilton, R. L., A. Moorehouse, and R. J. Havel.** Isolation and properties of nascent lipoproteins from highly purified rat hepatocytic Golgi fractions. *J. Lipid Res.* 1991. **32:** 529–543.

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The Golgi apparatus of rat hepatocytes contains lipid-rich particles of defined size (400–600 Å in diameter), which have been identified as precursors of plasma very low density lipoproteins (VLDL) (1). The size and composition of Golgi VLDL resemble those of VLDL that accumulate in perfusates of rat liver (2) and VLDL in blood plasma (3, 4). Golgi VLDL appear to originate in special cisternae of the endoplasmic reticulum and are transported to the Golgi apparatus where they accumulate in clusters in dilated ends of *trans* Golgi cisternae (1, 5–8). These dilated compartments have been considered to be forming secretory vesicles that pinch off from the cisternae and carry the nascent Golgi VLDL and other secretory proteins to the basolateral surface of the cell, where they discharge their contents into the space of Disse by exocytosis (1, 5–9).

Lipoprotein-filled vesicles found in the apical (bile canalicular) pole of hepatocytes were for many years thought to contain nascent VLDL exclusively. In radioautographic studies, however, it has been shown that plasma lipoproteins, labeled with radioiodine, are taken into hepatocytes by endocytosis and accumulate within non-Golgi organelles adjacent to Golgi membranes (10–12). These organelles, identified as multivesicular bodies (MVBs) by their internal bilayer vesicles (13, 14), represent late endosomes that acquire some of their lysosomal enzymes from the Golgi apparatus (14, 15). When rats are treated with ethinyl estradiol so as to induce hepatocytic low density lipoprotein (LDL) receptors manyfold and then injected intravenously with large amounts of LDL, MVBs become larger and more numerous within 20 min. This facilitates the isolation of MVBs in a highly purified state and the examination of the properties of their contained lipoproteins (13). Thus, by labeling newly synthesized triglycerides in the livers of these rats with [^{3}H]palmitate and endocytosed LDL with

Abbreviations: MVBs, multivesicular bodies; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; GF_{1+2}, Golgi light and intermediate combined fractions.

^{125}I, it could be shown that the MVB fraction contained little contaminating VLDL present in Golgi fractions isolated from the same livers. By contrast, the Golgi fractions were appreciably contaminated with endosomal remnant lipoproteins (13). This observation suggested that available data on the properties of nascent lipoproteins recovered from hepatocytic Golgi fractions require reevaluation.

In the current research, we determined the extent to which hepatocytic Golgi fractions prepared by the two most commonly used procedures are contaminated by endosomes containing catabolized lipoproteins. Our goal was to obtain Golgi fractions virtually free of endosomes in order to reexamine the properties of nascent lipoproteins as they exist within the terminal secretory compartment.

METHODS

Animals

Male Sprague-Dawley rats (250–350 g), were fed Purina standard chow ad libitum. In a few experiments, rats were injected subcutaneously with 17α-ethinyl estradiol (5 mg/kg) dissolved in propylene glycol (1 mg/ml) for 5 days to increase hepatocytic LDL receptors (16). In some experiments rats were given 0.6 g ethanol/100 g body weight in a 50% aqueous solution by stomach tube 90 min before livers were excised, according to the described procedure for isolating Golgi Gf_{1+2} fractions (8).

Radiolabeled markers

Human LDL (ϱ 1.025–1.055 g/ml) were isolated from blood serum by sequential centrifugation (17) and labeled with ^{125}I to a specific activity of ~85 μCi/mg protein by a modification (18) of the method of McFarlane (19). Palmitic acid [9,10(n)^{3}H] (28.5 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Four μmol unlabeled palmitic acid and 1.5 mCi labeled palmitic acid were complexed with 8 μmol bovine serum albumin in 0.15 M NaCl (20). Small chylomicrons were collected from the main intestinal lymph duct of rats, as previously described (21). To label chylomicrons, 6.67% glucose in 0.15 M NaCl containing 1.67 μCi [^{3}H]cholesterol (58 Ci/mmol, New England Nuclear, Boston, MA) per ml was infused into the duodenal cannula at a rate of 2.4 ml/h. Lymph chylomicrons were isolated by flotation for 2 h at 38,000 rpm through Krebs-Henseleit buffer in a 50.2-Ti rotor of a Beckman preparative ultracentrifuge (Beckman Instruments, Palo Alto, CA), followed by recentrifugation under the same conditions. The injected chylomicrons contained 5 to 9.6 $\times$ 10^6 dpm [^{3}H]cholesterol, 30–100 mg triglyceride, and 1.5–3.5 mg protein, and the injected palmitic acid contained 15 to 25 $\times$ 10^6 dpm ^{14}C. In separate experiments, chylomicrons were labeled with ^{125}I as previously described (18).

Isolation of Golgi fractions

Rats to be injected with radioisotopic markers for Golgi and endosomal compartments were anesthetized with diethyl ether, and the right and left femoral veins were exposed surgically. Radiolabeled small chylomicrons were first injected into one femoral vein and, 5 min later, labeled palmitic acid was injected into the opposite femoral vein. The skin wounds were clamped, and the rats were allowed to recover. Ten min later, they were anesthetized again with ether, the abdomen was opened, and 50 ml of ice-cold 0.9% sodium chloride in 0.1% EDTA was injected into the portal vein to chill and remove blood from the liver. The liver was then excised, rinsed in ice water, and quickly minced with scalpels.

Isolation of structurally "intact" Golgi compartments was based on the method of Morré et al. (22), with some modifications (4, 13). Portions of finely minced liver were mixed with ice-cold homogenizing medium (1.8–2.0 ml/g). The medium contained 0.1 M phosphate, pH 7.3 (Na_2HPO_4 + KH_2PO_4), 0.25 M sucrose, 1.0% dextran (M_r ~500,000, Sigma Chemical Co., St. Louis, MO) and 0.01 M $MgCl_2$. Homogenization was with a Polytron PT-10 (Brinkman Instruments, New York, NY) for 30 sec, with the rheostat setting at position 0.5. The homogenate was first centrifuged at 4°C in a Beckman SW-41 (13-ml tube) or SW-27 (17-ml tube) rotor at 2,000 rpm for 5 min, followed by 10,000 rpm for 30 min. Floating fat was aspirated and the supernatant fluid was decanted. The upper one-half to two-thirds of the pellet from each centrifuge tube was resuspended in either 2 or 3 ml of homogenizing medium with the tip of a 5-ml pipette, rapidly decanted, then layered (4 ml/tube) onto 9 ml of ice-cold 1.2 M sucrose and centrifuged at 4°C in an SW-41 rotor for 30 min at 25,000 rpm. The two closely spaced white bands at the interface were recovered by rapid aspiration in a small volume (~0.5 ml) with a Pasteur pipette. The pooled Golgi fractions were then diluted 5- to 20-fold with cold homogenizing medium, or in some experiments with cold distilled water, and the intact Golgi membranes were pelleted away from contaminating microsomes, lysosomes, and MVBs by sedimentation at 5,000 rpm for 20 min in an SW-41 or SW-27 rotor. In experiments in which the content nascent VLDL were analyzed, the intact Golgi fractions were diluted 25- to 30-fold with homogenizing medium to reduce endosomal contamination further (23).

