Isolation and Characterization of Multivesicular Bodies from Rat Hepatocytes: An Organelle Distinct from Secretory Vesicles of the Golgi Apparatus

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ABSTRACT Hepatocytes of estradiol-treated rats, which express many low density lipoprotein receptors, rapidly accumulate intravenously injected low density lipoprotein in multivesicular bodies (MVBs). We have isolated MVBs and Golgi apparatus fractions from livers of estradiol-treated rats. MVB fractions were composed mainly of large vesicles, ~0.55 μm diam, filled with remnantlike very low density lipoproteins, known to be taken up into hepatocytes by receptor-mediated endocytosis. MVBs also contained numerous small vesicles, 0.05–0.07 μm in diameter, and had two types of appendages: one fingerlike and electron dense and the other saclike and electron lucent. MVBs contained little galactosyltransferase or arylsulfatase activity, and content lipoproteins were largely intact. Very low density lipoproteins from Golgi fractions, which are derived to a large extent from secretory vesicles, were larger than those of MVB fractions and contained newly synthesized triglycerides. Membranes of MVBs contained much more cholesterol and less protein than did Golgi membranes. We conclude that two distinct lipoprotein-filled organelles are located in the bile canalicular pole of hepatocytes. MVBs, a major prelysosomal organelle of low density in the endocytic pathway, contain remnants of triglyceride-rich lipoproteins, whereas secretory vesicles of the Golgi apparatus contain nascent very low density lipoproteins.

Many different types of macromolecules, including hormones, growth factors, plasma glycoproteins and lipoproteins, and some viruses, are taken up by cells after binding to specific receptors in plasma membranes. The ligand–receptor complexes are concentrated in clathrin-coated pits and taken into the cell by endocytosis, forming primary endocytotic vesicles 0.05–0.08 μm in diameter. The complexes soon become associated near the cell surface with large, smooth-surfaced, polymorphic structures (1–4) which have been called sorting vesicles (5), endosomes (3), receptosomes (6), or compartment of uncoupling of receptor and ligand (CURL) (7). Later, in hepatocytes ligands are found in larger vesicular structures in the bile canalicular region of the cell, near the Golgi apparatus. These organelles, most of which contain small internal vesicles, are called multivesicular bodies (MVBs).[1] Like secretory vesicles of the Golgi apparatus in this cell, they contain structures with diameters of 300–800 Å that resemble plasma lipoproteins (8, 9).

In earlier research we identified MVBs as an intermediate compartment in the receptor-mediated catabolic pathway for low density lipoprotein (LDL) in hepatocytes (8, 9). We now report a method for isolating MVBs from livers of estradiol-treated rats. We have isolated MVBs and intact Golgi apparatus fractions from the same livers and have found that they can be distinguished by morphological and enzymatic criteria. Furthermore, the properties of the very low density lipoprotein (VLDL) content of these adjacent organelles are distinct and indicate that they comprise endocytosed and newly synthesized lipoproteins, respectively.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats, 300–350 g, were fed standard chow (Ralston Purina Co., St. Louis, MO). 17-α-ethinyl estradiol, dissolved in pro-

[1] *Abbreviation used in this paper:* MVB, multivesicular body.

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pylene glycol (1 mg/ml), was injected (5 mg/kg) subcutaneously for 5 d to induce a high rate of uptake of LDL into the liver (10, 11).

Radiolabeled Markers: Human LDL (ρ = 1.025–1.050 g/ml) was isolated from blood serum of normolipidemic adults by sequential ultracentrifugation (12) and labeled with ^{125}I to a specific activity of ~85 μCi/mg by a modification (13) of the method of McFarlane (14). [9,10 (*n*)-^{3}H]palmitic acid was obtained from Amersham Corp. (Arlington Heights, IL). 3 μmol (1.5 mCi) labeled and 4 μmol unlabeled palmitic acid were dissolved in warm 50% ethanol in 0.15 M NaCl containing an excess of $NaHCO_3$. The ethanol was removed by evaporation and 8 μmol bovine serum albumin in 0.15 M NaCl was added to form a palmitate–albumin complex.

Isolation of Multivesicular Bodies: Ethinyl estradiol-treated rats were anesthetized with diethyl ether, and radiolabeled LDL (1.0–1.5 mg protein) was injected into the femoral vein. 15 min later, the abdomen was opened, the vena cava below the liver was cut, and the livers were flushed by perfusion of 100 ml ice cold 0.15 M NaCl containing 0.1% EDTA through the portal vein. The livers, which contained ~35% of the injected ^{125}I, were then removed, minced with scalpels, and homogenized in 0.25 M sucrose (1:3 wt/vol) in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ) with five strokes of the loose-fitting pestle and one stroke of the tight-fitting pestle. Protease inhibitors (Sigma Chemical Co., St. Louis, MO) were added to the homogenate to give concentrations of 20 μM antipain, 5 μM pepstatin, and 0.8 mg/ml bacitracin. The procedure for isolation of MVBs is summarized in Fig. 1. The homogenate was first centrifuged in a SW 41 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 500 g_{av} for 10 min. The supernatant was then centrifuged in the SW 41 rotor at 3,500 g_{av} for 15 min. The second supernatant was transferred to a Beckman 50.2 Ti rotor and centrifuged at 12,000 *g*av for 20 min to yield a third supernatant which was diluted with isotonic Percoll, pH 7.4 (Pharmacia Fine Chemicals, Piscataway, NJ) to a density of 1.070 g/ml. This material was centrifuged in a Beckman 50.3 Ti rotor (eight tubes) for 45 min at 29,000 g_{av}. We fractionated the resulting gradients by pumping 60% sucrose into the bottom of the tube and collecting 0.25-ml samples from the top. The average density of the fractions in which ^{125}I was concentrated was ~1.035 g/ml, as estimated from marker beads (Pharmacia Fine Chemicals). These fractions (~10 ml) were diluted 1:2 with 0.15 M NaCl containing the described protease inhibitors, layered over a sucrose cushion (ρ = 1.074 g/ml), and centrifuged in a SW 41 rotor at 11,000 g_{av} for 30 min. The white band that appeared at the interface was then aspirated with a blunt, bent 12-gauge needle. This material (~8 ml) was pelleted without dilution at 29,000 g_{av} for 30 min in a 50.3 Ti rotor to separate organelles from soluble protein, membrane fragments, and most of the Percoll, and then washed by resuspension in 8 ml of 0.15 M NaCl and repelleted under the same conditions. Isolation of MVBs took ~6 h after removal of the liver; the yield was 2.5–3.5 mg protein from 16 g of liver.

Isolation of Golgi Apparatus: Isolation of structurally intact Golgi compartments was based on the method of Morré et al. (15) with some modifications (16). Portions of the same livers used to isolate MVBs were minced very fine with scalpel blades on an ice-chilled glass petri dish, then mixed with ice-cold homogenizing medium (1.77 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 (molecular weight 500,000; Sigma Chemical Co.) and 0.01 M $MgCl_2$.

