Isolation and characterization of three endosomal fractions from the liver of normal rats after lipoprotein loading

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Abstract In earlier research we isolated and characterized three endosomal fractions from livers of estradiol-treated rats (1987. Belcher et al. *Proc. Natl. Acad. Sci. USA.* **84:** 6785; 1989. Jäckle et al. *Proc. Natl. Acad. Sci. USA.* **86:** 1880). We now describe the isolation of comparable endosomal fractions from untreated rats. The fraction of lowest density was composed almost exclusively of lipoprotein-filled multivesicular bodies (MVBs); the intermediate density fraction was composed of smaller lipoprotein-filled vesicles that were also multivesicular and had more numerous membranous appendages; the fraction of highest density was composed of membranes resembling the appendages of the two vesicular fractions. The paucity of contamination of these fractions with nonendosomal organelles is supported by their ultrastructural characteristics and by the proteins, lipids, and marker enzymes of their membranes. Only small amounts of MVBs could be separated from untreated rats not given a load of lipoproteins. However, after injection of large amounts of β-very low density lipoproteins (β-VLDL) from cholesterol-fed rabbits, the mass of MVBs increased dramatically. Under these conditions radiolabeled β-VLDL and epidermal growth factor taken up by the liver accumulated in isolated endosomes at rates similar to those found for LDL in estradiol-treated rats. Although chylomicrons and chylomicron remnants were rapidly taken up by the liver of normal rats, chylomicrons and chylomicron remnants accumulated in endosomes at a lower rate than β-VLDL. These findings, which differ from earlier data in estradiol-treated rats (1989 Jäckle et al., *Proc. Natl. Acad. Sci. USA.* **86:** 1880) that showed equivalent rates of processing of chylomicron remnants and β-VLDL, suggest that extracellular processing of chylomicron remnants in the liver normally precedes endocytosis. —**Jäckle, S., E. Runquist, S. Brady, R. L. Hamilton, and R. J. Havel.** Isolation and characterization of three endosomal fractions from the liver of normal rats after lipoprotein loading. *J. Lipid Res.* 1991. **32:** 485–498.

Supplementary key words multivesicular bodies • Golgi apparatus • β-very low density lipoproteins • chylomicrons

The terminal catabolism of many macromolecules, including polypeptide hormones, lipoproteins, asialoglycoproteins, immunoglobulins, and transport proteins, occurs chiefly in the liver after receptor-mediated endocytosis (1). In earlier research, we have used estradiol-treated rats, in which low density lipoprotein (LDL) receptors are expressed at a high level, to define the intracellular pathway of several receptors and their ligands (2–6). Three distinct endosomal fractions were isolated from livers of estradiol-treated rats; the lipid composition and major proteins of their membranes were similar and distinct from those derived from the Golgi apparatus (2). Lipoproteins, epidermal growth factor, and other ligands accumulate first in the endosome fraction of intermediate density, designated compartment of uncoupling of receptor and ligand (CURL), first described by Geuze et al. (7) and subsequently in a fraction of low density (multivesicular bodies, MVBs), which appear to represent the immediate prelysosomal compartment (1, 8). The endosome fraction of highest density, the membranes of which resemble the appendages of the endosomes in the MVB and CURL fractions, was designated as a receptor recycling compartment (RRC), because of its high concentration of recycling receptors.

In estradiol-treated rats, separation of endosomal fractions was facilitated by loading with human LDL. Under these conditions, MVBs increased in size and could readily be separated on a density gradient from early endosomes in the CURL fraction (2). Such loading cannot be

Abbreviations: MVBs, multivesicular bodies; β-VLDL, β-very low density lipoproteins; LDL, low density lipoproteins; CURL, compartment of uncoupling of receptor and ligand; RRC, receptor recycling compartment; LRP, LDL receptor-related protein; EGF, epidermal growth factor; DAB, 3,3'-diaminobenzidine; DFP, diisopropylfluorophosphate; ASGP, asialoglycoprotein.

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achieved in untreated rats by injecting LDL, because of the much lower concentration of LDL receptors on hepatocytes. We have found that β-VLDL, which are readily obtained from the blood of cholesterol-fed rabbits and have a much higher affinity for the LDL receptor than human LDL (4), are readily taken up into the liver of untreated rats and can readily load hepatocytic endosomes. We have thus been able to obtain endosomal fractions from untreated rats that are comparable to those obtained previously from estradiol-treated animals. In our previous work, we also showed that the endosomal trafficking of β-VLDL and rat chylomicron remnants in estradiol-treated rats closely resembled that of LDL (4), consistent with uptake by the LDL receptor (9) or a similar endocytic receptor. Recent studies have raised the possibility that a newly discovered protein, the LDL receptor-related protein (LRP), may participate in chylomicron remnant uptake (5, 10–12). Indeed, we have found that the enrichment and relative distribution of LRP in hepatic endosomal membranes resembles that of the LDL receptor (5). Unlike the LDL receptor, however, the hepatic concentration of LRP is not affected by estradiol (5). We have therefore exploited our ability to separate endosomal fractions to evaluate the endosomal trafficking of chylomicron remnants in the liver of untreated rats. Unexpectedly, we obtained evidence that endocytosis of chylomicron remnants taken up by the liver occurs more slowly in these animals than in those treated with estradiol.

MATERIALS AND METHODS

Reagents

Epidermal growth factor (EGF) (isolated from mouse submaxillary glands by the method of Savage and Cohen (13)) was from Collaborative Research, Inc., Bedford, MA); 17-α-ethinyl estradiol was from Sigma (St. Louis, MO); and sodium ^{125}I iodide (carrier-free) was from Amersham/Searle (Arlington Heights, IL).

Animals

Male Sprague-Dawley rats (250–300 g) were fed Ralston Purina standard chow. Experiments were begun in the morning. Male New Zealand white rabbits weighing 2.5–3.0 kg were fed Purina Lab rabbit chow containing 1% cholesterol by weight for 3–4 weeks.