Golgi fractions GF_{1+2} were isolated as described (8, 24). A portion of the finely minced liver used to isolate the intact Golgi fraction described above was homogenized in 0.25 M sucrose with 6–8 strokes of a motor-driven Teflon pestle (No. B or C, A. H. Thomas Co., Philadelphia, PA)

at 2,800 rpm in a glass homogenizer to give a 20% (wet weight of liver/vol) homogenate. We initially tried pressing the finely minced tissue through a garlic press with 1-mm pores to mimic the tissue press step described (8), but this did not alter the results and was therefore omitted in most cases. All manipulations were carried out on ice, and all centrifugal steps at 2–4°C. The homogenate was first centrifuged in a Beckman 60 Ti rotor for 10 min at 10,000 g_{av}. The supernatant was diluted to the original volume with 0.25 M sucrose and centrifuged in the same rotor for 90 min at 105,000 g_{av} to obtain microsomal pellets. These pellets were resuspended in 0.25 M sucrose by 3–5 strokes in the same homogenizer at the same initial speed. The suspension was mixed with 2.0 M sucrose to give a refractive index of 1.3920, as measured with a refractometer (Bausch and Lomb, Rochester, NY). Ten-ml portions of this microsomal suspension were layered under a discontinuous sucrose gradient with steps of 1.5 M, 0.86 M, and 0.25 M. This gradient was centrifuged in a SW-27 rotor for 120 min at 25,000 rpm. The white band, concentrated at the 0.86 M/0.25 M sucrose interface, was collected by aspiration with a blunted needle. This GF_{1+2} fraction was diluted to a refractive index of 1.3450 with distilled water and pelleted by centrifugation for 60 min at 105,000 g_{av} in a 60 Ti rotor (8, 24).

Isolation of VLDL from intact Golgi fractions

All steps were carried out with ice-cold materials or at 4°C. Intact Golgi pellets were resuspended in 4.0 ml of distilled water (pH 6.0–6.5) by repeated aspiration into a Pasteur pipette. This resuspended fraction was rapidly passed through an iced French pressure cell two times at 16,000 psi to rupture the membranes. D_2O (0.4 ml) was added to raise the density to ~1.010 g/ml in order to layer the sample below 2 ml of 0.15 M sterile sodium chloride (Travenol, Deerfield, IL). Golgi VLDL were floated through the salt solution by ultracentrifugation for 16 h at 35,000 rpm in a 40.3 Beckman rotor. Space was left between the top of the saline and the centrifuge cap so that the VLDL could be collected by aspiration with a Pasteur pipette. The clear solution occupying most of the remaining tube contents was removed, and the sedimented proteins seen beside the ruptured membrane pellet were aspirated in a volume of 0.5 ml.

Electron microscopy

Pellets of intact Golgi and Golgi GF_{1+2} fractions were punctured several times with the tips of Pasteur pipettes in order to sample the membrane fractions randomly but thoroughly. About 1–2 mm cores were obtained in the pipette tip, which were delivered below the surface of a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4. The pellets were transferred to 3% osmium tetroxide in veronal acetate buffer, where they were stored for 24–48 h at 4°C. They were then stained en bloc with aqueous uranyl acetate for 1–2 h at 37°C, dehydrated in acetone, and embedded in Epon. Thin sections were doubly stained in uranyl acetate and lead citrate and photographed in a Siemens 101 electron microscope at 80 KV with a 60-μm objective aperture (Siemens Corp, Iselin, NJ).

Analyses

Protein was measured by the method of Lowry et al. (25) in lipoproteins and by the method of Peterson in membrane fractions (26). Lipids were extracted from lipoproteins as described by Folch, Lees, and Sloane Stanley (27). Phospholipids in the extract were estimated from content of lipid P (28). Cholesterol and cholesteryl esters were measured by a manual method (29) in extracts or directly in lipoprotein fractions by an automated enzymatic technique based upon the method of Allain et al. (30). Triglycerides were estimated in extracts from content of glyceride-glycerol (31) or directly in lipoprotein fractions by an automated enzymatic technique based upon the method of Bucolo and David (32). The electrophoretic mobility of lipoprotein fractions was evaluated by the method of Noble (33). Proteins in contents of Golgi fractions were separated by $NaDodSO_4$/PAGE in a 3–15% polyacrylamide gradient (34) or by isoelectric focusing gel electrophoresis (35) and stained with Coomassie Blue R250. Two-dimensional gel electrophoresis of apolipoproteins was carried out as described (36); for the first dimension, the pH gradient was 3.5–7.0; $NaDodSO_4$/PAGE for the second dimension was in 3–25% polyacrylamide. Gels were stained with Coomassie Blue R250. Proteins were transferred to nitrocellulose (37) and immunoblotting was carried out as described (38) with monospecific polyclonal antiserum against rat apoA-I prepared in a rabbit (39). Bound antibodies were visualized with ^{125}I-labeled protein A. Apolipoprotein E and apoA-I were quantified by radioimmunoassay in solutions containing sodium decyl sulfate as described (39, 40).

^{125}I was measured by gamma scintillation spectrometry. Radioactive lipids ([^{14}C]palmitic acid, largely in triglycerides and phospholipids, and [^{3}H]cholesterol and cholesteryl esters) were extracted from whole homogenates and subcellular fractions by the method of Bligh and Dyer (41). For thin-layer chromatography, a portion of the chloroform phase was then mixed with 10 μl carrier lipids (triolein, oleic acid, cholesterol, and cholesteryl laurate, each 5 mg/ml) and applied to a precoated glass plate of silica gel 60, 0–25 mm, 20 × 20 cm (EM Science, Gibbstown, NJ). The plates were developed with 200 ml of hexane–ether–acetic acid 83:16:1 for 30 min, air dried, and visualized with I_2. Regions corresponding to the carrier lipids were transferred to a funnel containing filter paper and eluted with 3 ml of chloroform–methanol 1:2 into a counting vial. The sample was dried under N_2, and

^{14}C and ^{3}H were assayed by liquid scintillation spectrometry.

RESULTS

Golgi GF_{1+2} fractions are enriched in endocytosed plasma lipoproteins

We found that intact Golgi fractions from ethinyl estradiol-treated rats, injected with ^{125}I-labeled LDL 15 min before livers were perfused, contained considerable radioisotope, which reflected the presence of MVBs containing endocytosed LDL (13). We therefore sought another procedure for isolating Golgi apparatus in order to obtain Golgi VLDL. We chose the GF_{1+2} fraction obtained by the procedure described by Ehrenreich et al. (8) because this vesicular fraction appeared, by electron microscopy, to be enriched in VLDL particles. This procedure has been used by others for studies of nascent lipoproteins (24, 42–52).

Our first experiments were done with estradiol-treated rats injected with human ^{125}I-labeled LDL, because we had considerable experience with this model (10, 11, 13–16). These rats were also intubated with ethanol 90 min before livers were homogenized, according to the described procedure for isolating GF_{1+2} fractions (8, 24). Under these conditions, the GF_{1+2} fraction was highly enriched in endocytosed LDL, as shown in **Table 1**. The enrichment of ^{125}I over homogenate (50- to 75-fold) approached that obtained in MVB fractions (~100-fold), which contain highly purified MVBs by several criteria (13). Electron microscopic examination of these GF_{1+2} fractions showed that the predominant organelles had properties more characteristic of MVBs than of Golgi secretory vesicles: internal bilayer structures (some of which were vesicular), heterogeneity of content lipoproteins, and large membranous appendages.

Because livers of estradiol-treated rats may not be suitable for isolating GF_{1+2} fractions, we carried out similar experiments in which untreated rats were injected with ^{125}I-labeled chylomicrons. These rats were also given ethanol as described (8, 24). Fifteen min before their livers were flushed, the rats received the labeled chylomicrons intravenously. In these experiments, the GF_{1+2} fractions were similarly enriched in ^{125}I over homogenate (40- to 80-fold), suggesting that estradiol treatment did not cause the contamination with MVBs (Table 1).

