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Autoradiographic localization of the sites of uptake, cellular transport, and catabolism of low density lipoproteins in the liver of normal and estrogen-treated rats

(hepatic receptors/endocytosis/multivesicular body-like structures/lysosomes)

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ABSTRACT **The hepatic uptake and catabolism of low density lipoproteins are stimulated severalfold in rats treated with large amounts of 17α-ethinylestradiol. To determine the sites within the liver at which these processes occur, ^{125}I-labeled human low density lipoproteins were injected intravenously into intact control and estradiol-treated rats or added to perfusates of their isolated livers. The livers were fixed by perfusion and processed for light and electron microscopic autoradiography. Distribution of autoradiographic silver grains was estimated qualitatively in light micrographs and quantitatively in electron micrographs. Many more silver grains were seen in livers from estradiol-treated than from control rats, but the processing of labeled low density lipoprotein was indistinguishable. Three minutes after intravenous injection or perfusion of livers, the grains were concentrated over the microvillous surface of parenchymal cells bordering the space of Disse. Many of these grains were within two half-distances from endocytic pits. Only 5–15% of the grains were seen over endothelial and Kupffer cells. Silver grains were also observed over vesicles beneath the plasma membrane whose size and shape suggested that they were derived from fusion of endocytic vesicles. By 15 min, grains were predominantly located in structures like multivesicular bodies in the region of the GERL (Golgi complex–endoplasmic reticulum–lysosomes) near the bile canaliculi. These bodies were packed with small vesicle-like structures and a few larger vesicles, the latter possessing a unit membrane. Between 15 and 30 min, when proteolysis of low density lipoproteins is known to begin, the initially clear matrix of the multivesicular body-like structures became dark and the structures frequently had a dense tail-like appendage. At the same time, silver grains began to appear over secondary lysosomes. These and other results indicate that the hepatic uptake of low density lipoproteins that is stimulated in rats given large amounts of estradiol follows a pathway that closely resembles that of the well-defined "LDL receptor" in cultured cells. In the liver these lipoproteins appear to be transported in endocytic vesicles; the vesicles fuse to form multivesicular body-like structures that acquire lysosomal enzymes and are converted to secondary lysosomes as the lipoproteins are degraded.**

In the rat the terminal catabolism of the triglyceride-rich lipoproteins [chylomicrons and very low density lipoproteins (VLDL)] occurs mainly in parenchymal cells of the liver (1–3), probably by receptor-mediated endocytosis (4–6). The liver also takes up and catabolizes some of the cholesterol-rich lipoproteins [low density lipoproteins (LDL) and high density lipoproteins (HDL)] (7, 8), but the mechanisms of uptake have not been defined. Recent studies have shown that the hepatic uptake and catabolism of both rat and human LDL is stimulated manyfold in rats treated with pharmacological amounts of 17 α-ethinylestradiol (9). The stimulated uptake is associated with comparably increased numbers of high affinity binding sites on cell membranes within the liver (10). In the present research, we have used autoradiography at the light and electron microscopic levels to determine the cell responsible for the enhanced hepatic uptake and to define the pathway by which the protein moiety of LDL is brought to the site of catabolism within the cell.

METHODS

Treatment of Animals. Male Sprague–Dawley rats, weighing 300–350 g, were fed standard Purina Chow. 17α-Ethinylestradiol, prepared as described (9), was injected (5 mg/kg) subcutaneously daily for 5 days. Control rats received equal volumes of diluent.

Preparation of Lipoproteins. Human LDL (1.024 g/ml < density < 1.063 g/ml) were isolated from plasma by sequential ultracentrifugations (7) and labeled with ^{125}I to a specific activity of 86 μCi/mg (1 Ci = 3.7×10^{10} becquerels) by a modification of McFarlane's method (11), as described (7). Unreacted ^{125}I was removed by dialysis for 24 hr against 0.15 M NaCl containing 0.01% Na_2EDTA, pH 7.4.