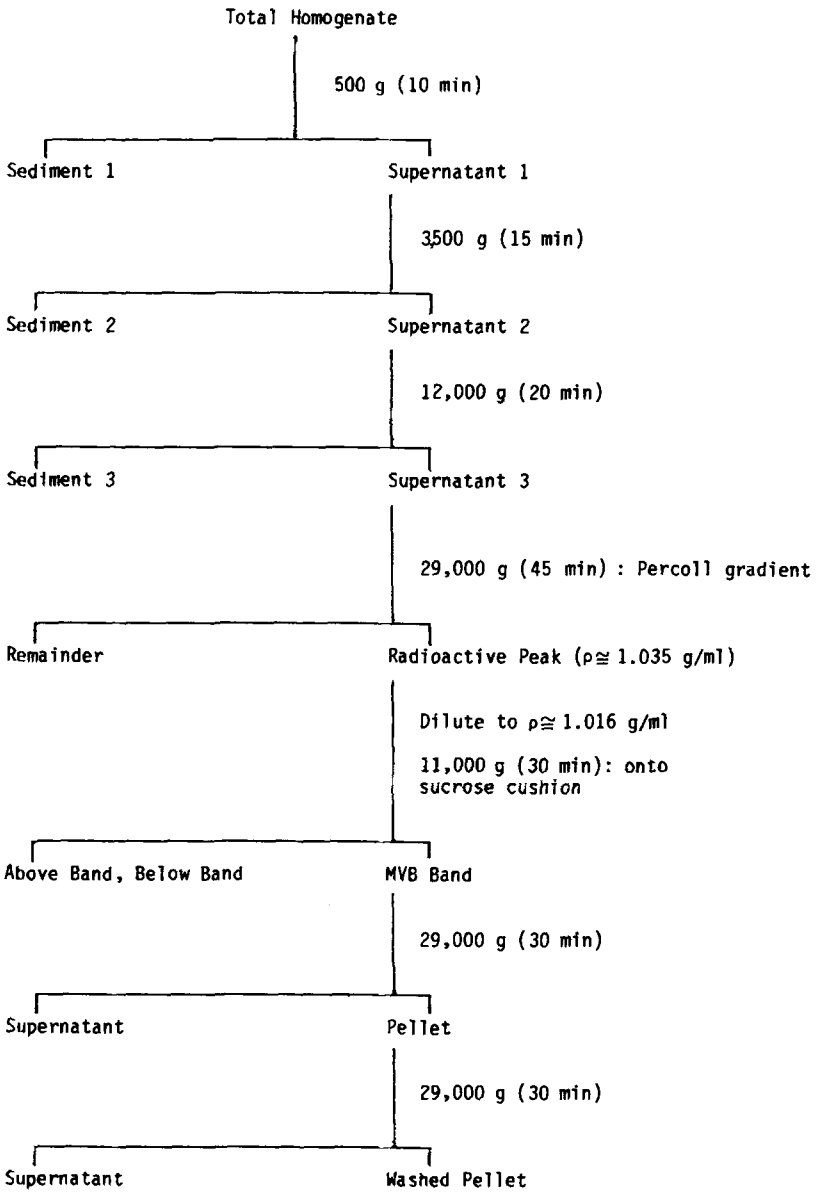

FIGURE 1 Procedure for isolation of MVBs from homogenate of livers (16 g) from two estradiol-treated rats.

Homogenization was done with a Polytron PT-10 (Brinkman Instruments, Inc., Westbury, NY) set at 0.5–1.0 for 25–30 s. The homogenate was first centrifuged at 4°C in a SW 41 rotor at 2,000 rpm for 5 min and 10,000 rpm for 30 min. The upper two-thirds of the pellets were resuspended in 2.0–3.0 ml homogenizing medium by rotation of the tip of a 5-ml glass pipette against the tube wall. This was pooled and carefully layered upon 9.0 ml of 1.2 M chilled sucrose cushions, and centrifuged in a SW 41 rotor at 25,000 rpm for 30 min. The white bands ("Golgi rugs") were recovered at the interface by rapid aspiration of the bands in a small volume (~0.5 ml/tube) into a Pasteur pipette. The pooled Golgi rugs (~3–4 ml) were diluted 8–10-fold with iced 0.15 M NaCl containing the described antiproteases, and the Golgi membranes were pelleted away from contaminating lysosomes and microsomal membranes by a slow speed sedimentation at 5,000 rpm in a SW 41 rotor for 20 min. The isolation of Golgi pellets took 3.5–4 h after removal of the liver; the yield was 4–8 mg protein from 16 g liver.

Labeling of MVBs and Golgi Compartments: In experiments performed to distinguish content lipoproteins of MVBs from those of Golgi fractions on the basis of their concentrations of ^{125}I-LDL and [^{3}H]palmitate, ^{125}I-LDL (1.01.5 mg protein, ~500 μCi) was injected intravenously 15 min before the liver was flushed, and [^{3}H]palmitate (~150 μCi) was injected 5 min later. MVB and Golgi fractions were then isolated from the same livers. 15 min after injection a substantial portion of the ^{125}I-LDL has entered the liver of estradiol-treated rats but it has not yet begun to accumulate in the lysosomal compartment and therefore is concentrated in MVBs (8, 9). 10 min after injection of the [^{3}H]palmitate–albumin complex, about one-third of the ^{3}H had been incorporated into hepatic lipids (mainly phospholipids and triglycerides), but the much smaller fraction that was incorporated into nascent lipoproteins had not yet been secreted (control experiments, data not shown). Therefore, the lipids of nascent VLDL should be labeled, but those of VLDL taken up into the liver by endocytosis should not.

Characterization of Membranes and Lipoproteins of MVBs and Golgi Compartments: MVB and Golgi fractions were resuspended in 4 ml distilled water and then disrupted by passage twice through a French pressure cell at 16,000 psi. Alternatively, the fractions were resuspended in 20 ml ice-cold 0.1 M Na_2CO_3 containing the protease inhibitors (final pH 11.2). The suspension was homogenized in a Dounce homogenizer with five strokes of the tight pestle and then mixed by vortexing. This process was repeated after 15 min (17). Content lipoproteins were separated from membranes by ultracentrifugation at densities of 1.008, 1.063, and 1.21 g/ml (12). To examine the LDL label released from MVBs, the fraction of $\rho < 1.063$ g/ml was subjected to electrophoresis into 0.1% SDS/3% polyacrylamide gels and visualized by Coomassie Brilliant Blue staining (18). Gels were then sliced into 2-mm sections and ^{125}I content was determined by scintillation spectrometry.

Electron Microscopy: Pellets of intact MVB and Golgi membrane fractions were fixed in situ in 40.3 rotor centrifuge tubes (Beckman Instruments, Inc.) in 3% OsO_4 in veronal acetate buffer at 4°C overnight. They were then stained en bloc with aqueous uranyl acetate for 1–2 h at 37°C, dehydrated in acetone (which facilitates the separation of membrane pellets from the plastic tubes), and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and photographed in a Siemens 101 electron microscope at 80 kV with a 60-μm objective aperture (Siemens Corp., Medical Industrial Groups, Iselin, NJ). Negative stains of isolated membrane fractions and of plasma lipoproteins were made with 2% potassium phosphotungstate, pH 6.45, on carbon-coated grids (19) and photographed as for thin sections.

Analyses: To measure protein in samples containing Percoll, 100 μl of sample was mixed with 50 μl 4 M NaOH and centrifuged at 11,000 *g* for 7 min. The resulting supernatant (100 μl) was mixed with 900 μl water, and protein concentration was determined by the method of Peterson (20). For other samples, we applied the Peterson procedure directly. Apolipoproteins were separated by SDS PAGE (18, 21) or by isoelectric focusing electrophoresis (22).

Cholesterol and cholesteryl esters (23), phospholipids (24), and triglycerides (25) were measured in lipoprotein and membrane fractions of MVB and Golgi fractions. Lipid classes were separated by chromatography on columns of silicic acid (16). ^{3}H was measured by beta-scintillation spectrometry after addition of 10 ml Aquasol (New England Nuclear, Boston, MA) to aqueous homogenate fractions. ^{125}I was measured by gamma-scintillation spectrometry. Glucose-6-phosphatase (27), arylsulfatase (28), and galactosyltransferase (29) were assayed in all homogenate fractions. For these assays, the protease inhibitors were added as described above and the fractions were frozen at −70°C until analyses were performed. Recovery of activities in the homogenate fractions exceeded 90% of that in the total homogenate in each case.