Comparison of endosomal contamination of Golgi fractions prepared by two procedures

Chylomicrons labeled with ^{125}I may not be an ideal marker for MVBs, because some of the radioactivity is associated with exchangeable phospholipids and apolipoproteins (12). We therefore pursued these experiments with a nonexchangeable endogenous marker of chylomicrons. In addition, we labeled VLDL in Golgi fractions in vivo to estimate the relative amounts of endocytic and exocytic lipoproteins present in Golgi fractions prepared by two procedures: *1*) the procedure originally described by Ehrenreich and associates (8) and Howell and Palade (24), which yields Golgi fraction GF_{1+2}; and *2*) the procedure originally described by Morré et al. (22) and modified by Hornick and associates (13) which yields an "intact" Golgi fraction.

Small chylomicrons can be collected from the mesenteric lymph of rats infused duodenally with solutions lacking fat. These particles are synthesized in the enterocytes of the small intestinal mucosa from endogenous lipids derived primarily from bile. Such small chylomicrons, which contain the same lipids and apolipoproteins and are metabolized like larger ones produced during ab-

TABLE 1. Enrichment of endocytosed radioiodinated lipoproteins in GF_{1+2} fractions from livers of ethinyl estradiol-treated and untreated rats

Experiment	Homogenate	GF_{1+2}	Enrichment
	cpm/mg protein (× 10^{-3})		*-fold*
^{125}I-labeled LDL (estradiol-treated rats)			
1	29	1,490	52
2	8.2	529	65
3	5.6	393	70
4	1.7	1,252	74
5	11	570	52
^{125}I-labeled chylomicrons (untreated rats)			
1	11	911	83
2	20	816	41
3	109	6,331	58

Radioiodinated lipoproteins were injected into ether-anesthetized rats 15 min before livers were perfused with 0.1% EDTA in 0.15 M NaCl to wash out receptor-bound lipoproteins and homogenized, as described in Methods. Rats were intubated with ethanol 90 min before livers were taken (8).

sorption of dietary fat, have a distinct advantage for these studies because they contain much more cholesteryl esters relative to triglycerides than large chylomicrons (53). Most dietary cholesterol appears in chylomicrons as cholesteryl esters (54), a core lipid that does not undergo exchanges or transfers between other plasma lipoproteins in rats, which lack cholesteryl ester transfer protein (55). Small chylomicron remnants, produced during lipolysis of chylomicrons, undergo receptor-mediated endocytosis into hepatocytes and are concentrated in MVBs 15 min after intravenous injection into intact rats (12, 14). In the current experiments, most of the [^{3}H]cholesterol of small chylomicrons from intestinal lymph was in cholesteryl esters (range 72–82%). The labeled chylomicrons were injected intravenously 15 min before livers were perfused and taken for isolation of cell fractions.

In previous research, we found that intravenously injected radiolabeled palmitic acid was rapidly incorporated into hepatocytic Golgi VLDL (13). We therefore injected [^{14}C]palmitate as an albumin complex intravenously 10 min before livers were taken for isolation of cell fractions. At this time VLDL triglycerides in the Golgi apparatus are well labeled, but virtually none of the nascent VLDL has been secreted into the blood. Thus, no labeled Golgi VLDL should enter endocytic compartments in hepatocytes.

The results of experiments in which the two Golgi isolation procedures were compared in order to measure contamination with endosomes are summarized in **Table 2**. The ratio of newly synthesized triglycerides labeled with [^{14}C]palmitate (a marker of Golgi VLDL) to [^{3}H]cholesteryl esters (a marker of remnant lipoproteins within contaminating endosomes) was much higher in intact Golgi pellets than in pellets containing GF_{1+2} fractions. Only the former were enriched in ^{14}C-labeled triglycerides relative to [^{3}H]cholesteryl esters, as compared with liver homogenate. Pretreatment of rats with ethanol did not alter these results. In these experiments, flotation of the GF_{1+2} fractions in the sucrose step gradient was limited to 2 h. In other experiments, the GF_1 and GF_2 fractions were isolated separately by a 3-h centrifugation of the microsomal fractions obtained from rats fasted overnight and intubated with ethanol, as described for the original procedure (8). The endosomal contribution to the separated GF_1 and GF_2 fractions was even greater (Table 2). In the final step of isolation of GF_1 and GF_2 fractions, the sucrose-containing bands are diluted with water to a refractive index equivalent to that of 0.25 M sucrose before the organelles are pelleted by centrifugation (8). To determine the effect of the dilution medium used for the final pelleting of the intact Golgi fraction, we compared the recoveries of the labeled lipids after dilution with homogenizing medium, 0.9% sodium chloride, water, and 0.25 M sucrose. When water was used, there was a large loss of radioactive triglycerides into the supernatant, yielding a lower ^{14}C-labeled triglyceride to [^{3}H]cholesteryl ester ratio in the intact Golgi pellet (Table 2). Virtually the same results were obtained with 0.25 M sucrose (data not shown), whereas dilution with 0.15 M NaCl (data not shown) produced results comparable with those achieved with homogenizing medium (Table 2).

TABLE 2. Tracers labeling Golgi (^{14}C) and endocytic (^{3}H) compartments in two Golgi fractions isolated from homogenates of rat livers

Experimental Protocol[a]	Number of Experiments	^{14}C/^{3}H (Golgi Pellet) / ^{14}C/^{3}H (Homogenate)[b]
Fed rats		
Intact Golgi, no ethanol (homogenizing medium)[c]	6	14.40 ± 2.96
Intact Golgi, with ethanol[d] (homogenizing medium)	3	13.20 ± 5.20
Intact Golgi, no ethanol (water)	6	4.78 ± 3.63
GF_{1+2}, with ethanol[d]	4	0.97 ± 0.90
Fasted rats (fasted overnight)		
GF_1, with ethanol	4	0.42 ± 0.3
GF_2, with ethanol		0.10 ± 0.07

[a]Rats were injected intravenously with small chylomicrons containing ^{3}H-labeled cholesteryl esters to mark endosomes 15 min before livers were homogenized and with [^{14}C]palmitate 5 min later to mark Golgi VLDL triglycerides. Lipids were separated by thin-layer chromatography and assayed by liquid scintillation spectrometry.
[b]Ratio of cpm in ^{14}C-labeled triglycerides to [^{3}H]cholesteryl esters in final pellet of Golgi fraction divided by that ratio in liver homogenate.
[c]Medium used for final pelleting of intact Golgi fraction.
[d]Data obtained from same livers.