Uptake of ^{125}I-Labeled LDL (^{125}I-LDL) by Intact Rats. A measured volume (about 1 ml) of radioiodinated LDL, containing 7 mg of apo-LDL, was injected into femoral veins of rats anesthetized with diethyl ether. At 3-, 10-, and 30-min intervals, the rat livers were perfused through the portal vein with 36 ml of 0.15 M NaCl at 4°C and then fixed by perfusion with a biocarbonate-buffered glutaraldehyde/formaldehyde mixture (12), both at a rate of 12 ml/min. The tissue was then processed for light and electron microscopic autoradiography (13).

Uptake of ^{125}I-LDL in Isolated Perfused Rat Livers. The procedures of isolated liver perfusion were the same as previously described (7, 9). The livers were first perfused in the recirculating mode for 30 min. Fresh medium containing ^{125}I-LDL (0.3 mg of protein per ml of perfusate) was then passed through the livers without recirculation at a rate of 12 ml/min. After 3, 5, 7, 10, or 15 min, the livers were flushed with 36 ml of ice-cold saline and fixed as above.

Autoradiography. Thick sections (0.5 μm) and thin sections (900 Å) were coated with Kodak NTB-3 and Ilford L-4 emulsions, respectively, for light and electron microscopic autoradiography (13). The exposed thin sections were developed in Ko-

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Abbreviations: VLDL, LDL, and HDL are very low density, low density, and high density lipoproteins; GERL, Golgi complex–endoplasmic reticulum–lysosomes; MVBLS, multivesicular body-like structures.

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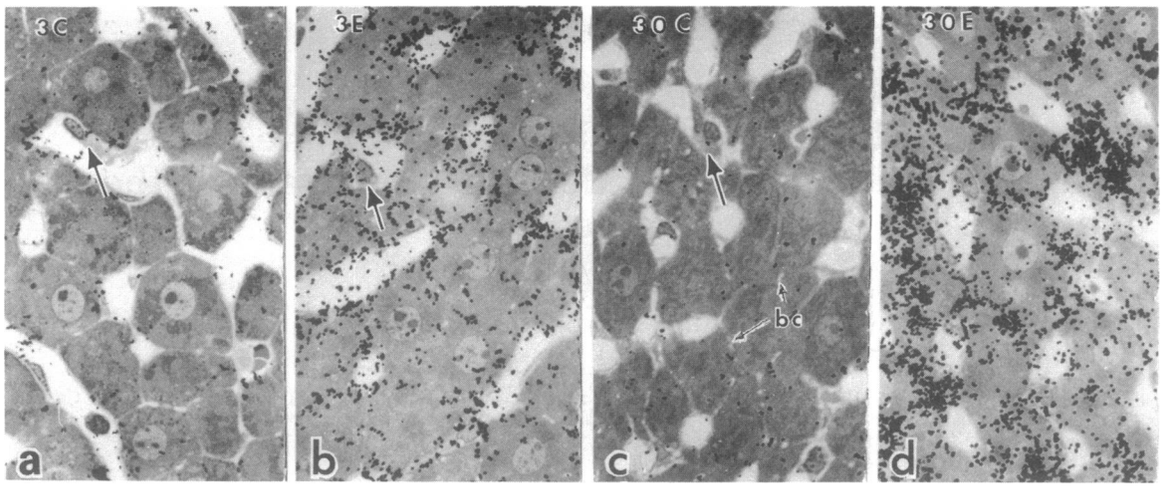

FIG. 1. Light microscopic autoradiographs of control (*a*) and estradiol-treated (*b*) rat livers 3 min after intravenous injection of ^{125}I-LDL. The small dense silver grains representing ^{125}I-LDL are in far greater numbers in *b*, but in each case the grains are predominantly located along the sinusoidal surfaces of the hepatocytes. By 30 min after injection of ^{125}I-LDL, the grains are largely confined to the interior of the hepatocytes (*c* and *d*). Note the heavy concentration of grains in the region of the bile canaliculus in the estradiol-treated liver (*d*). (*c*) Control liver. Large arrow, Kupffer cell; bc, bile canaliculi. (×200.)