RESULTS

Isolation of MVBs

MVBs were isolated from livers of ethinyl estradiol-treated rats 15 min after intravenous injection of ^{125}I-LDL. We expected intact MVBs to contain a high concentration of the ^{125}I-LDL, to be of low density, and to be identifiable by electron microscopy. For the method that we developed (Fig. 1 and Table I), the homgenate was centrifuged three times to yield a supernatant fraction containing about one-half of the ^{125}I and one-third of the protein originally present. When this fraction was centrifuged in Percoll, one-half of the ^{125}I it contained was concentrated near the top of the gradient at a density of 1.035 g/ml (Fig. 2). To concentrate the larger organelles containing ^{125}I-LDL and separate them from smaller organelles, membrane fragments, and soluble proteins, we diluted this material with 2 vol 0.15 M NaCl and centrifuged it onto a cushion of 0.56 M sucrose to yield ~12% of the ^{125}I and 1% of the protein in the homogenate (Fig. 1 and Table I). We achieved further purification by centrifuging the fraction to yield a pellet, which contained almost all of the ^{125}I in the fraction but only ~15% of the protein. Overall purification was ~100-fold (^{125}I/protein). Washing the pellet produced only a slight additional purification. When antiproteolytic agents were added to the original homogenate, <5% of the ^{125}I in all fractions was soluble in 10% trichloracetic acid.

TABLE I

Purification of MVBs Containing ^{125}I-LDL from Livers of Estradiol-treated Rats

	^{125}I	Protein	Specific activity	Purification
	cpm × 10^{-6}	*g*	*cpm × 10^{-6} g^{-1}*	*fold*
Total homogenate	142.6	3.10	46.0	
Supernatant 1	117.0	1.73	67.6	1.47
Supernatant 2	113.8	1.41	80.7	1.75
Supernatant 3	78.8	1.02	77.3	1.68
Radioactive peak	35.8	0.22	163.0	3.54
MVB band	17.8	0.03	593.0	12.9
Band pellet	14.8	0.0036	4111.0	89.3
Washed pellet	14.3	0.0029	4931.0	107.0

Values represent recovery from 16 g rat liver.

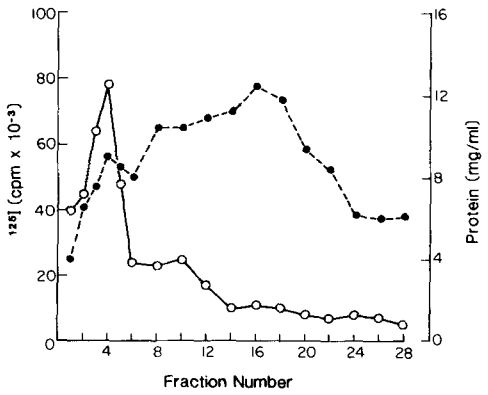

FIGURE 2 Distribution of ^{125}I (○) and protein (●) in fractions from Percoll gradient. Fractions 1–5 typically contained approximately one-half of the ^{125}I and 20% of the protein in the fractions (supernatant 3) added to the Percoll.

Morphologic Characterization of Isolated MVBs: Comparison with Golgi Fractions

At low magnification (× 15,000), the predominant organelles in thin sections made near the middle of MVB pellets were vesicles, 0.5–0.6 μm in diameter, of variable electron density (Fig. 3, *top*), similar to those reported earlier for MVBs within hepatocytes (8, 9). At this magnification the contents were indistinct, but each vesicle contained at least one and often several electron-dense structures. At higher magnification, these dense structures were seen to be bilayer vesicles, 0.05–0.07 μm in diameter (Fig. 4, *top*), thereby permitting designation of the organelles as MVBs. In addition, the pellets contained variable numbers of membranous structures, many of which were clearly attached to individual MVBs (Figs. 3, *top* and 4, *top*). At least two forms of these membranous appendages were seen: double bilayer sacs, some of which were almost as large as MVBs, but which lacked visible contents; and smaller, fingerlike protrusions that contained electron-opaque material (Figs. 3, *top* and 4, *top*). These appendages were more numerous near the bottom of the MVB pellet, whereas the MVBs were slightly larger near the top of the pellet (not shown). In occasional MVBs, the surface membrane was indented, suggesting an endocytic event (Fig. 4, *top*). The only other structures seen commonly in sections of the pellet were smaller, smooth-surfaced vesicles, which lacked electron-dense contents. Images of typical secondary lysosomes were rare.

By contrast, the Golgi membrane fraction from these livers was predominantly composed of flattened stacks of cisternae and secretory vesicles (Fig. 3, *bottom*). The secretory vesicles were smaller (~0.2–0.3 μm) than MVBs (Figs. 3, *bottom* and 5, *bottom*) and the bilayer vesicles that characterized MVBs were not seen. The flattened cisternae of the isolated Golgi fraction often occurred in stacks of three or four (Fig. 3, *bottom*), as occurs in hepatocytes of the ethinyl estradiol–treated animals (9).

In negatively stained preparations, the structure of MVBs was also distinctive. Morphologically intact MVBs had the same diameter as those in thin sections and they also had two distinct appendages that may correspond to those observed in thin sections (Figs. 4, *bottom* and 5, *top*). The content lipoproteins were not visible in the intact MVBs (Fig. 4, *bottom* and Fig. 5, *top*), but they were clearly visible in MVBs whose membrane had ruptured, permitting the phosphotungstate stain to enter (Figs. 4, *bottom* and 6). The diameter of the great majority of the content lipoproteins was 250–850 Å, the range expected for remnants of triglyceride-rich lipoproteins. In addition, a small number of particles ~200 Å in diameter was seen in ruptured MVBs, which could represent the injected ^{125}I-labeled LDL. The character of the different inclusions is better illustrated at higher magnification, as shown in Fig. 6. The larger, electron-lucent particles resemble triglyceride-rich lipoproteins obtained from blood plasma. The smaller, slightly darker particles resemble the injected LDL. Darker structures, 600–800 Å in diameter, which could be distinguished from the more numerous lucent particles, were consistently seen (Figs. 4, *bottom* and 6, white arrowheads). These darker structures probably are the bilayer vesicles that characterize MVBs in thin sections but that collapse in negatively stained preparations. We identified 10 to 25 of these structures in each of 20 collapsed MVBs, in which the internal vesicles were clearly distinguished from content lipoproteins (Figs. 4, *bottom* and 6). In addition, ruptured MVBs typically contained discoidal structures (Fig. 6). Like MVBs, ruptured secretory vesicles of Golgi fractions contained electron-lucent particles that resembled plasma VLDL but we did not see LDL-sized particles, bilayer vesicles, and discoidal structures (Fig. 5, *bottom*). The lipoproteins of $\rho < 1.008$ g/ml released from Golgi and MVB fractions by either the French pressure cell or by Na_2CO_3 were similar in shape and staining properties (Fig. 5, *top* and *bottom*), but those from Golgi fractions were somewhat larger (> 90% of particles were 300–700 Å in diameter) than those from MVB fractions (90% of particles were 200–600 Å in diameter).