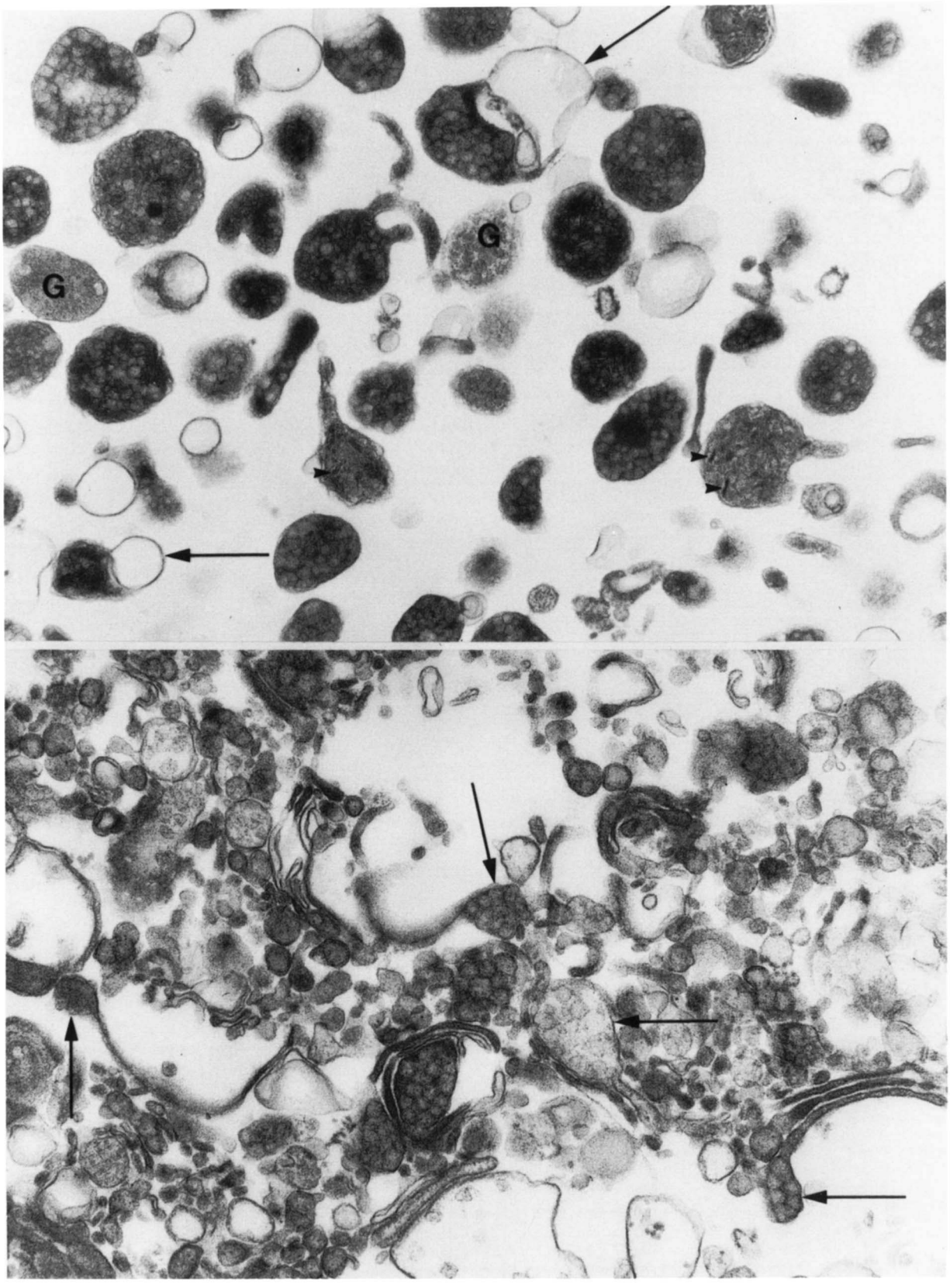

TABLE 3. Composition (mass %) of nascent (Golgi) and plasma VLDL

	Golgi (A)[a]	Golgi (B)[a]	Golgi (C)[a]	Serum[a]	Plasma[b]
Unesterified cholesterol	0.6 (±0.09)	1.4 (±0.2)	1.8 (±0.4)	2.9 (±0.3)	2.7
Phospholipids	23.4 (±1.07)	18.0 (±1.0)	14.0 (±1.4)	11.4 (±1.2)	12.2
Protein	7.1 (±1.10)	7.4 (±0.3)	10.4 (±0.7)	9.9 (±0.8)	11.1
Cholesteryl esters	6.1 (±0.84)	7.0 (±1.10)	6.4 (±2.3)	5.4 (±0.6)	5.8
Triglycerides	62.8 (±2.23)	66.3 (±2.5)	67.6 (±3.5)	70.5 (±1.2)	68.2

(A) VLDL isolated from intact Golgi fractions prepared by modified procedure (little endosomal contamination); (B) VLDL isolated from intact Golgi fractions prepared by original procedure (moderate endosomal contamination); (C) VLDL from (B) were mixed with plasma HDL ($1.065 < d < 1.21$ g/ml), and reisolated by ultracentrifugation. Unesterified cholesterol, cholesteryl esters, and triglycerides in Golgi (A) and plasma VLDL were analyzed by enzymatic techniques, and in Golgi (B), Golgi (C), and serum VLDL by manual methods (see Methods).

[a]Mean of 6 experiments ± SD.

[b]Mean of 2 experiments.

The morphologic characteristics of the intact Golgi fractions were compared with those of the GF_{1+2} fraction isolated from the same livers (**Fig. 1**). The GF_{1+2} fraction consisted largely of spherical vesicles, up to 0.5 μm diameter, filled with lipoprotein particles that were heterogeneous in size and staining characteristics. In some organelles, bilayer vesicles were evident and many contained a large membranous appendage. These characteristics identified the majority of the vesicles in GF_{1+2} fractions as MVBs (13–15, 56). Only a few vesicles in the GF_{1+2} pellet were tentatively identified as Golgi secretory vesicles, based upon the more homogeneous size and staining properties of content lipoproteins and absence of bilayer vesicles and appendages. Intact Golgi fractions were characterized by flattened stacks of cisternae, to some of which forming secretory vesicles containing VLDL particles were attached. Numerous small dark vesicular profiles, representing the Golgi tubular network (22) cut in various planes of sections, surrounded the stacks.

Preparation of highly purified intact Golgi fractions

The foregoing experiments showed the intact Golgi fraction to be a more suitable source for studying the properties of nascent VLDL than GF_{1+2} fractions. These experiments also demonstrated the presence of some endosomal lipoproteins in intact Golgi fractions from livers of untreated rats, confirming observations with intact Golgi fractions from livers of estradiol-treated rats (13). We found that endosomal contamination of our intact Golgi fractions was variable. When a large amount of material was obtained in the prefinal step of the procedure, the final Golgi pellet appeared to have greater endosomal contamination, as measured by chylomicron cholesteryl ester content. Because the Golgi material from the prefinal step was diluted to a constant final volume (based on centrifuge tube volume) in these experiments, we considered the possibility that endosomes were entrained in the complex tubules of the intact Golgi apparatus and were thus pelleted together with the Golgi membranes at low centrifugal force. To test this hypothesis, intact Golgi fractions from the prefinal step were diluted normally (~5-fold) with homogenizing medium or 15-fold before final pelleting. The ratio of ^{14}C-labeled triglycerides to [^{3}H]cholesteryl esters was then measured in the final pellets and in the overlying supernatants. The larger dilution of the Golgi membranes released considerably more [^{3}H]cholesteryl esters but no more ^{14}C-labeled triglycerides from the Golgi pellet into the supernatant. As compared with the smaller dilution volume, the larger dilution reduced contamination of the Golgi pellet with the endosomal marker approximately 5-fold with no loss of the Golgi marker (data not shown). This result is consistent with the hypothesis of endosomal entrainment.

Properties of VLDL released from intact Golgi fractions

The composition, as percent mass, of the major components of various VLDL fractions is shown in **Table 3**. The composition of VLDL, obtained from intact Golgi fractions by the modified procedure, which minimized endosomal contamination, was compared with that of plasma VLDL. Major differences were evident in the surface components: nascent VLDL contained about one-fifth as much unesterified cholesterol, twice as much phospholipids, and about 70% as much protein as plasma VLDL.

The composition of VLDL isolated from intact Golgi fractions by the original procedure is also shown in Table

Fig. 1. Electron micrographs of Golgi fractions. Top: GF_{1+2} fraction (8) from livers of rats taken 15 min after injection of small chylomicrons. The majority of vesicles are MVBs identified by the great heterogeneity of content lipoproteins, large membranous appendages (arrow) and internal bilayer vesicles (arrowheads). Few Golgi secretory vesicles (G), identified by the greater homogeneity of content lipoproteins and by absence of appendages and internal bilayer vesicles, are present in these fractions. Bottom: Intact Golgi fractions, isolated from the same liver homogenates as above, are characterized by flattened cisternae with attached secretory vesicles containing nascent VLDL (arrows). The number of cisternal stacks is reduced because of ethanol pretreatment (8). The numerous circular profiles surrounding the stacks of cisternae represent the complex tubular network characteristic of the Golgi apparatus in situ (22).