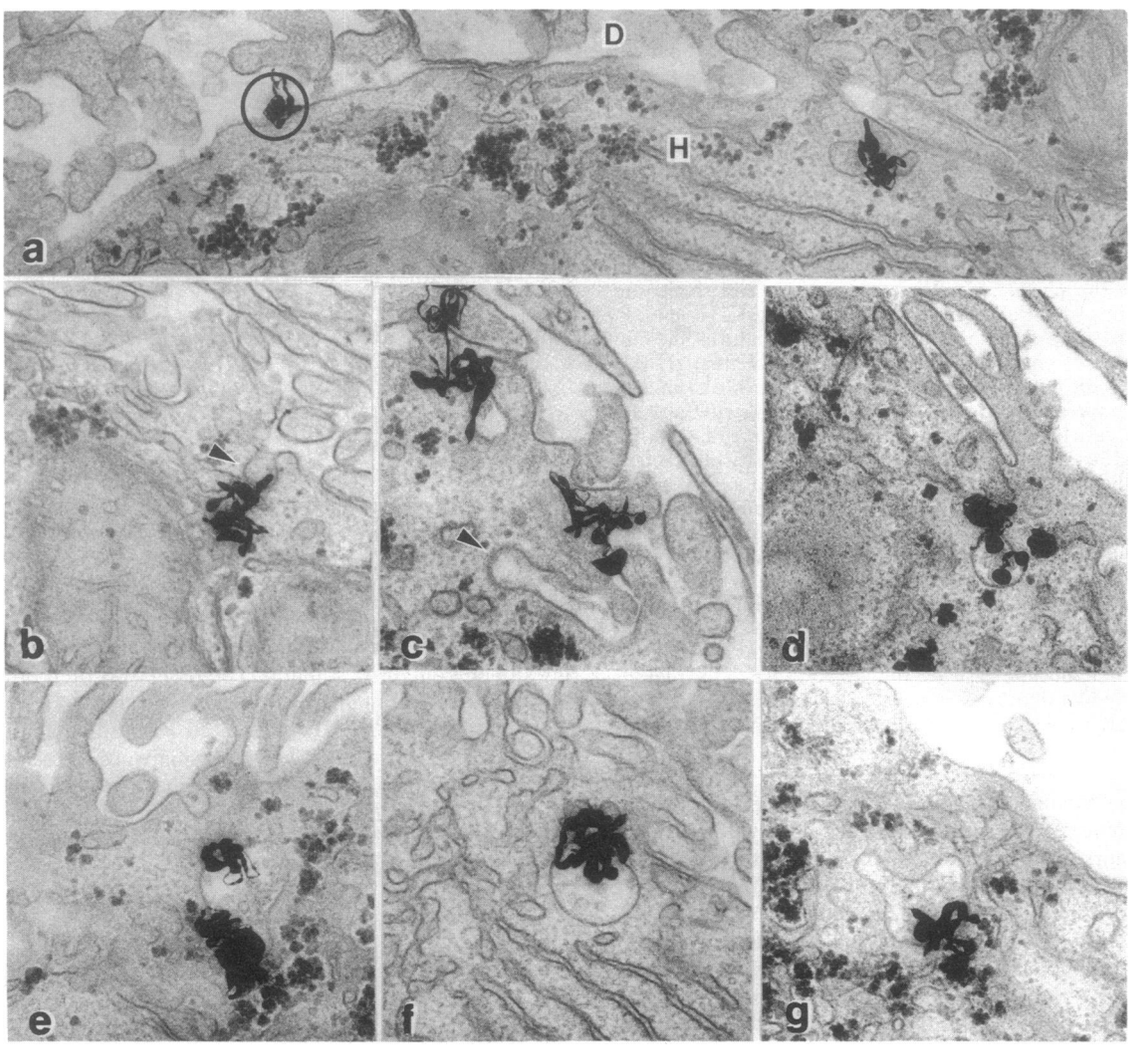

FIG. 2. Electron microscopic autoradiographs showing silver grains (circle, *a*) of ^{125}I-LDL along the sinusoidal cell surface of hepatocytes (H) in estradiol-treated (*a–d* and *f*) and control livers (*e* and *g*). D, space of Disse. Images such as these are particularly prominent 3–7 min after exposure to ^{125}I-LDL, and suggest that the lipoproteins are first located in regions of endocytic pits (*b* and *c*), some with "coats" (arrowheads), and are then taken up by endocytosis. Fusion of these endocytic vesicles appears to take place just beneath the cell surface (*a*, *d*, *e*, and *g*). The particulate material within the vesicles may represent LDL. (≈×28,000.)

dak D-19 developer and stained with lead citrate. The resolution half-distance of ^{125}I was determined to be 850 Å (13). Grains were quantified by using a concentric circle analysis (13).

RESULTS

Light Microscopic Autoradiography. Three minutes after intravenous injection of ^{125}I-LDL into intact control rats or perfusion of isolated livers from control rats, silver grains representing the labeled B apoprotein of LDL were seen mainly near the sinusoidal surface of the hepatocytes. There was no evidence of concentration of grains in Kupffer cells, but an association with endothelial cells could not be excluded at this level of resolution. In identical experiments with estradiol-treated rats, the distribution of grains was similar but many more grains were seen (Fig. 1 *a* and *b*). By 30 min most of the grains were within the hepatocytes in both control and estradiol-treated rats (Fig. 1 *c* and *d*). An extraordinary concentration of grains was seen in the region of the bile canaliculus of estradiol-treated animals (Fig. 1*d*).