Distinction of Content Lipoproteins and Membranes of MVBs from Those of the Golgi Apparatus

To distinguish lipoproteins of MVBs from those of the Golgi apparatus of hepatocytes, we isolated these organelles from livers of ethinyl estradiol–treated rats injected with ^{125}I-LDL and [^{3}H]palmitate, as described in Materials and Methods. As shown in Table II, MVBs contained 12% of the ^{125}I and 0.9% of the ^{3}H present in the original liver homogenate. When MVBs were ruptured by passage through the French pressure cell or treatment with sodium carbonate, 82–92% of the ^{125}I remained in the supernatant after the membranes were pelleted by centrifugation at 5.9×10^6 g_{av} min. VLDL, isolated from the supernatant by ultracentrifugation at $\rho = 1.008$ g/ml, contained only ~4% of the ^{3}H present in the MVB fraction (Table II).

Golgi apparatus–rich fractions contained 1.6% of the ^{125}I and 4% of the ^{3}H present in the original homogenate (Table II). As with MVB fractions, ~85% of the ^{125}I was released by rupture of membranous compartments of Golgi fractions. However, in contrast to MVBs, ~36% of the ^{3}H in Golgi fractions was associated with VLDL (Table II). In these Golgi VLDL, 90% of the ^{3}H was in neutral lipids, 7% in phospholipids, and 3% in cholesteryl esters. These data indicate that VLDL from MVB fractions contained very small amounts of the newly synthesized triglycerides found in nascent VLDL from Golgi fractions, whereas Golgi fractions, contained appreciable amounts of ^{125}I, a presumptive marker for contaminating MVBs.

To evaluate the extent of contamination of membranes of MVB fractions by membranes of the Golgi apparatus, we assayed galactosyltransferase activity. Whereas Golgi fractions contained almost one-half of the galactosyltransferase activity present in the original homogenate, MVB fractions contained < 0.1% (Table III), consistent with the lack of newly synthesized lipids in their content VLDL. In the procedure for purifying MVBs, only about 10% of the galactosyltransferase activity found in the original homogenate remained in the material added to the Percoll (see Fig. 1) and ~90% of this was found below fraction 5 in the Percoll gradient (Fig. 2). Both MVBs and Golgi fractions were depleted of arylsulfatase activity, as compared with the original homogenate (Table III). MVBs, but not Golgi fractions, were slightly enriched in glucose-6-phosphatase activity.

Chemical Characterization of VLDL and Membranes of MVB and Golgi Fractions

VLDL isolated from MVBs contained less triglycerides and more cholesteryl esters than those from Golgi fractions (Table

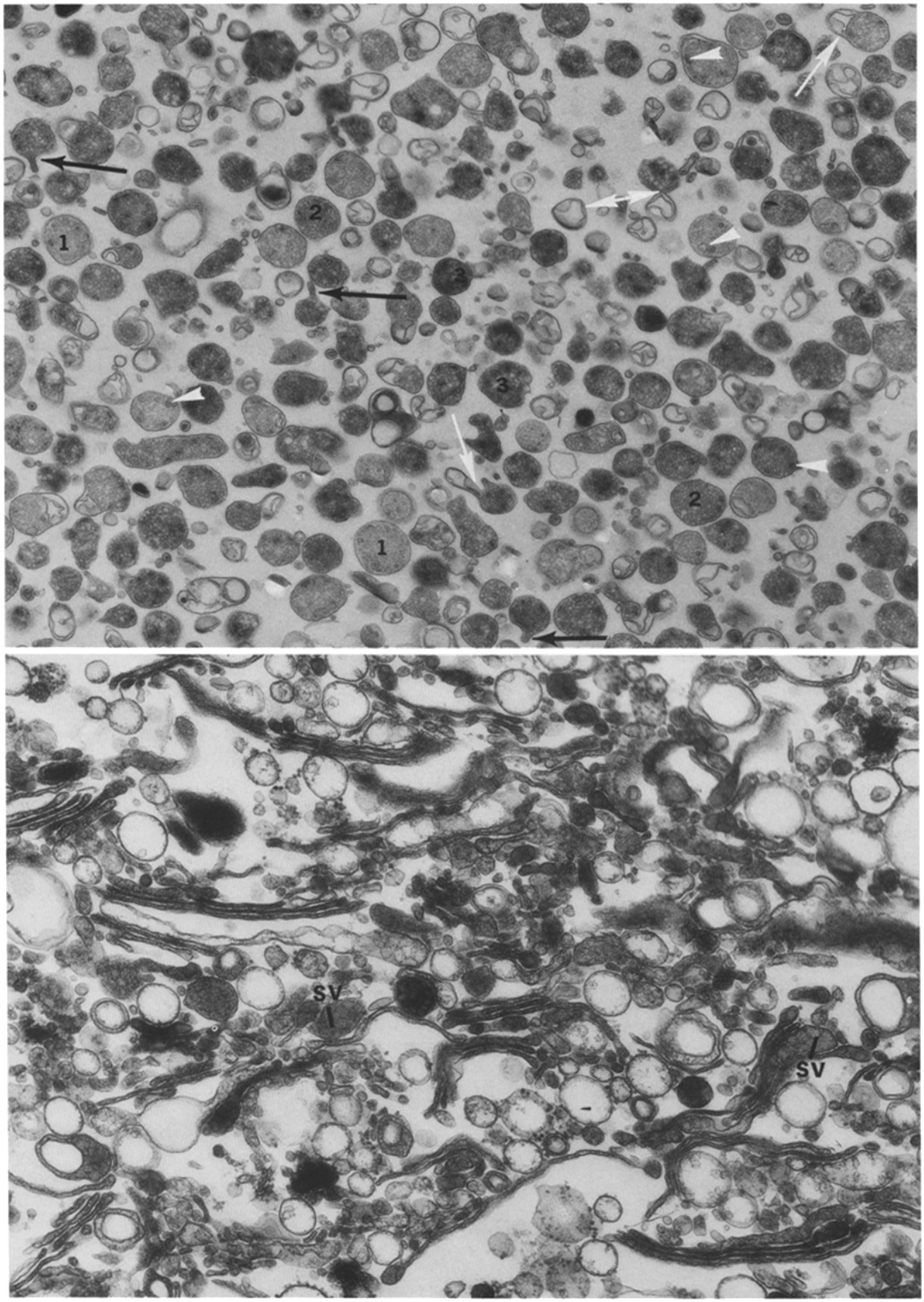

FIGURE 3 (*Top*) Thin section of field representative of the bulk of MVB pellet from estradiol-treated rats. Individual MVBs vary in diameter (0.4–0.75 μm) and electron density (e.g., *1*, *2*, and *3*). Each MVB contains from one to six small electron dense vesicles (white arrowheads). Additional membranous structures appear between MVBs; many of these are attached. Some of these appendages (white arrows) appear as large, double bilayer, apparently empty sacs. A second type of appendage is a smaller fingerlike projection with electron dense contents (black arrows). (*Bottom*) Thin sections of intact Golgi membrane compartment from estradiol-treated rat. The number of flattened cisternae that make up the Golgi stacks (three or four) is larger than in Golgi fractions from normal rat livers. The secretory vesicles (*SV*) that contain nascent VLDL particles are smaller than in normal livers and much smaller (diameter ~0.2–0.3 μm) than MVBs. (*Top*) × 15,000. (*Bottom*) × 30,000.