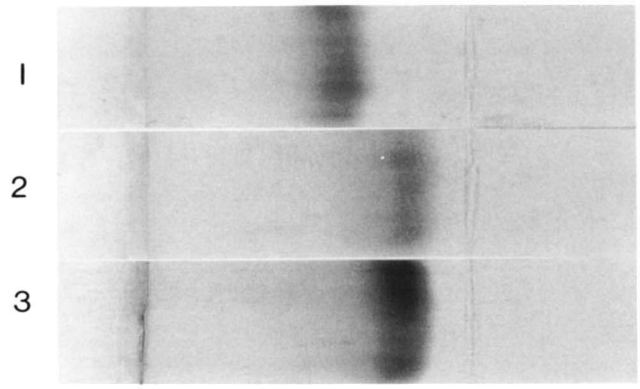

Fig. 2. Agarose electrophoretogram. Lane 1. VLDL (d < 1.010 g/ml) from intact Golgi apparatus have a lower mobility than serum VLDL shown in lane 3. Lane 2 shows that the mobility of Golgi VLDL increases to that of serum VLDL after the particles (0.2–0.3 mg protein) were mixed with rat serum HDL (0.5 mg protein) at 0°C and immediately re-isolated by flotation at a density of 1.006 g/ml.

3, and is compared with the composition of serum VLDL from the same animals. These experiments were carried out in 1972 before we were aware of the possibility of endosomal contamination. The most striking difference between the composition of these VLDL and that obtained with the improved technique is the higher unesterified cholesterol content found earlier. Hepatocytic endosomes are filled with remnants of triglyceride-rich lipoproteins (14), which are also enriched in unesterified cholesterol (53). Also shown in Table 3 is the effect of exposure of Golgi VLDL to serum HDL. Golgi VLDL took up unesterified cholesterol and proteins (mainly C apolipoproteins) and lost phospholipids. The altered composition was accompanied by increased electrophoretic mobility, comparable to that of serum VLDL (**Fig. 2**). The mobility of VLDL from liver perfusates (2) was comparable to that of Golgi VLDL (data not shown).

The distribution of diameters of Golgi VLDL, pooled from livers of eight rats, was measured in electron photomicrographs of negatively stained fractions in five experiments. The mean diameter of Golgi VLDL was 443 Å (SD ± 19.7 Å). The distribution of particle diameters was near Gaussian (**Fig. 3**). In all of the samples examined 75–85% of the diameters were between 400 and 600 Å. The mean diameter of plasma VLDL was similar to that of Golgi VLDL, but the distribution of diameters was more heterogeneous. Twice as many plasma VLDL particles were between 200 and 400 Å in diameter and more particles exceeding 600 Å in diameter were seen.[1]

[1]We have found that the procedure for preparing negative stains can yield an artifactually low number of larger particles (600–4000 Å in diameters) on the carbon-coated grids. Such particles, presumably representing chylomicrons, can be separated from plasma VLDL of our fed animals, but not from Golgi VLDL, by brief ultracentrifugation. Their presence presumably accounts for the higher triglyceride content of plasma VLDL than that of Golgi VLDL.

Apolipoproteins of lipoproteins released from intact Golgi fractions

After rupture of the membranes of intact Golgi fractions by passage through a French pressure cell, the entire fraction was subjected to ultracentrifugation at a density of 1.010 g/ml. VLDL layered at the top of the centrifuge tube and the membranes formed a pellet at the bottom. Just above and beside the membrane pellet was a slightly yellow solution containing sedimented Golgi content proteins; the remainder of the tube was clear and uncolored. The proteins of the VLDL and the sedimented protein material were visualized with Coomassie Blue after SDS

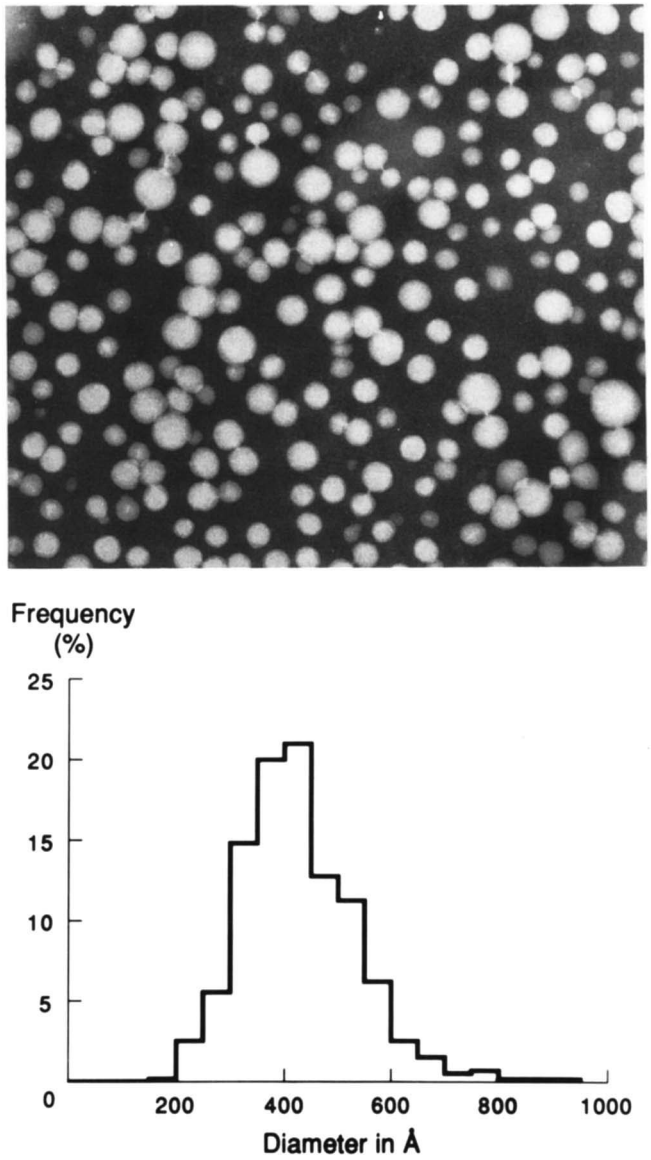

Fig. 3. Structure of Golgi VLDL. The histogram shows the distribution of diameters of 600 Golgi VLDL particles measured in electron photomicrographs of a negatively stained preparation (example of the image used is shown at the top). The mean diameter of particles in this sample was 435 Å.

gel electrophoresis (**Fig. 4**). Golgi VLDL contained components corresponding to apoB-100, apoB-48, apoE, apoA-I, and C apolipoproteins present in the d < 1.21 g/ml fraction of blood serum (apoA-IV was not seen in Golgi VLDL). Golgi VLDL also contained some minor components with mobilities between apoA-I and the C apolipoproteins. By radioimmunoassay, apoE accounted for 22.1 ± 5.5% (n = 9) and apoA-I for 0.86 ± 0.36% (n = 4) by mass of the protein component of four preparations of Golgi VLDL. ApoE comprised 23.3 ± 11.9% of protein in plasma VLDL (n = 4). The proteins in the sedimented contents material were composed principally of components corresponding to serum albumin and urate oxidase with trace amounts of apoE (both have molecular masses of ~34–35 kDa), together with a prominent components of ~14 kDa. The latter protein was tentatively identified as hepatic fatty acid-binding protein by immunoblotting (Dempsey, M., R. L. Hamilton, and R. J. Havel, unpublished data). By radioimmunoassay, most of the apoE and apoA-I was in the VLDL or remained with the pellet of Golgi membranes (**Table 4**). These membranes also contained a small fraction (10–25%) of the triglycerides present in the total Golgi fraction. Very little of the apoE and apoA-I in the Golgi fraction was present in the sedimented content proteins or the clear supernatant between the VLDL and sedimented proteins. These fractions also contained very little of the Golgi lipids. Examination of negative stains of these two fractions by electron microscopy revealed essentially no discernible particles that resembled spherical lipoproteins such as LDL or the nascent discoidal HDL found in liver perfusates (2). Attempts to visualize such particles after flotation from these fractions at densities of 1.055 and 1.25 g/ml were also unsuccessful.