Preparation of labeled proteins and lipoproteins

Human low density lipoproteins (LDL) ($1.025 < d < 1.050$ g/ml) were isolated from blood of normolipidemic adult humans by sequential ultracentrifugation (14) and purified by recentrifugation at the upper density limit. Beta-very low density lipoproteins (β-VLDL) ($d < 1.006$ g/ml) were isolated from the blood of cholesterol-fed rabbits (15). The core of these lipoproteins is composed chiefly of cholesteryl ester (about 65% of particle mass) with little triglyceride (about 5% of mass) (16). The apoB of these particles is mainly B-100 with little B-48 (17) and apoE is the other major apolipoprotein, usually comprising about 50% of the protein mass (R. J. Havel and L. Kotite, unpublished data). The molar ratio of apoE to apoB is 10–20:1. Particle diameters range from 300 to 800 Å (median ~400 Å). Uptake of β-VLDL into the liver does not require prior lipolysis in extraheptic tissues. To obtain small chylomicrons from rats with an intestinal lymph fistula, 50 ml of 0.15 M NaCl containing 5 g glucose was infused into the duodenum for 25 h while lymph was collected (18). Chylomicrons were isolated by centrifugal flotation at 3×10^7 *g*-min (19). Chylomicron remnants were produced in vivo by injecting lymphatic chylomicrons into a femoral vein of functionally eviscerated rats (19) or in vitro by incubation of lymphatic chylomicrons with the $d > 1.019$ g/ml plasma fraction obtained after injection of heparin (9). Unlike β-VLDL, the core of small chylomicrons is composed chiefly of triglycerides (about 73% of particle mass) with little cholesteryl ester (about 4% of mass) (18). The apoB of these particles is exclusively B-48 (20) and apoE comprises about 4% of protein mass (18). The particle diameters range from 250 to 1000 Å (median ~500 Å). Chylomicron remnants are also triglyceride-rich particles (21), but apoE is a prominent protein, comprising up to 45% of the protein mass (22). They are smaller than their parents (median diameter is ~400 Å). Hepatic uptake of chylomicrons is limited, requiring prior hydrolysis of triglycerides and phospholipids in extrahepatic tissues to form remnants, whereas chylomicron remnants are readily taken up by the liver (19).

Lipoproteins were labeled with ^{125}I by the iodine monochloride method of McFarlane (23) as previously described (24). To obtain isotopically labeled chylomicrons, 10 μCi [^{3}H]cholesterol dispersed in 10 ml 0.15 M NaCl containing 0.5 g bovine serum albumin was infused into the duodenum of rats prepared with an intestinal lymph fistula (19). EGF was labeled with ^{125}I by a modification of the method of Hunter and Greenwood (25).

Isolation of organelles

For the isolation of endosome fractions from untreated rats, food was withdrawn 20 h before the experiment. Routinely, three rats were anesthetized with diethyl ether, and lipoproteins were injected into the femoral vein. At

TABLE 1. Recovery of ^{125}I in endosomes 15 min after injection of labeled lipoproteins or EGF

Fraction	^{125}I-β-VLDL + LDL[a]	[^{3}H]Chylomicrons + Chylomicrons[b]	[^{3}H]Chylomicron Remnants + Chylomicron Remnants[c]	^{125}I-β-VLDL + β-VLDL[d]	^{125}I-EGF + Chylomicrons[e]	^{125}I-EGF + β-VLDL[f]
			% of liver homogenate			
Total endosomes	7.3	5.4	6.9	9.1 ± 2.5	10.0 ± 2.9	14.5
MVBs	0.18 (15)[g]	0.24 (21)	0.61 (48)	2.58 ± 1.06 (123)	0.13 ± 0.06 (7.9)	2.00 (72)
CURL	3.65 (91)	1.78 (63)	1.78 (53)	2.82 ± 0.77 (75)	1.50 ± 0.92 (35)	4.00 (64)
RRC	1.51 (22)	1.08 (22)	1.67 (45)	0.59 ± 0.17 (12)	3.90 ± 1.2 (42)	2.40 (19)

[a]Injected 5.8 mg LDL protein into each of three rats.
[b]Injected 1.6 mg protein into each of three rats.
[c]Injected 0.47 mg protein into each of three rats.
[d]Injected 1.7–3.4 mg protein into each of three rats (10 experiments; mean ± SD).
[e]Injected ^{125}I-EGF (0.5 μg protein) and 0.3–1.8 mg chylomicron protein into each of three rats (three experiments; mean ± SD).
[f]Injected ^{125}I-EGF (0.5 μg protein) and 3.5 μg β-VLDL into each of three rats.
[g]Values in parentheses are fold purification over homogenate, based on total protein of endosomes (membranes + content).

specified times thereafter, the livers were perfused through the portal vein with 50 ml 0.15 M NaCl, then removed, homogenized, and pooled. Endosomes and endosomal membranes were isolated exactly as described for estradiol-treated rats (2). To prevent rebinding of lipoproteins to endosomal membranes, 1 mM suramin was added before endosomal vesicles were ruptured in a French pressure cell (2). Golgi-rich fractions and lysosomes were isolated from rat livers as described previously (3, 26).

Analytical procedures

Protein was measured by the method of Petersen (27). Phospholipids (28), cholesterol (29), sialyltransferase (30), glucose-6-phosphatase (31), and arylsulfatase (32) were measured in membrane fractions. Membrane proteins were separated by NaDodSo$_4$/PAGE (33) and stained with Coomassie Blue.

Electron microscopy

Pellets of intact endosome fractions were prepared for thin sectioning and negative staining, as described previously (3).

RESULTS

Endosomal uptake of lipoproteins in normal rat liver

The increased number of hepatocytic LDL receptors in rats treated with estradiol results in a high rate of receptor-mediated endocytosis, which facilitates the isolation of endosomes from liver with little contamination with Golgi elements, endoplasmic reticulum, and lysososmes (2). The separation of endosomes from other organelles is based on the low density of the lipoprotein-filled endosomal vesicles. After injection of large amounts of LDL, the size of multivesicular bodies (MVBs), representing late endosomes, increases, permitting their separation from smaller early endosomes.

In a preliminary experiment, we isolated endosomes from livers of three rats injected with a large amount of human LDL (5.8 mg protein) and trace amounts of ^{125}I-labeled rabbit β-VLDL. A substantial fraction (29%) of the injected radioiodine was recovered in livers taken 15 min after injection. Recovery of ^{125}I in the combined endosomal fraction, obtained by sedimenting low density membranes obtained from the Percoll gradient step of our procedure, was 7.3% of that present in the liver. This is somewhat lower than the value of 12.4% found in this fraction when radioiodinated LDL was injected with a load of 1.0-1.5 mg of LDL-protein into estradiol-treated rats (3).[3] When the endosomes were fractionated on a discontinuous sucrose gradient, very few endosomal vesicles were recovered at the lowest density interface characteristic of MVBs in estradiol-treated rats, and these vesicles contained only 2% of the endosomal ^{125}I (**Table**

[3]In estradiol-treated rats given a load of LDL, one-third to one-half of the hepatic radioiodine is recovered in the supernatant fraction (supernatant 3) applied to the Percoll gradient, in which endosomes are recovered in the top portion of the tube after centrifugation (3). The Percoll top fraction contains most of radioiodine present in supernatant 3, but less than one-half of this is recovered in the sedimented endosomes, termed "MVB band" in reference 3. Any lipoproteins free in the Percoll top fraction (released from ruptured endosomes or originally associated with cell surface) will fail to sediment with the endosomes, resulting in considerable losses at this step. Similar losses occurred with this and other protocols used to load endosomes in untreated rats in the current study.