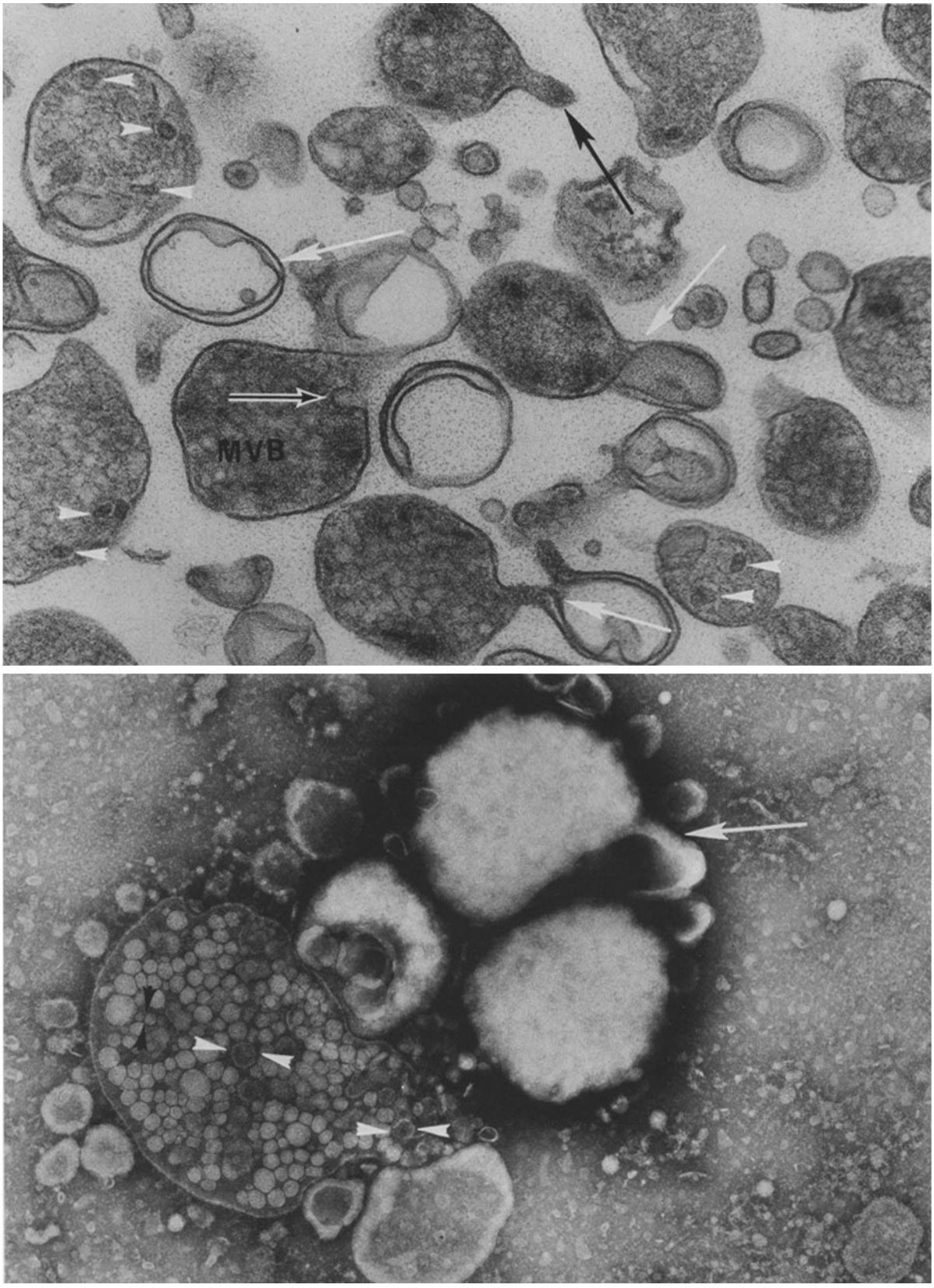

FIGURE 4 (*Top*) Higher magnification of a thin section of the MVB fraction shows lipid bilayer vesicles within each MVB (white arrowheads). The larger of the two types of membranous appendages appears between and attached to individual MVBs (white arrows). The smaller appendage contains electron dense material (black arrow). The bulk of the contents are remnants of triglyceride-rich lipoproteins from plasma that are indistinctly stained (see text). An occasional image shows an indentation of the MVB membrane (black and white arrow). (*Bottom*) Negative stain of MVB fraction shows two different images: intact MVBs are not penetrated with phosphotungstate and appear almost uniformly electron lucent; and ruptured MVBs show their contents due to entry of stain. The intact MVBs have membranous appendages (white arrow) that may correspond to the larger double bilayer structures seen in thin sections (*top*). The content lipoproteins are mostly particles in the size range of remnant VLDL and chylomicrons (diameter 250–800 Å), but some particles resembling LDL (diameter ~200 Å) can also be seen (black arrowheads). In addition, MVBs characteristically contain darker structures (white arrowheads) that correspond to collapsed bilayer vesicles. × 60,000.