TABLE 4. Distribution of apolipoproteins E and A-I among Golgi compartments

Compartment	% ApoE	% ApoA-I
Nascent VLDL	53.1 (5.0)	33.1 (6.1)
Membranes	42.3 (2.6)	58.0 (4.2)
Supernatant	2.6 (4.4)	8.8 (5.)

Values are mean (SD); n = 4.

In isoelectric focusing gel electrophoretograms the distribution of isoforms of apoE in Golgi VLDL closely resembled that of serum VLDL (**Fig. 5**). This was confirmed in two-dimensional gel electrophoretograms (**Fig. 6**). Although Golgi VLDL contained some apoA-I (Table 4), the pI of the major protein that focused in the region of apoA-I on gel electrophoresis was 5.95 (Fig. 5), whereas that of the major component of apoA-I in plasma HDL is 6.02. This was confirmed by immunoblotting (**Fig. 7**), which demonstrated that the major component in Golgi VLDL corresponded to proapoA-I (57). Immunoblots from SDS gel electrophoretograms showed that none of the proteins with mobilities between apoA-I and the C apolipoproteins reacted with anti-apoA-I (**Fig. 8**). Although the relative amounts of apolipoproteins C-II and C-III in Golgi VLDL resembled those of plasma VLDL (Fig. 5), Golgi VLDL invariably contained a

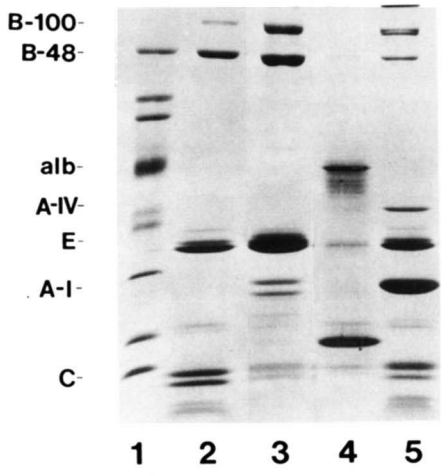

Fig. 4. SDS-PAGE of proteins of Golgi VLDL. Proteins were separated on a 3–15% polyacrylamide slab gel and stained with Coomassie Blue. From left: lane 1, molecular weight standards; lane 2, serum VLDL; lane 3, Golgi VLDL; lane 4, Golgi fraction contents that sedimented during ultracentrifugation; lane 5, rat serum HDL. ApoB-100 and apoB-48 are present in Golgi VLDL, as are apoE isoforms, apoA-I, and apolipoproteins C-II and C-III. An unidentified component is seen below apoA-I. The content proteins that were concentrated at the bottom of the tube after ultracentrifugation (lane 4) appeared to be mainly serum albumin and a large amount of a protein of ~14 kDa, identified as hepatic fatty acid-binding protein (see text).

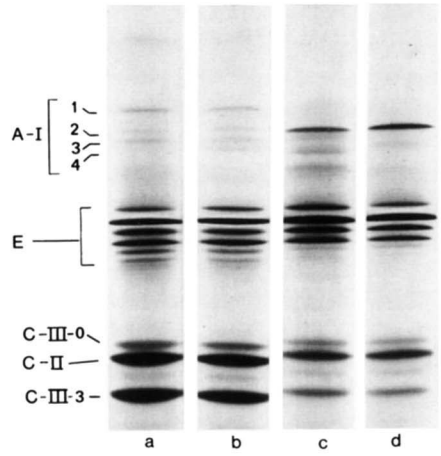

Fig. 5. Isoelectric focusing gel electrophoretogram, run under reducing conditions, of two preparations of proteins of serum VLDL (lanes 1 and 2) and Golgi VLDL (lanes 3 and 4); 50–60 μg protein was applied to each gel. Note the prominent component in the region of apoA-I in the case of Golgi VLDL, an the more cationic band in this region in the case of serum VLDL.

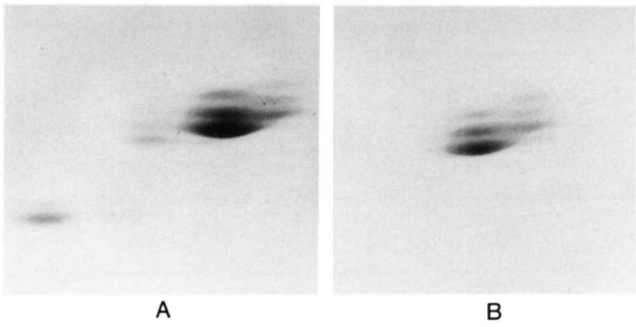

Fig. 6. Two-dimensional gel electrophoretogram showing apoE components of Golgi VLDL and serum VLDL (B). First dimension, isoelectric focusing gel (50 μg of VLDL protein applied), pH 3.5–7.0, right to left; second dimension, 3–25% SDS gel. The major components, as well as most of the minor components of apoE appear to be represented in the same proportions in VLDL from each source. The minor component at the lower left in gel A is proapoA-I, seen only in Golgi VLDL.

smaller amount of C apolipoproteins, relative to apoE, than serum VLDL. The increased electrophoretic mobility of Golgi VLDL that had been exposed to serum HDL (Fig. 2) was accompanied by a substantial increase in the relative amount of C apolipoproteins (data not shown).

DISCUSSION

In the current research, we have found that the composition of nascent VLDL from hepatocytic Golgi apparatus fractions (shown to be almost free of endosomes) differs substantially from that obtained by us previously, as well as by others, which were obtained with Golgi fractions that were either moderately (intact Golgi) or severely (GF_{1+2}) contaminated with endosomes. Most, if not all, of this endosomal contamination reflects the co-isolation of MVBs which, like Golgi fractions, have a low density, caused in part by the high content of triglyceride-rich lipoproteins. In both cases, VLDL and chylomicron remnants become mixed with newly synthesized VLDL, such that the properties of the particles studied do not represent exclusively, or even mainly, the nascent VLDL.

The extent to which Golgi fractions GF_{1+2} are contaminated with endosomes can be estimated from electron microscopic examination of pellets of these fractions: more than one-half of the lipoprotein-filled organelles in these pellets can be identified morphologically as MVBs. Intact Golgi pellets obtained by our original technique, were about 7% as contaminated with endocytosed lipoproteins (Table 2). Thus, if comparable masses of lipoproteins are present in vesicles derived from the Golgi apparatus and in MVBs, no more than 3–4% of the isolated organelles in intact Golgi pellets would represent MVBs. Our modified method for isolation of intact Golgi fractions yields a value only one-fifth as great as this

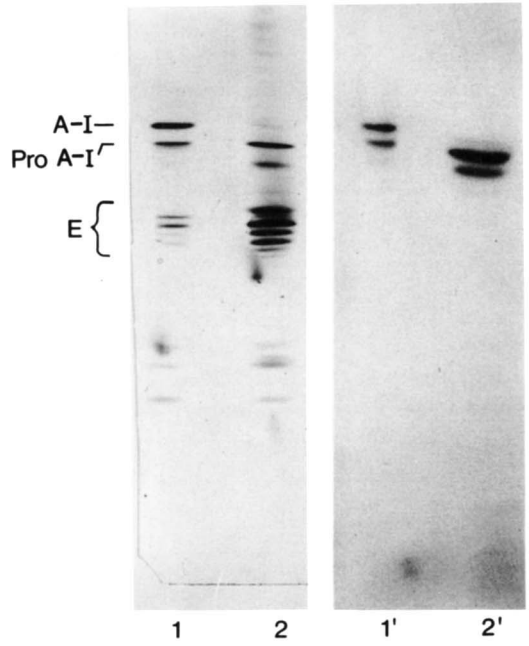

Fig. 7. Isoelectric focusing gel electrophoretogram of proteins of < 1.21 g/ml serum lipoproteins (lane 1, 3 μg applied) and Golgi VLDL (lane 2; 80 μg applied). Lanes 1' and 2' show immunoblot prepared from this gel against rat apoA-I antiserum (1:500 dilution). Bound antibodies were detected with ^{125}I-labeled protein A; exposure was for 45 min at −70°C.