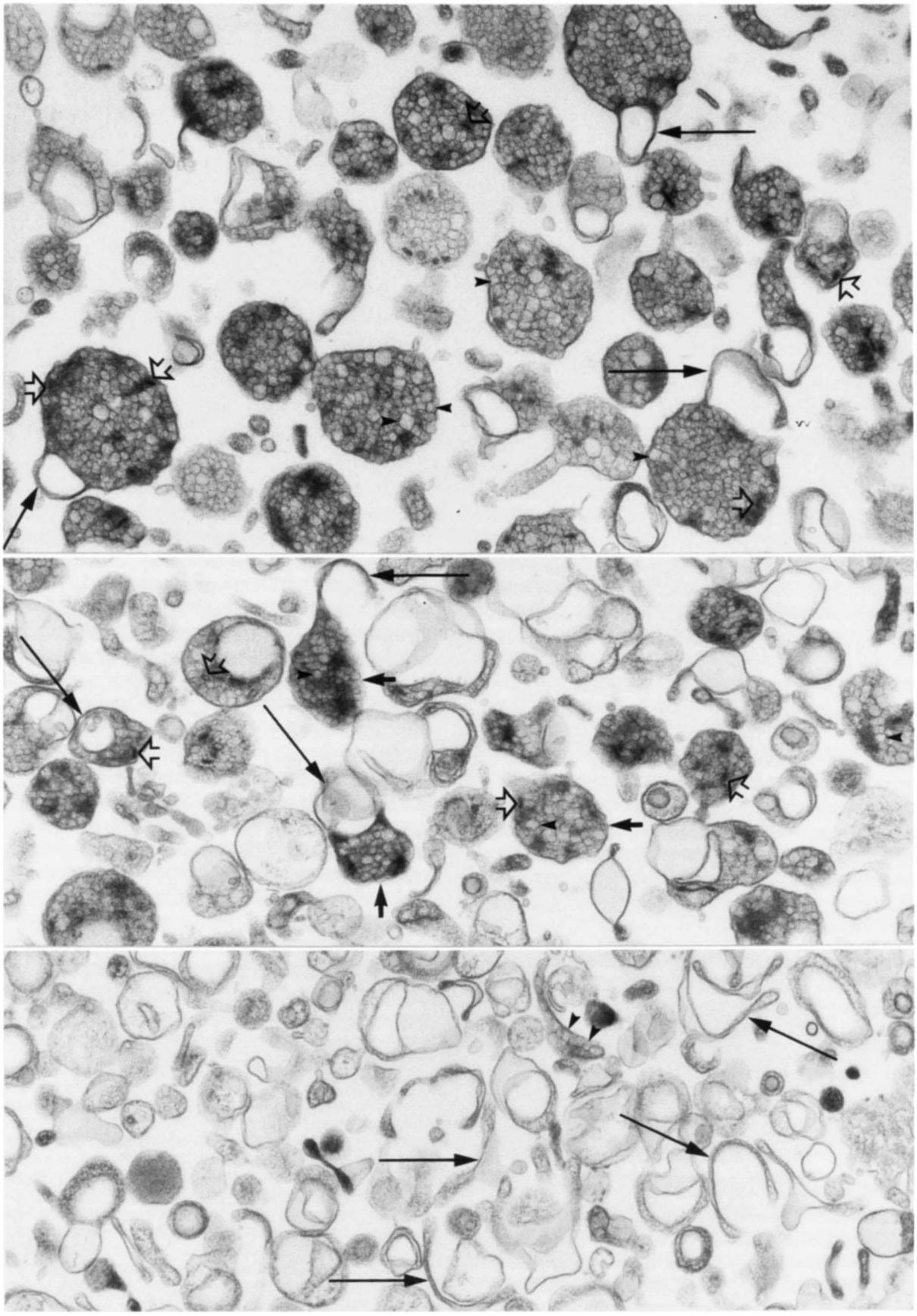

TABLE 2. Recovery of ^{125}I and protein in endosomal fractions after injection of ^{125}I-labeled β-VLDL

Fraction	Low Density (MVBs)		Intermediate Density (CURL)		High Density (RRC)	
Min after injection	15[a]	30[b]	15[a]	30[b]	15[a]	30[b]
^{125}I (% of homogenate)	2.58 ± 1.06 (123)	4.89 ± 0.42[c] (128)	2.82 ± 0.77 (75)	2.85 ± 0.62 (61)	0.59 ± 0.17 (12)	0.59 ± 0.05 (10)
Total endosomal membrane protein (μg)	205 ± 90	515 ± 80[c]	476 ± 130	862 ± 133[c]	844 ± 312	1151 ± 100

Results are given as mean ± SD. Values in parentheses indicate fold purification over homogenate, based on total protein of endosomes.
[a]Injected 3.0 ± 0.7 mg protein; n = 10.
[b]Injected 3.6 mg protein; n = 4.
[c]Significantly different from 15 min value ($P < 0.005$ by two-tailed t test).

1). Most of the recovered ^{125}I was in the endosomal fraction of intermediate density, and the remainder was in the high density fraction. The intermediate density fraction, by electron microscopy, was composed mainly of lipoprotein-filled vesicles, and the high density fraction was composed of membranous fragments and small vesicles (not shown). When rats were injected with a large amount of rat [^{3}H]cholesterol-labeled chylomicrons (1.6 mg protein/rat) or [^{3}H]cholesterol-labeled chylomicron remnants (0.47 mg protein/rat), 38 and 55%, respectively, of the injected ^{3}H was recovered in livers taken 15 min later. Recovery of ^{3}H in the total endosomal fraction was 5.4% and 6.9%, respectively, of that present in the liver. Less than 10% of the endosomal ^{3}H was recovered in the low density fraction (Table 1).

When increasing amounts of ^{125}I-labeled β-VLDL were injected, the fraction of ^{125}I recovered in the combined endosomal fraction tended to rise and a progressively larger amount was present in the lowest density (MVB) fraction. With injection of ^{125}I-labeled β-VLDL containing 1.7–3.4 mg protein, only about 10% of the ^{125}I was in the liver after 15 min; 9% of hepatic ^{125}I was in the total endosome fraction, and 28% of the endosomal ^{125}I was recovered in MVBs; 31% was in the intermediate density fraction (CURL) and 6% was in the high density fraction (RRC) (Table 1). These results can be compared with those obtained in similar experiments carried out earlier in estradiol-treated rats (4) in which lipoprotein loading was achieved with LDL (3 mg protein). Recovery of labeled LDL in total endosomes was somewhat higher (15%) 15 min after injection, and 41% of the endosomal radioiodine was recovered in MVBs.

Thirty min after injection of ^{125}I-labeled β-VLDL (3.6 mg protein), substantially more of the endosomal ^{125}I was in the MVB fraction (46%). Concomitantly, the recovery of endosomal membrane protein increased substantially in the MVB and CURL fractions, but changed relatively little in the RRC fraction (**Table 2**).