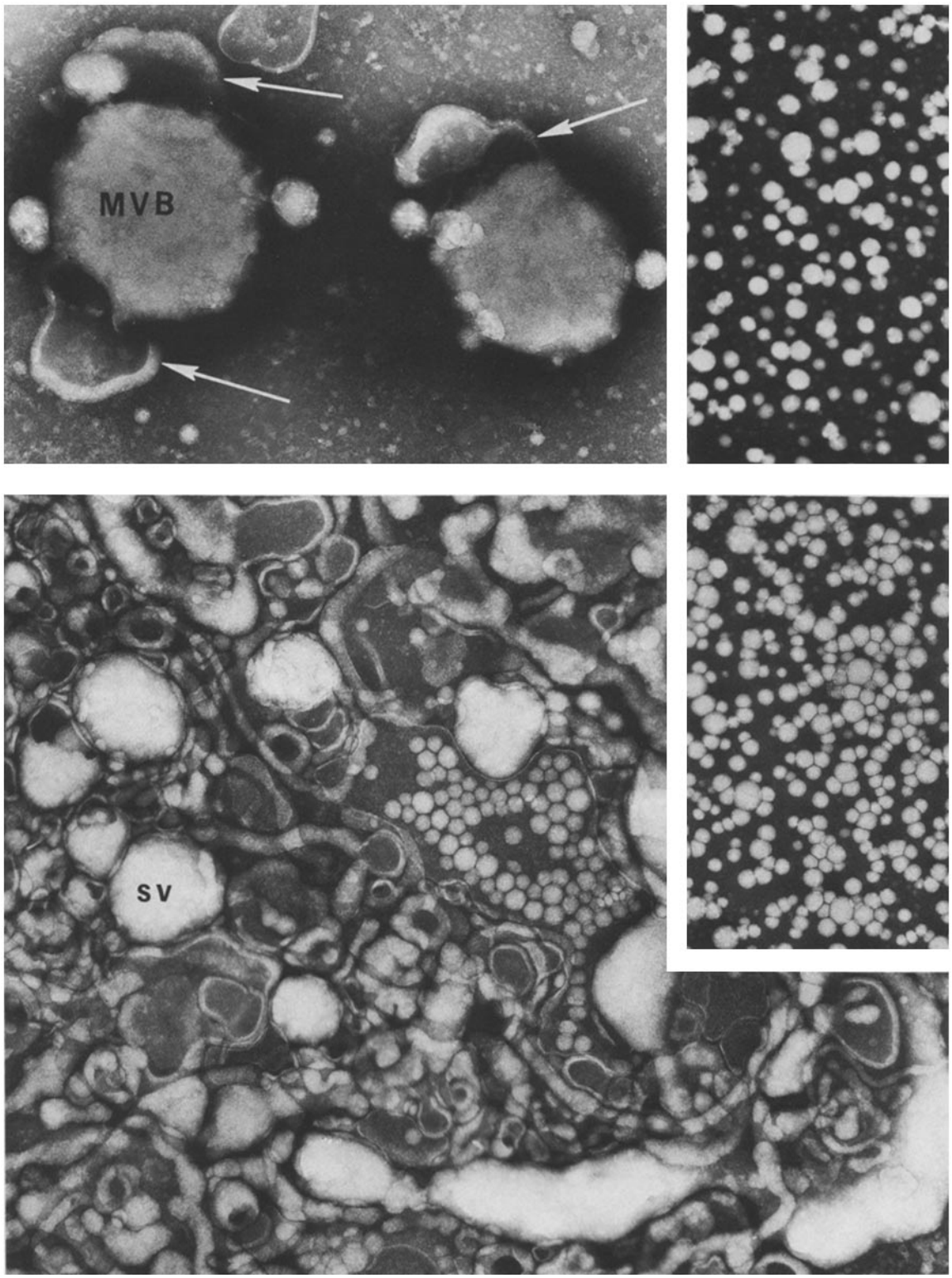

FIGURE 5 (*Top left*) Negatively stained, intact, isolated MVBs often contain two different membranous appendages. The larger ones (white arrows) may correspond to the double bilayer sacs of thin-sectioned preparations, whereas the smaller bleb-like attachments may correspond to the fingerlike projections of thin-sectioned MVBs. (*Top right*) The $\rho < 1.008$ g/ml lipoproteins released from MVB fractions by either French pressure cell of Na_2CO_3 treatment appear much like VLDL (average diameter ~390 Å). (*Bottom left*) Negatively stained intact Golgi fractions from livers of estradiol-treated rats appear similar to those from normal rat livers. Intact secretory vesicles (*SV*) are not penetrated by the stain (like intact MVBs) but are much smaller (diameter 0.2–0.3 μm) than MVBs (*top*) that average ~0.55 μm. A ruptured secretory vesicle contains only nascent VLDL particles (average diameter ~450 Å) and lacks the 200 Å LDL-sized particles and the collapsed vesicles that typify MVB contents. (*Bottom right*) The $d <$ 1.008 g/ml lipoprotein fraction released from Golgi membranes by the French pressure cell contains particles (nascent VLDL) that closely resemble plasma VLDL. × 60,000.

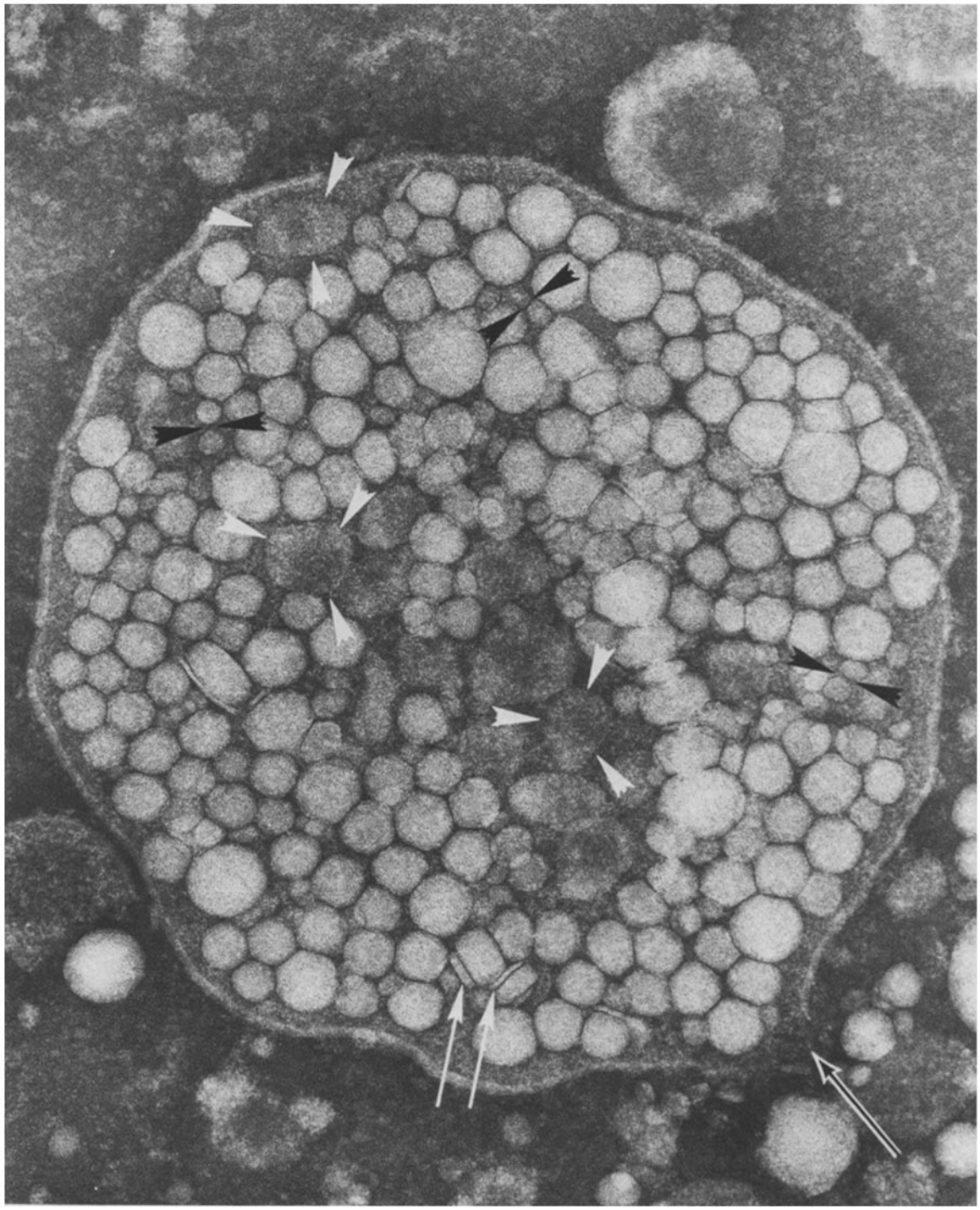

FIGURE 6 A higher magnification shows the rupture (black and white arrow) of the membrane of a single MVB which reveals the contents clearly in this negatively stained preparation. Most of the contents are electron-lucent particles 250–800 Å in diameter, typical of remnants of triglyceride-rich plasma lipoproteins. Several 200 Å, slightly darker particles of LDL size are also evident (black arrowheads). A number of disc-shaped structures also characterize MVB contents (white arrows). However, the most distinctive morphologic characteristic of ruptured MVBs are darker structures that represent collapsed vesicles (diameter 500–800 Å) unique to MVBs (white arrowheads). × 180,000.

IV). Content of phospholipids was similar, but VLDL from MVBs contained much more (free) cholesterol and more protein. In SDS gel electrophoretograms, both forms of apolipoprotein (apo) B (apo B-100 and B-48) could be identified in VLDL from both sources, together with a component with the mobility of apo E (not shown). The isoelectric focussing pattern of apo E in the two VLDL preparations was similar (Fig. 7). The bulk of the ^{125}I released by treatment in the French pressure cell or by treatment with sodium carbonate was isolated between densities of 1.008 and 1.063 g/ml. After solubilization in SDS, most of this ^{125}I had the electrophoretic mobility of apo B-100 (Fig. 8).

The composition of the membranes from MVB and Golgi apparatus fractions was determined after release of contents

as described in Materials and Methods. MVB membranes contained much more (free) cholesterol and less protein than did Golgi membranes (Table IV). The molar ratio of cholesterol to phospholipids in MVB membranes was 0.46:1, whereas that in Golgi membranes was 0.17:1.