(~1%), which we consider adequate for evaluation of nascent lipoproteins.

Considered in the light of our finding that unesterified cholesterol content of Golgi VLDL is about one-fifth that of plasma VLDL (Table 3), published data on the composition of putative nascent VLDL released from GF_{1+2} fractions (42–45) also indicate that the content lipoproteins characterized were predominantly remnants of triglyceride-rich particles rather than nascent VLDL.

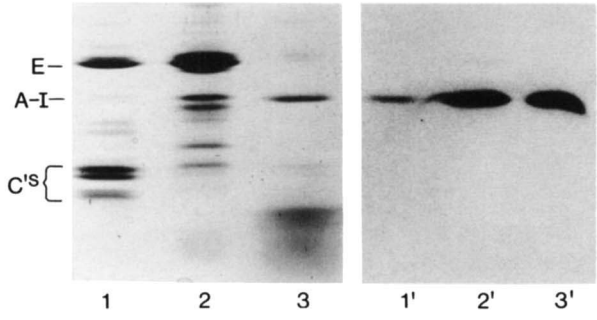

Fig. 8. SDS gel electrophoretogram of Coomassie Blue-stained proteins of serum VLDL (lane 1; 50 μg applied), Golgi VLDL (lane 2; 50 μg applied); and d < 1.21 g/ml serum lipoproteins (lane 3; 5 μg applied). Lanes 1', 2', and 3' show immunoblot prepared from this gel against rat apoA-I antiserum (1:500 dilution). Bound antibodies were detected with ^{125}I-labeled protein A; exposure was for 45 min at −70°C.

This is shown by the 2- to 3-fold higher content of both unesterified and esterified cholesterol of the intracellular particles released from GF_{1+2} fractions than those of serum VLDL. The percentage mass of unesterified and esterified cholesterol in remnants of VLDL and chylomicrons in rat plasma is 2- to 10-fold higher than that of their parent fractions (53). Although one group has reported quite different lipid compositions for nascent VLDL isolated from GF_{1+2} fractions in two reports (43, 44), this discrepancy was unexplained, and the more recent data still indicate a large endosomal contamination. The latter report also states that these putative nascent VLDL completely lack C apolipoproteins and are poor in apoE, which are acquired by transfer from plasma HDL (44). Our data show that apoC-II and the isoforms of apoC-III are present and that apoE is a major protein component of nascent VLDL.

In addition to the data on unesterified cholesterol and cholesteryl esters described above, the distribution of phospholipid species can also provide a marker for the extent of endosomal contamination. We recently reported that Golgi VLDL contain fourfold more phosphatidylethanolamine than plasma VLDL and that the ratio of PE to sphingomyelin differs by a factor of about 12 between Golgi VLDL and plasma VLDL (23). In VLDL isolated from GF_{1+2} fractions, the phosphatidylethanolamine:sphingomyelin ratio was less than twofold higher than that of plasma VLDL (42). The actual ratios found for plasma VLDL were the same in both reports (23, 42).

These results and our current data strongly indicate that GF_{1+2} fractions, as prepared in several laboratories, are composed predominantly of endosomal organelles rather than Golgi secretory vesicles. Based on this observation, we modified our original method for isolating hepatocytic MVB fractions (13) by flotation of a subcellular fraction (obtained differently than the microsomal fraction used as the source of GF_{1+2}), through the same sucrose step gradient used in the final stages of isolation of GF_{1+2} fractions (8, 24). This modification resulted in two distinctive improvements. First, it improved our purification of MVBs from about 100-fold to more than 200-fold over the homogenate; second, it led to the separation of two additional endosomal compartments from our low density, endosome-rich fraction (14, 56, 58).

The wide use of the GF_{1+2} isolation procedure to isolate putative nascent plasma lipoproteins has led to the concept that the hepatocytic Golgi apparatus contains immature precursors of LDL and HDL (42–47). In these studies fractions that were largely endosomal in origin were used, and the contents were released (in some cases at high pH ($\sim$11.5), and then centrifuged sequentially to isolate VLDL, LDL, and HDL fractions. Because radioisotopes were used in some cases to label newly synthesized apolipoproteins and lipids, it was assumed that radioactive compounds present in the final fraction represented only nascent material. The validity of this assumption, however, depends upon the conditions of labeling. When immunoblots are used to estimate the mass of proteins of such fractions (52), conclusions about lipoprotein synthesis will inevitably be biased, as will data on nascent lipoprotein structure and composition. In the current research, in which endosomal contents were shown to be largely eliminated from the Golgi fraction from which content lipoproteins were released, we found little or no lipoprotein other than Golgi VLDL. Both apoE and apoA-I either floated with the released Golgi VLDL or remained bound to (or trapped within) the Golgi membranes. Thus, it appears, at least in normal fed rats, that Golgi VLDL are virtually the only particulate plasma lipoproteins secreted from the liver into blood plasma. Immature HDL particles or recognizable precursors are not found as such, consistent with the extracellular assembly of these lipoproteins by a complex series of events (59). Moreover, particles of LDL size and density do not appear to be secreted to any extent by normal rat hepatocytes, as shown also by liver perfusion experiments in rats, guinea pigs, and rabbits (2, 60, 61). This does not mean that under abnormal conditions, such as the cholesterol-induced fatty liver, particles of LDL size and density cannot be syntheized and secreted (60). These abnormal particles differ from plasma LDL in their high content of apoE (60). The observation that human hepatoma cells (Hep G2) secrete apoB-100 largely in particles of LDL and HDL size and density may be an expression of the inability of these tumor cells to lipidate apoB-100 normally (62, 63). Under normal metabolic conditions, apolipoproteins destined to appear in plasma HDL evidently are bound to Golgi VLDL and become dissociated soon after secretion of nascent VLDL into the blood. ApoA-I does not require nascent VLDL to be secreted, however. In the fatty liver of orotic acid-fed rats that secrete no VLDL, normal amounts of apoA-I together with some apoE accumulate in liver perfusates in discoidal HDL particles (64).

How can we explain the failure to recognize earlier the endosomal contamination of Golgi fractions, especially in the GF_{1+2} fractions? First, when the GF_{1+2} isolation procedure was developed (8), receptor-mediated uptake of plasma lipoproteins by hepatocytes had not been described. Second, lipid-staining particles within hepatocytes were thought to represent nascent VLDL particles exclusively (65). Third, electron microscopic images of this subcellular fraction resembled Golgi-derived secretory vesicles containing clusters of nascent VLDL (8). Fourth, it was not widely appreciated that the composition and size of remnants of VLDL and chylomicrons do not differ drastically from those of plasma VLDL. Even though some investigators were aware of this resemblance (53) and that the liver removes remnants from the blood plasma, the magnitude of lipoprotein traffic into the liver,

which dwarfs the uptake of other ligands entering hepatocytes by receptor-mediated endocytosis (14), was largely unappreciated. In addition, the endocytic mechanisms that lead to the accumulation of remnant lipoproteins in MVBs were not fully recognized.