The effect of lipoprotein loading upon the endosomal distribution of an unrelated ligand was evaluated by injecting ^{125}I-labeled EGF together with unlabeled chylomicrons (0.3–1.8 mg protein) or β-VLDL (3.5 mg protein). Hepatic uptake of ^{125}I-labeled EGF was independent of the lipoprotein injected (22.7 ± 2.1% with chylomicrons and 21.8 ± 6.7% with β-VLDL), and recovery in the total endosome fraction was also similar (Table 1). Tenfold more of the endosomal ^{125}I was recovered in the MVB fraction, however, when β-VLDL were injected than when chylomicrons were injected (Table 1). Recovery of endosomal membrane protein was also greatly affected. With concomitant chylomicron injection, recovery of proteins in MVB, CURL, and RRC membranes was 95 ± 3.5, 285 ± 77, and 1007 ± 163 μg, respectively; with concomitant β-VLDL injection, recovery of protein in the same fractions was 298 ± 81, 628 ± 281, and 953 ± 282 μg.

To compare the endosomal recovery of the lipoproteins taken up rapidly by normal rat liver under comparable conditions of lipoprotein loading, tracer amounts of ^{131}I-labeled chylomicrons or ^{131}I-labeled chylomicron remnants were injected with ^{125}I-labeled β-VLDL and unlabeled β-VLDL. Results are summarized in **Table 3**. In each case, 8–10% of the injected radioiodine was recovered in livers taken 30 min after injection. Mean recovery of radioiodine in the combined endosomal fraction was 5.8%, 6.4%, and 10.6% of that present in the liver for chylomicrons, chylomicron remnants, and β-VLDL, respectively. Recovery of radioiodine in the MVB

Fig. 1. Thin sections of representative fields of the three endosomal fractions from livers of normal rats injected with β-VLDL. Top, MVBs; middle, CURL; bottom, RRC. (× 40,000). MVBs average about 0.55 μm in diameter and are characterized by internal bilayer vesicles (open arrows), many content lipoproteins (predominantly β-VLDL with characteristically low electron density; black arrowheads), and large membranous appendages (long black arrows). CURL is composed of smaller, irregular vesicles (short arrows), more numerous appendages (long black arrows), internal bilayer vesicles (open arrows), and β-VLDL (black arrowheads). The RRC consists mainly of the large membranous appendages (long black arrows), with a few small vesicles containing β-VLDL (black arrowheads).

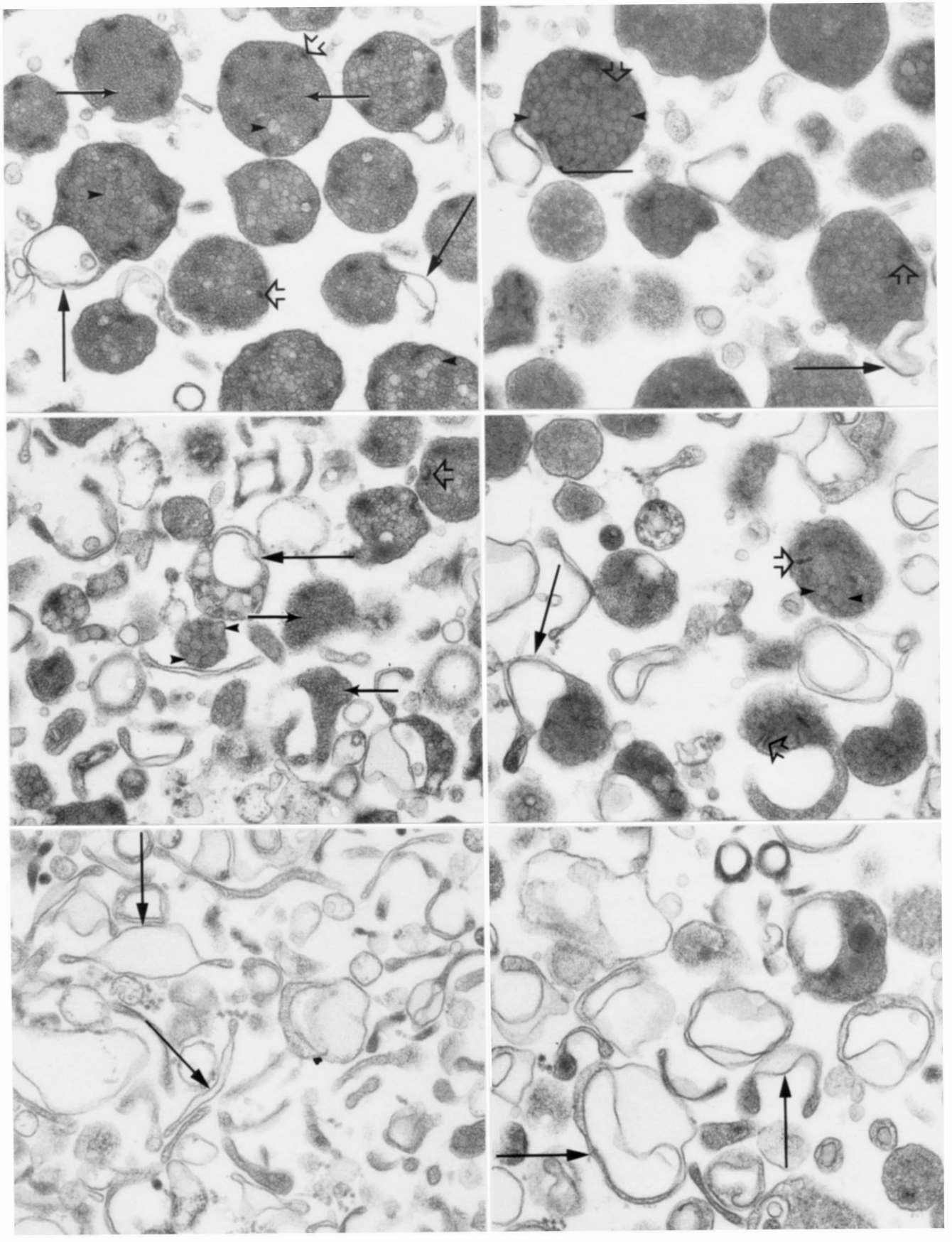

TABLE 3. Recovery of radioiodine in endosomes 30 min after simultaneous injection of ^{131}I-labeled chylomicrons or ^{131}I-labeled chylomicron remnants with ^{125}I-labeled β-VLDL during loading of endosomes with unlabeled β-VLDL (3.6 mg protein)

Fraction	Chylomicrons + β-VLDL		Chylomicron Remnants + β-VLDL	
	Chylomicrons	β-VLDL	Chylomicron Remnants	β-VLDL
	% radioiodine in liver homogenate			
Total endosomes	5.53, 6.09	11.69, 12.09	5.00, 7.74	7.4, 11.20
MVBs	1.12, 1.32 (37)	4.47, 5.52 (146)	2.47, 2.95 (54)	4.59, 5.28 (109)
CURL	0.82, 0.86 (19)	3.07, 3.46 (73)	1.25, 1.77 (27)	2.00, 2.88 (48)
RRC	0.37, 0.44 (7.9)	0.60, 0.66 (12)	0.56, 0.56 (7.2)	0.54, 0.57 (8.0)

Individual values from two experiments. Values in parentheses indicate fold purification over homogenate, based on total protein of endosomes (mean values for two experiments).

and CURL fractions was much higher when labeled β-VLDL was injected (7–8%) than was the case when chylomicrons (~2%) or chylomicron remnants (~4.4%) were injected. This result can be compared with observations in estradiol-treated rats, injected with chylomicron remnants alone (~100 μg protein) labeled with ^{125}I or [^{3}H]cholesterol. After only 15 min, 13% of the hepatic radioactivity was in the total endosomal fraction; of this 8% was in MVBs and 3% was in CURL (4).