DISCUSSION

MVBs are a Distinct Organelle of the Receptor-mediated Endocytic Pathway

The morphologic and functional properties of MVBs isolated in this research confirm our earlier autoradiographic observations (8, 9) on the role of MVBs as a major prelysosomal organelle in the pathway of degradation of LDL in hepatocytes of estradiol-treated rats. The MVBs isolated here appear to be analogous to those identified in the pathway of catabolism of macromolecules in a variety of cells (30–39), all of which are characterized by internal vesicles 500-800 Å in diameter.

The paucity of newly synthesized palmityl esters in VLDL isolated from ruptured MVBs and the low activity of galactosyltransferase in MVB fractions indicate that the MVB fractions are almost free from Golgi contamination. Both negatively stained and fixed thin-sectioned preparations of MVB fractions show that MVBs, which resemble those identified in intact livers of estradiol-treated rats, are the predominant organelle present. Secretory vesicles of the Golgi apparatus resemble MVBs morphologically, but they lack the characteristic internal vesicles, which can be seen in most of the lipoprotein-filled vesicles in MVB fractions. Golgi secretory vesicle fractions from livers of normal rats are enriched in galactosyltransferase as compared with Golgi cisternae (40). This would seem to exclude significant contamination of MVB fractions with secretory vesicles, unless the content of the enzyme in secretory vesicles is drastically reduced by treatment of rats with ethinyl estradiol. Contamination of MVB fractions with secondary lysosomes is also very small, as indicated by the low levels of arylsulfatase activity and the rarity of typical secondary lysosomes in electron photomicrographs. The smaller vesicles seen in MVB fractions (Fig. 3, *top*) may represent contaminating smooth endoplasmic reticulum or other prelysosomal compartments of the endocytic

TABLE II

Recovery of ^{125}I-LDL and ^{3}H-Palmitate in Organelles and VLDL

Fraction	Isotope in total homogenate*	
	^{125}I	^{3}H
	%	
MVB	12.1 ± 4.1	0.9 ± 0.3‡
VLDL from MVB fraction	0.16 ± 0.05	0.034 ± 0.01
Golgi	1.6 ± 0.8	4.0 ± 0.6‡
VLDL from Golgi fraction	0.020 ± 0.01	1.43 ± 0.3

* Values are mean ± SD for six experiments.
‡ Represents ^{3}H in membranes (mainly phospholipids) as well as contents of organelle.

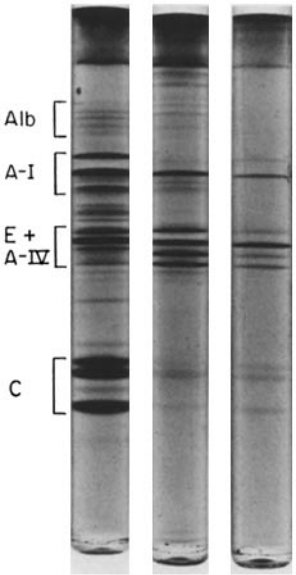

FIGURE 7 Isoelectric focussing gel electrophoretograms of proteins of (from left) chylomicrons from rat lymph; $\rho < 1.008$ g/ml lipoproteins from Golgi apparatus fraction; and $\rho < 1.008$ g/ml fraction from MVBs. In the middle and right gels, the multiple components in the region labeled E + A-IV are typical of rat apo E, as isolated from blood plasma (80). *Alb*, albumin.

TABLE III

Marker Enzyme Activity in Homogenate and Isolated Fractions

Fraction	Galactosyltransferase		Arylsulfatase		Glucose-6-phosphatase	
	Specific activity	% Total	Specific activity*	% Total	Specific activity	% total
Homogenate	10.16 ± 3.0 (5)‡	100	1.64 ± 0.096 (4)	100	0.051 ± 0.04 (4)	100
MVB	1.19 ± 1.7 (5)	0.08 ± 0.11	0.073 ± 0.017 (4)	0.035 ± 0.01	0.090 (2)	0.19
Golgi	630 ± 112.8 (3)	45.9 ± 18.7	0.055 ± 0.269 (4)	0.078 ± 0.05	0.025 (2)	0.12

* Specific activities are expressed as follows: galactosyltransferase, nanomoles galactose transferred per hour per milligram protein; arylsulfatase, micromoles paranitrocatechol per hour per milligram protein; glucose-6-phosphatase, micromoles phosphate released per minute per milligram protein.
‡ Number of preparations is indicated in parentheses.

TABLE IV

Composition of VLDL and Membranes (% Mass)

Fraction	Cholesteryl esters	Triglycerides	Cholesterol	Phospholipids	Protein
VLDL					
MVB ($n = 4$)	18.3 ± 3.6	36.7 ± 4.5	5.4 ± 1.5	20.1 ± 2.3	19.2 ± 2.9
Golgi ($n = 3$)	12.8 ± 2.4	53.4 ± 2.2	1.5 ± 0.58	21.5 ± 1.0	10.6 ± 0.26
Membranes					
MVB ($n = 3$)	0.91 ± 0.01	7.1 ± 2.4	13.0 ± 0.8	56.2 ± 4.0	22.6 ± 1.0
Golgi ($n = 3$)	0.57 ± 0.15	5.6 ± 3.2	3.7 ± 0.4	43.5 ± 2.4	46.8 ± 1.5

Values are mean ± SD. Contents were released by disruption of fractions in a French pressure cell except for one preparation of the MVB fraction, which was treated with sodium carbonate.

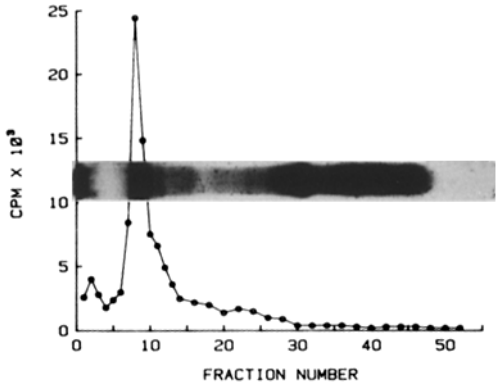

FIGURE 8 Distribution of ^{125}I in sections (2 mm) cut from SDS gel electrophoretogram of proteins of $\rho < 1.063$ g/ml lipoproteins isolated from MVB ruptured in a French pressure cell. The values for ^{125}I are shown in relation to the photograph of a duplicate electrophoretogram of the proteins stained with Coomassie Brilliant Blue. Human LDL (20 μg protein) was added to the lipoproteins to mark the mobility of apo B-100. Most of the ^{125}I is associated with the apo B-100 band. In this experiment, no protease inhibitors were used.

pathway. The latter could contain some of the ^{125}I-LDL, but the volume of such vesicles must be much smaller than that of the typical large MVBs. Such vesicles may account for a larger fraction of the ^{125}I in higher density regions of the Percoll gradients (Fig. 2).

The reasons that MVBs have not been identified consistently in the receptor-mediated catabolic pathway of macromolecules are unclear but could be related to inadequate staining of these internal bilayer vesicles, their absence from the plane of section, or classification of MVBs as secondary lysosomes. In hepatocytes of estradiol-treated rats, MVBs are consistently larger and more numerous than in untreated rats, and they are filled with lipoproteins with properties consistent with those of remnants of VLDL and chylomicrons that are known to be taken up by receptor-mediated processes in rat liver (41). The presence of these particles, 250-800 Å in diameter, facilitates the identification of MVBs by electron microscopy of thin sections of fixed tissues. The same pathway has also been observed for LDL (8) and for chylomicrons and VLDL (42) in autoradiographic studies of livers of untreated rats, with no qualitative or quantitative differences from the pathway of LDL in estradiol-treated animals. In all cases, MVBs contain 20–35% of the ^{125}I-labeled lipoprotein in the liver 15-30 min after injection and are observed predominantly, but not exclusively, in the bile canalicular region of the cell, typically adjacent to the trans face of Golgi stacks. Very recently, MVBs have also been identified as a major prelysosomal compartment in the receptor-mediated endocytic pathway of galactosylated serum albumin coupled to colloidal gold in cultured rat hepatocytes (43).