Radioautographic localization of injected radioiodinated lipoproteins first led to the realization that MVBs in the Golgi/bile canalicular (apical) pole of hepatocytes contain endocytosed particles that are easily confused with Golgi secretory vesicles (10–12). This led to the development of a procedure to isolate highly pure MVB fractions, which brought to light subtle morphologic characteristics that can be used to distinguish MVBs and Golgi secretory vesicles (13). A third advance was the use, in the latter research, of isotopes that differentially labeled Golgi VLDL and endosomal remnants. These measurements showed, for the first time (13), that intact Golgi fractions from livers of estradiol-treated rats are contaminated to a sufficient extent ($\sim$15% of content lipoproteins) as to cast doubt on the validity of our previous analyses (Table 3) of the composition of nascent VLDL. At almost the same time, a report was published showing that a large amount of endocytosed insulin is recovered in GF_{1+2} fractions, which could be separated from vesicles that contained the *trans* Golgi marker enzyme, galactosyltransferase (66). The latter report, taken together with our current findings, may help to reconcile some of the spurious results obtained with GF_{1+2} fractions and other confusing observations in the literature. GF_{1+2} fractions contain little galactosyltransferase activity (8), leading to the erroneous conclusion that this enzyme occurs in the *cis* and middle, but not the *trans* Golgi membranes (67), in contradistinction to the immunocytochemical localization of the enzyme in intact rat hepatocytes (68). GF_{1+2} fractions stain for acid phosphatase histochemically (69) to an extent (15–20%) comparable to that of isolated hepatocytic MVB fractions (15). An antiserum to NADPH-cytochrome-C reductase (supposedly an enzyme localized in the endoplasmic reticulum) was found to immunoprecipitate a large fraction of the vesicles in the GF_{1+2} fraction, whereas most galactosyltransferase-containing vesicles failed to bind to this antiserum (70). Images of immunosorbed vesicles shown in this report resemble MVBs. Recent studies have shown that reticulocyte endosomes contain an NADH-ferricyanide reductase that is required for transport of endocytosed iron through the vesicle membrane to the cytosol (71). This observation is supported by an earlier report that two types of lipoprotein-containing vesicles occur in rat hepatocytic Golgi areas, one of which stains cytochemically for NADH-ferricyanide reductase (72). These vesicles probably are MVBs, because they contained smaller lipoprotein particles than unstained Golgi vesicles. Novikoff and Yam (73) first recognized that there are two lipoprotein-containing vesicles of the rat Golgi apparatus and that one of these is degradative. They speculated, however, that the degradative vesicles containing lipoprotein particles and acid hydrolases (i.e., lysosomal enzymes) are both derived directly from the endoplasmic reticulum (73).

These observations may now be reinterpreted in the light of our present findings, as follows. Golgi secretory vesicles containing nascent VLDL dissociate from the Golgi apparatus and discharge their contents into the space of Disse by exocytosis. Both VLDL from hepatocytes and chylomicrons from intestinal absorptive cells (which are produced in the postabsorptive as well as the postprandial state) undergo processing by lipoprotein lipase in capillary beds, which transforms them into triglyceride-rich remnants (14). Most remnants are taken up into hepatocytes by receptor-mediated endocytosis, a process involving repeated fusions of endocytic vesicles and recycling of receptors, during which remnants are concentrated in endosomes, which become MVBs. MVBs are translocated via microtubules to the Golgi apparatus, at which point they may fuse with coated blebs of the *trans* Golgi network or reticulum (74), which discharge lysosomal enzymes into MVBs. Acid hydrolases (particularly cathepsin D and acid lipase; Runquist, E., and R. J. Havel, unpublished results) eventually digest the lipoprotein contents of MVBs, which become secondary lysosomes. Because MVBs containing triglyceride-rich lipoprotein remnants and forming Golgi secretory vesicles are of similar size, are filled with lipoproteins of similar size, and are concentrated at the bile canalicular pole of hepatocytes, they are often incorrectly identified in electron micrographs. Furthermore, they co-isolate in centrifugal fractions of livers homogenized by shear forces, which probably dissociate forming Golgi secretory vesicles from the intact organelle. By contrast, when livers are homogenized with a Polytron apparatus operated at low speed, and in a homogenizing medium that does not dissociate the forming Golgi secretory vesicles, the complicated and large tubular Golgi network (22) sediments at low centrifugal force. Some MVBs are entrained with (or possibly physically bound to) the Golgi network. With greater dilution of homogenizing medium, the forming secretory vesicles remain attached, but the entrained or more loosely enjoined MVBs dissociate and thereby remain buoyant at the low centrifugal forces that pellet the intact Golgi apparatus.

Our observations demonstrate that the content of core components (triglycerides and cholesteryl esters) of Golgi VLDL resembles closely those of plasma VLDL, but the major surface components differ widely. The nascent particles contain much more phospholipids (particularly phosphatidylethanolamine) (23)), only about one-fifth as much unesterified cholesterol, and considerably fewer C apolipoproteins than plasma VLDL. Nascent VLDL rapidly acquire C apolipoproteins from HDL and, by transfer to HDL or as a result of lipase action, lose a large

fraction of their phosphatidylcholine and phosphatidylethanolamine. These changes are analogous to those that occur when nascent chylomicrons enter intestinal lymph and, subsequently, the blood (75, 76). Nascent VLDL also acquire a considerable amount of unesterified cholesterol in blood plasma. Although the proximate source of this cholesterol is unclear, it likely is derived ultimately from the plasma membrane of cells. One such cell is the erythrocyte: exposure of plasma VLDL to erythrocytes has been shown to increase the mass of component unesterified cholesterol almost twofold (77). Whatever the source, the cholesterol transferred to nascent VLDL would appear, in a matter of minutes, to be taken up by the liver with VLDL remnants. The magnitude of this traffic could be considerable. Transport of VLDL cholesteryl esters though the blood of rats has been estimated to be about 2000 μg/h based upon a content of 6% of VLDL mass (77). Transport of acquired VLDL unesterified cholesterol then would be about one-third as great (670 μg/h). This amount is approximately equivalent to the rate at which cholesteryl esters are produced by lecithin:cholesterol acyltransferase in rat blood plasma (78). If the unesterified cholesterol acquired by nascent VLDL is obtained from tissues other than the liver, transport of this unesterified cholesterol could make a major contribution to reverse cholesterol transport in the rat.

Our observations raise new questions about the determinants of surface composition of nascent VLDL. The low content of unesterified cholesterol[2] and sphingomyelin (23), as well as the high content of phosphatidylethanolamine may reflect the composition of the inner leaflet of membranes of the Golgi apparatus and endoplasmic reticulum (79). Some data suggest that phosphatidylethanolamine may be added in the Golgi apparatus itself (80). The B apolipoproteins probably join primordial particles destined to become mature nascent VLDL in the endoplasmic reticulum (81), but the site at which apoE is added is also unknown. Our immunocytochemical data suggest that some apoE present in Golgi cisternae is not associated with nascent VLDL (82), and it is possible that apoE was acquired by our nascent VLDL when the Golgi contents were mixed after the Golgi membranes were ruptured in the French pressure cell. These considerations make evident the complexity of the issues surrounding our attempts to understand even the final stages of lipoprotein assembly in the Golgi apparatus. Our current results, however, provide a firmer basis for designing new experimental approaches to the assembly of VLDL and the formation of nascent HDL.

[2]Because triglyceride-rich remnants may contain up to tenfold more unesterified cholesterol (53) than the values we have found for nascent VLDL, the true content of this lipid in nascent VLDL could be even lower than 0.6% of mass.

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