Ultrastructural characteristics of endosomes and content lipoproteins

Endosomal fractions obtained from livers taken 15 min after injection of β-VLDL (3–4 mg protein) resembled those from estradiol-treated rats injected with human LDL (**Fig. 1**; compare with **Fig. 2**, left panels). The fraction of highest density, thought to contain predominantly membranes participating in receptor recycling, was characterized by double bilayer membranes that resemble the membranous appendages of vesicular endosomes. Additional membranous fragments were sometimes found as well (not shown in Fig. 1 and 2), which could represent contaminants from other organelles. The lipoprotein contents of the MVB and CURL endosomes from normal rats loaded with β-VLDL included many particles with the characteristic appearance of the injected β-VLDL, whereas the contents of these endosomes from estradiol-treated rats included many particles resembling the injected human LDL (Figs. 1 and 2). Endosomes isolated from livers of normal uninjected rats differed from those found after injection of β-VLDL only in the smaller number of large MVBs and the lack of particles resembling β-VLDL (compare Fig. 2, right panels with Fig. 1). The lipoprotein particles in these endosomes resembled, in size and staining properties, the VLDL-sized particles found in endosomes from estradiol-treated rats. The appearance of the content lipoproteins under the three experimental conditions can be seen more clearly in endosomes photographed at higher magnification in **Fig. 3**, which also illustrates negatively stained images of the injected lipoproteins.

In thin sections of livers from untreated rats taken 15 min after injection of large amounts of β-VLDL, MVBs were prominent in the Golgi/lysosome region of hepatocytes (**Fig. 4**). MVBs could be distinguished from secretory vesicles of the Golgi apparatus by the typical appearance of their content β-VLDL. The size and appearance of these MVBs in situ resembled those of isolated MVBs.

Properties of endosomal membranes

The chemical properties of endosomal membranes obtained after injection of β-VLDL (**Table 4**) resembled those from estradiol-treated rats (2). As compared with liver homogenate, these membranes were depleted of glucose-6-phosphatase and contained comparable arylsulfatase activity. The activity of sialyltransferase in MVB membranes was similar to that of liver homogenate, but the activity was enriched four- to fivefold in membranes of CURL and RRC. The cholesterol:phospholipid ratio of

Fig. 2. Thin sections of representative fields of three endosomal fractions (×40,000) from the liver of estradiol-treated rats injected with human LDL (left panel) contrasted with the same fractions isolated from the liver of an uninjected normal rat (right panel). Top, MVBs; middle, CURL; bottom, RRC. These endosomal fractions are remarkably similar to those described in Fig. 1 and are characterized by internal bilayer vesicles in MVBs (open arrows), large membranous appendages (long black arrows); and content lipoproteins (black arrowheads). The major differences observed are the numerous LDL particles within MVBs and CURL of the estradiol-treated rat (left top and middle; black on white arrows) and the somewhat less homogeneous fractions of CURL and RRC from the liver of the uninjected normal rat (right middle and bottom). The content lipoproteins in the uninjected vesicles resemble rat plasma VLDL (more electron-dense than β-VLDL) and presumably are VLDL and chylomicron remnants.