In normal rat hepatocytes the location of MVBs adjacent to the Golgi apparatus and the similarity of their size and staining properties to those of secretory vesicles of Golgi cisternae, together with the similar lipoprotein contents of the two organelles, makes it difficult to distinguish the two organelles in thin sections of liver. MVBs in this region of hepatocytes appear to have been described as components of GERL (44) or as residual bodies (45).

A number of investigators have concluded, from autoradiographic and cell-fractionation studies of endocytosed ligands, that the Golgi apparatus is a way-station between endosomes and lysosomes in the macromolecular catabolic pathway in liver (46) and various cultured cells (47, 48). More recently, Bergeron et al. (49) suggested that the lipoprotein-filled vesicles in the trans-Golgi region of hepatocytes may be a separate organelle of the endocytic pathway.

In normal rats, > 90% of VLDL particles secreted from the liver are taken up into the liver a few minutes later as VLDL remnants (41). Chylomicron remnants are also taken up almost entirely by the liver. The presence of two lipoprotein-filled vesicular structures in liver must be considered in relation to reported properties of hepatic Golgi fractions. Images of some vesicular structures in light Golgi fractions (GF_1 and GF_2) isolated from livers of ethanol-intoxicated rats (50) closely resemble MVBs. Those Golgi fractions are separated by sucrose-density centrifugation of liver homogenates prepared in a motor-driven homogenizer (51) which dissociates secretory vesicles from Golgi cisternae. By contrast, the preparation of Golgi fractions by the method of Morré et al. (15) begins with rupture of the plasma membrane by a Polytron apparatus operated at slow speed, which leaves Golgi apparatus largely intact. Even so, our Golgi fractions, prepared by a modification of this method, appeared to be contaminated to some extent with MVBs. The extent to which GF_1 and GF_2 and Golgi fractions prepared by other methods are contaminated by MVBs remains to be determined by experiments analogous to those reported here, in which the secretory and endocytic compartments are labeled differentially.

Like VLDL from liver perfusates of estradiol-treated rats (52), the lipoprotein fractions ($\rho < 1.008$ g/ml) from the Golgi and MVB fractions alike contained much more cholesteryl ester than did the fraction, perfusate, and plasma VLDL from normal rats (16, 53). The enrichment of VLDL from MVBs with cholesterol, cholesteryl esters, and protein, as compared with nascent Golgi VLDL, is expected from the prior exposure of the former to cholesterol-rich membranes in the blood (such as those of erythrocytes) and the action of lipoprotein lipase upon them to form "remnants." The small difference in size of the VLDL fractions from MVB and Golgi fractions may be explained by the presence of larger chylomicron remnants as well as VLDL remnants in the MVB fraction. As expected, VLDL from both organelles contained relatively more apo E than C apolipoproteins. The latter are known to be added to nascent VLDL after secretion and to be removed from them in blood as remnants are formed (41).

Chemical Properties of MVB Membranes

The high cholesterol–phospholipid ratio in MVB membranes is consistent with their derivation from the sinusoidal plasmalemma (54, 55). Recent studies suggest that the absence of digitonin or filipin marking of membranes of coated pits (56, 57) may reflect the presence of associated clathrin rather than the absence of cholesterol (58). Other studies show that coated vesicles and endosomes contain appreciable amounts of cholesterol (6, 59–62). By whatever mechanisms plasma membrane is brought into the cell by endocytosis, some investigators think that a portion recycles through the Golgi apparatus, mainly via trans-cisternae (4, 63). Such recycling is not incompatible with the low ratio of cholesterol to phospholipid that we found for the total Golgi fraction. Orci et al. (64) found that trans-cisternae contain more cholesterol than do cis-cisternae.

Relative to membrane lipids, the MVB fraction contained considerably less protein than did sinusoidal plasmalemma (54, 55) or Golgi fractions, as we observed in this study. This can account in part for the low density of MVBs, although the low density of content lipoproteins presumably contributes as well. In recent years, prelysosomal organelles of low density have been described frequently as intermediate structures of the receptor-mediated endocytic pathway (39, 48, 65–71). Our observations suggest that these may include MVBs as well as earlier prelysosomal structures (endosomes, receptosomes, sorting vesicles) located mainly near the cell surface. Very recently, a density-shift method has been used to purify a low density fraction from rat liver homogenates that contained galactosylated bovine serum albumin conjugated to horseradish peroxidase (72, 73). The most highly purified fractions were poor in lysosomal enzyme markers and galactosyltransferase and contained mainly spheroidal organelles, ~0.35 μm in diameter. Their contents could not be discerned by electron microscopy, probably because they were obscured by dense reaction product, but we believe that these organelles from normal rat livers are MVBs. This preparation (72) and ours were separated from liver homogenates at low density. This procedure may yield mainly MVBs that have not received an appreciable complement of lysosomal enzymes. ^{125}I-LDL in our homogenates may be present in denser organelles (see Fig. 2), which might be recognized as MVBs morphologically but in which appreciable degradation of the ligand has occurred.

Significance of the Morphologic Features of MVBs

Several morphologic features that characterize MVBs are noteworthy. The internal vesicles are thought to be formed from the limiting membrane of MVBs by pinocytosis (30, 33). Our observations of occasional images that suggest an endocytic event in isolated MVBs are consistent with such a process.

The interior of MVBs can be presumed to have a pH close to 5 (74), as has also been observed for all other identified compartments of the receptor-mediated endocytic pathway (75, 76). The remnant lipoproteins that fill MVBs may have dissociated from the LDL receptor, as predicted from the low pH in the vesicle. However, they appear to have undergone little proteolytic or lipolytic breakdown. The isoforms of apo E observed in VLDL from Golgi and MVB fractions, which are thought to reflect primarily variable degrees of sialylation of the parent protein (77), were virtually indistinguishable; this indicates that neither the carbohydrate nor the polypeptide backbone of apo E has been hydrolyzed. The B-100 moiety of the ^{125}I-LDL injected to mark MVBs was also found to be largely intact, as judged by its mobility in polyacrylamide gels, even when protease inhibitors were not added during the isolation procedure. Thus, the MVBs that we have isolated evidently contain uncoupled ligands (VLDL and chylomicron remnants and LDL) which have not been subjected to extensive action of acid hydrolases. Our previous autoradiographic observations, however, suggest that such hydrolysis begins during the next 15 to 45 min (8, 9).

The two types of appendages of isolated MVBs are particularly intriguing. The fingerlike appendages, whose contents are electron opaque, have been observed in intact liver (8), but the larger saclike appendages were not seen. Similar sacs or flaps have been described in incubated lysosomal fractions from rat liver (78, 79), and it has been suggested that they are related to microautophagy. One or both of these appendages could be related to CURL (compartment of uncoupling of receptor and ligand), as described recently in rat hepatoma cells endocytosing asialoglycoproteins, by Geuze et al. (7, 38). However, CURL tubules have been found mainly in the subsinusoidal region, not in the bile canalicular region. Application of the techniques described by Geuze et al., together with measurements of binding of LDL and other ligands to MVBs, should provide information about this possible function of the appendages.

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