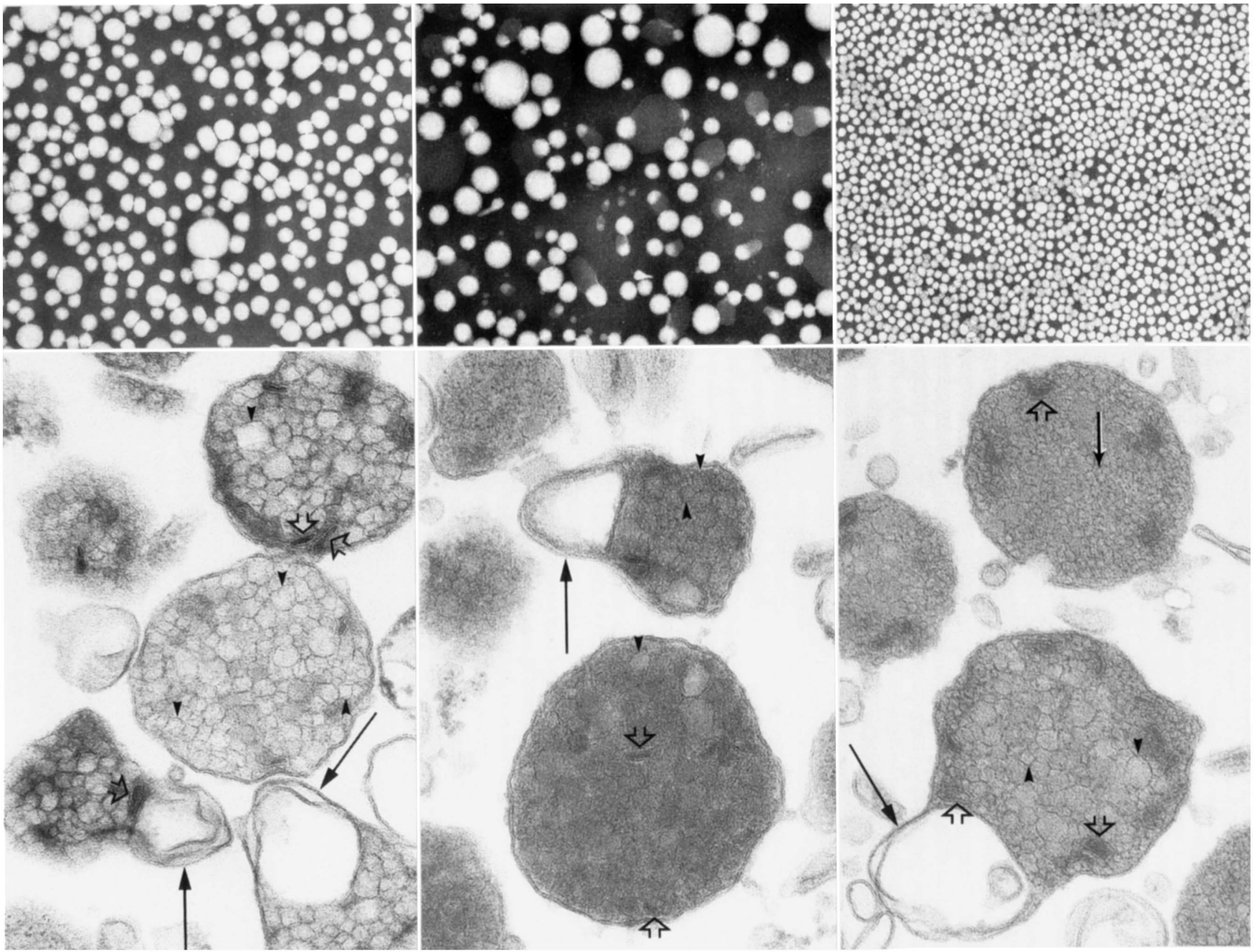

TABLE 4. Enzymatic activity and composition of membranes

	Sialytransferase (nmol × h^{-1} × mg^{-1})	Glucose-6-Phosphatase (nmol × min^{-1} × mg^{-1})	Arylsulfatase (nmol × min^{-1} × mg^{-1})	Protein/Phospholipids (mg/mg)	Free Cholesterol/Phospholipids (mg/mg)
Homogenate	2.26 ± 0.43 (3)	77.0 (1)	14.2 (1)		
MVBs	3.43 ± 0.57 (3)	32.9 ± 2.91 (3)	14.1 (1)	1.47 ± 0.27 (3)	0.31 ± 0.06 (3)
CURL	11.7 ± 7.83 (3)	59.2 ± 26.2 (3)	15.5 (1)	1.23 ± 0.22 (3)	0.30 ± 0.14 (3)
RRC	9.80 ± 3.08 (3)	50.0 ± 36.9 (3)	7.32 (1)	1.03 ± 0.22 (3)	0.26 ± 0.05 (3)
Golgi	80.5 ± 14.9 (3)				
Lysosomes			1147 (1)		

Values are means ± SD; number of experiments is indicated in parentheses.

the membranes of each fraction was similar (Table 4). The protein components of the three endosome membranes were also similar to those from estradiol-treated rats (2), as shown by the protein patterns seen in $NaDodSO_4$ gel electrophoresis (**Fig. 5**). There were, however, some additional prominent proteins in the CURL and RRC fractions from normal rats with molecular weights of 45–52 kDa. Protein bands corresponding to the LDL receptor, with an apparent molecular weight of 135,000 and to the injected apoB-100 (~500,000) were evident in the fractions from estradiol-treated rats, and a band corresponding to LRP (~600,000) was evident in CURL and RRC membranes in both cases (5). As shown in reference 5, as assessed by ^{45}Ca blotting and immunoblotting, LRP is enriched manyfold in endosome membranes as compared with crude liver membranes of untreated rats as well as rats treated with estradiol, and its concentration appears to be highest in RRC membranes, intermediate in CURL membranes, and lowest in MVB membranes. The residual apoB-100 in endosomal membranes (usually < 10% of that present in intact endosomes) probably is trapped within membranes that reseal following rupture in the French pressure cell.

DISCUSSION

The three endosomal fractions that we have isolated from livers of untreated rats have strikingly similar characteristics to those obtained from estradiol-treated rats. As in estradiol-treated rats, separation of endosomes from secretory components of the Golgi apparatus is facilitated by differences in size of these organelles. With the homogenization procedure that we use, most Golgi complexes evidently stay intact and are separated from endosomes by sedimentation at a very low centrifugal force. In estradiol-treated rats, late endosomes (MVBs) are largely separated from early endosomes by virtue of their larger size and lower density (1–3); the mass of isolated MVBs could be increased about twofold by injecting a large amount of LDL. In untreated rats, however, we were unable to increase the size and mass of the MVB fraction by injecting large amounts of LDL or chylomicrons. With these ligands, tracer amounts of injected β-VLDL or EGF were recovered almost entirely in the endosomal fractions of higher density. After injection of a large amount of β-VLDL, however, both tracer β-VLDL and EGF could be recovered from the three endosomal fractions to an extent similar to that found for LDL, β-VLDL, and chylomicron remnants in estradiol-treated rats (4). The amount of labeled β-VLDL or EGF recovered in the MVB fraction was increased more than ten times that found in rats not given a β-VLDL load (Table 1). As in estradiol-treated rats, the highest purification of the injected ligands was found in MVBs and the lowest in RRC. Evidently, without injection of the cholesteryl ester-rich β-VLDL, the size of the late prelysosomal endosomes is smaller and their density is higher than in estradiol-treated rats; therefore, late endosomes (MVBs) are recovered mostly in the intermediate density (CURL) fraction. After injection of large amounts of β-VLDL, recovery of the labeled ligands in the RRC fraction fell (Table 1), suggesting that without injection of a β-VLDL load, some early endosomes also have higher densities and are therefore isolated in the RRC fraction.

Between 15 and 30 min after injection of large amounts of β-VLDL (approaching saturation of hepatic uptake), the recovery of the labeled β-VLDL and the mass of mem-

Fig. 3. Thin sections of representative MVBs (left column) from livers of three rats (× 80,000). Top left, normal rat injected with β-VLDL; middle left, normal rat, no injection; bottom left, estradiol-treated rat injected with LDL. The right hand columns show negatively stained fractions of the lipoproteins injected or contained in rat blood plasma. Top right, β-VLDL; middle right, plasma VLDL; bottom right, human LDL. (× 80,000). The predominant endocytosed particles reflect the injected fraction or, in the case of the uninjected rat, particles resembling plasma VLDL. Lipid bilayer vesicles (open arrows); large double bilayer membranous appendages (long black arrows); content VLDL or β-VLDL (black arrowheads); human LDL (black on white arrow).

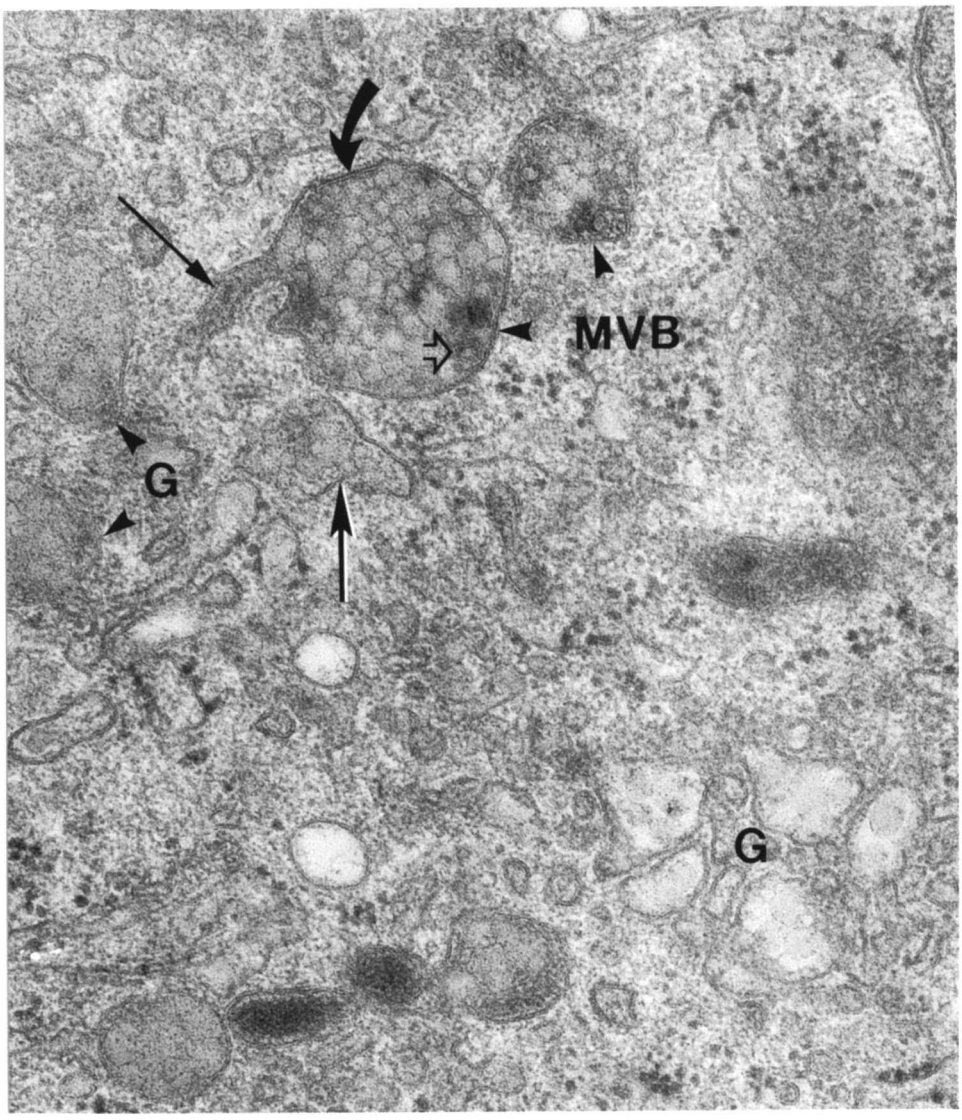

Fig. 4. Electron micrograph of Golgi/lysosome region of hepatocyte 15 min after injection of β-VLDL into a normal rat fasted for 16 h. MVBs can usually be distinguished from nearby Golgi secretory vesicles (G) by the greater variation in size of their content lipoproteins, which under these conditions are less electron dense than nascent Golgi VLDL. The larger MVBs frequently have other distinguishing characteristics: internal bilayer vesicles (open arrow), membranous appendages (long black arrow), and a thickened plaque-like region of the limiting membrane (curved arrow). The black on white arrow points to a vesicle in which none of these characteristics or the structure of the content lipoproteins permits its identification. (× 60,000). The typical characteristics of MVBs shown here within a hepatocyte are retained in isolated MVBs, as shown in Fig. 1.

brane proteins in MVBs almost doubled, suggesting that between 15 and 30 min the size of MVBs increased further (Table 2). The fraction of endosomal β-VLDL in MVBs increased similarly during this inverval. These observations are consistent with data from estradiol-treated rats showing that the CURL fraction contains mainly "early" endosomes, and the MVB fraction consists predominantly of "late" endosomes (2, 6). In addition, the total mass of endosomal membrane protein recovered increased between 15 and 30 min after injection of β-VLDL from 1525 to 2528 μg. Similarly, when β-VLDL rather than chylomicrons were given with labeled EGF, total endosomal membrane protein increased from 1387 to 1879 μg 15 min after injection. These observations suggest that lipoprotein loading also has a broader effect upon endosomal membrane trafficking.

The comparability of the three endosomal fractions that we have isolated from livers of normal rats to those from livers of estradiol-treated rats is strongly supported by their morphological characteristics. When β-VLDL were injected in large amounts, the content lipoproteins of the vesicular endosomes resembled β-VLDL in size,

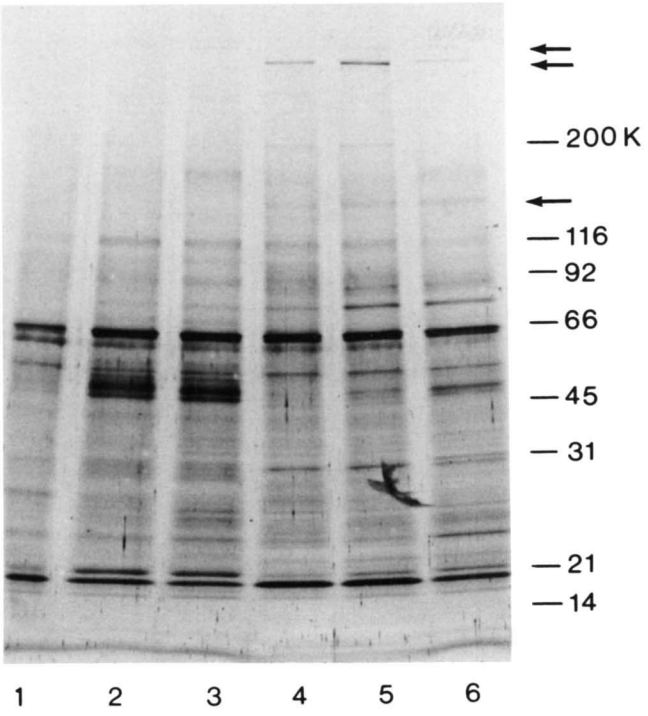

Fig. 5. $NaDodSO_4$ gradient (3–20%) gel electrophoretograms (unreduced) showing proteins of endosomal membranes (50 μg protein) from normal rats injected with β-VLDL (lanes 1–3) and estradiol-treated rats (lanes 4–6) (Coomassie Blue staining). Lanes: 1 and 4, MVB membranes, 2 and 5, CURL membranes, 3 and 6, RRC membranes. Numbers on right represent $M_r \times 10^{-3}$ of marker proteins. Bold arrows, from top, correspond to the LRP, apoB-100, and the LDL receptor.

shape, and staining characteristics. This observation clearly demonstrates the rapid uptake and movement of β-VLDL through endosomal pathway followed by LDL in estradiol-treated rats (Fig. 3). The fact that isolated MVBs in this situation resemble, by electron microscopy, those found at the bile canalicular pole of hepatocytes in thin sections of intact liver indicates that these organelles are little modified during the isolation procedure (Fig. 4). Previous injection of β-VLDL can thus facilitate identification of the hepatic exocytic and endocytic pathways in situ.

The paucity of contamination of the three endosomal fractions by nonendosomal organelles is supported not only by their ultrastructural characteristics (Figs. 1–3), but also by the protein and lipid composition of their membranes and the low activities of glucose-6-phosphatase and sialyltransferase (Table 4, Fig. 5). The enrichment of the membranes of all three endosomal fractions with cholesterol (cholesterol:phospholipid molar ratio ~0.6) is almost exactly the same as that observed previously with endosomal membranes from livers of estradiol-treated rats (2).

Sialyltransferase activity is concentrated in the *trans* cisternae and associated *trans* tubular network of the Golgi apparatus (34, 35). The membranes of CURL and RRC, but not MVBs, were somewhat enriched in sialyltransferase, and some proteins of 45–52 kDa not seen in MVBs, which could reflect proteins found in Golgi membranes (2), were prominent in $NaDodSo_4$ gels of CURL and RRC membranes. Therefore, some contamination of these two fractions with Golgi vesicles, and possibly other organelles, is likely. Although endosomal membranes from estradiol-treated rats are enriched six- to tenfold in 5'-nucleotidase (2), a plasma membrane marker, appreciable contamination of the MVB and CURL fractions with plasma membranes is unlikely from our ultrastructural observations. More likely, this enzyme is intrinsic to early and late endosomes. Such contamination is more difficult to exclude in the case of the RRC fraction.

Endosomal fractions have been separated from rat livers or isolated rat hepatocytes by a number of investigators. Existing methods are mostly based on density gradient centrifugation using sucrose (2, 36–38), Percoll (2, 3), Nycodenz (39, 40), Ficoll (41), or Metrizamide (42, 43). Free-flow electrophoresis has also been used to separate early and late endosomes (37, 39). To separate endosomes from contaminating organelles, several density shift techniques have also been applied. Courtoy, Quintart, and Baudhuin (44) and Quintart, Courtoy, and Baudhuin (45) increased the density of endosomes after injection of horseradish peroxidase, covalently linked to an injected ligand, by incubation with 3,3'-diaminobenzidine (DAB) and H_2O_2. Wall and Hubbard (43) took advantage of the high cholesterol content of endosomal membranes, which bind more digitonin than other organelles and can therefore shift by incorporation of digitonin to higher densities. Endocytic coated vesicles could be separated from exocytic coated vesicles, after inactivation of endogenous cholinesterase with diisopropylfluorophosphate (DFP) and injection of ligand-linked cholinesterase, by a cholinesterase-mediated density shift technique (46).

Immunological methods have been used by several investigators. Mueller and Hubbard (47) purified asialoglycoprotein (ASGP) receptor-rich endosomes by immunoadsorbtion to antibodies to the ASGP receptor. Gruenberg, Griffiths, and Howell (48) implanted the *trans*-membrane glycoprotein G of vesicular stomatitis virus into plasma membranes of baby hamster kidney cells and immunoisolated endosomes after internalization.

With our isolation procedure, sufficient amounts of highly purified early and late endosomes, together with receptor-recycling membranes, can be obtained for detailed analysis from livers of normal rats with a single intervention: injection of a high affinity ligand (β-VLDL) for lipoprotein receptors. The two lipoprotein-filled endosomal fractions that we have isolated appear to be comparable to those found in situ, as demonstrated by immunogold labeling (7), and fit very well with the dis-

crete structure of endosomal vesicles described for baby hamster kidney cells (49). The RRC fraction, which is largely derived from the membranous appendages of the two other fractions, bears some resemblance to tubules found in the oocyte of *Drosophila melanogaster*. Some of these tubules are associated with nascent yolk granules and they appear to recycle to the plasma membrane (50).

In studies of the processing of chylomicron remnants by rat liver, Jones et al. (51) showed by electron microscopic autoradiography that the intracellular pathway followed by protein-labeled rat lymph chylomicrons in normal rats resembles that observed for LDL in estradiol-treated rats. Thus, label was concentrated first in endosomal structures near the sinusoidal border of hepatocytes and, after 10–15 min, over MVBs. The fraction of autoradiographic grains over MVBs 15 and 30 min after injection was, however, lower after injection of chylomicron remnants into normal rats (11–22%) than after injection of LDL into estradiol-treated rats (28–47%). The rate and extent of uptake of β-VLDL into the CURL and MVB fractions in our current studies of normal rats closely resemble that of LDL in livers of estradiol-treated rats; however, chylomicron and chylomicron remnant uptake into these vesicular endosome fractions was comparatively reduced, and they did not induce substantial enlargement of late endosomes, as occurred with β-VLDL. Even when endosomes were loaded with β-VLDL, the accumulation of labeled chylomicrons and chylomicron remnants in vesicular endosomes 30 min after injection was substantially lower than that of β-VLDL itself (Table 3). These observations contrast with our earlier findings in estradiol-treated rats, in which the uptake of LDL, β-VLDL, and chylomicron remnants into endosomal fractions was virtually the same 15 min after injection (Table 3 and ref. 4).

These observations show that chylomicron remnants taken up into the liver of estradiol-treated rats are rapidly endocytosed to an extent comparable to that of LDL and β-VLDL. By contrast, the extent to which chylomicron remnants taken up into the liver accumulated in hepatocytic endosomes of normal rats was lower than that observed for β-VLDL. This difference could reflect a more rapid degradation of chylomicron remnants in normal rats; however, we have observed virtually no degradation of cholesteryl esters or apoB-100 in hepatocytic endosomes 15 min after injection of LDL into estradiol-treated rats (E. A. Runquist and R. J. Havel, unpublished data) and, in the current study, endosomal radioactivity from cholesterol-labeled and radioiodinated chylomicrons was comparably reduced as compared with radioiodine from β-VLDL (Tables 1 and 3). Thus, our data suggest that there is a delay in the delivery of chylomicron remnants into the endocytic pathway after initial rapid uptake into the liver of normal rats. The most obvious difference between estradiol-treated and untreated rats that could account for the differing behavior of chylomicron remnants in the two conditions is the 10- to 20-fold increase in hepatocytic LDL receptors in rats treated with estradiol (52). We thus suggest that endocytosis of chylomicron remnants in estradiol-treated rats is mediated predominantly by the induced LDL receptor. The reason for the apparent delay in delivery of chylomicron remnants into the endocytic pathway in normal rats remains to be established. As discussed elsewhere (53), chylomicron remnants may bind to one or more surface components of the sinusoidal plasma membrane of hepatocytes, such as heparan sulfate, hepatic lipase, or LRP (5, 10). Additional modifications may take place and perhaps are required before endocytosis occurs. These could include enzymatic attack by hepatic lipase or the acquisition of additional apoE, which is concentrated on the sinusoidal plasma membrane (53). Whether the LDL receptor (1, 4, 9) or yet another receptor (5, 10–12) predominantly mediates the endocytosis of chylomicron remnants in normal rats is unclear.

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