Electron Microscopic Autoradiography. The processing of labeled LDL as determined by autoradiography in control and estradiol-treated rat livers was indistinguishable. The only apparent difference was the much greater number of silver grains in sections from estradiol-treated rats. At 3 min, most of the grains were observed at the surface of parenchymal cells, although some grains could be seen in endothelial and Kupffer cells (Fig. 2). Quantitative analysis showed that approximately half of the grains were on the plasma membrane (Fig. 3). Many of these grains were within two half-distances from endocytic pits. Occasionally, these pits resembled the clathrin-containing coated pits known in other systems to be responsible for the uptake of LDL (Fig. 2 *b* and *c*). The diameter of these pits, with or without evident coats, was 1000 Å or greater. Grains were also seen, within 1–2 μm of the plasma membrane, in vesicles considerably larger than the pits, suggesting derivation from fusion of endocytic vesicles (Fig. 2 *a*, *d*, and *g*). This phenomenon was evident at 3 min and was quite frequent at 5 and 7 min. These larger vesicles usually contained particulate material of various sizes, but the diameter of most of the particles was within the expected range of LDL (200–230 Å).

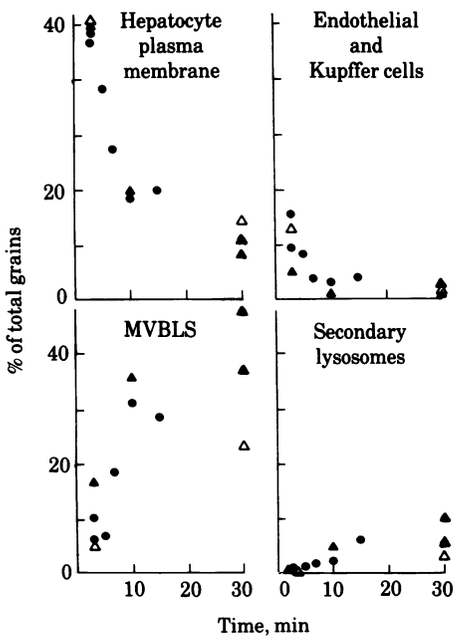

FIG. 3. Distribution of grains associated with specific liver cells and organelles of hepatocytes at intervals after intravenous injection of ^{125}I-LDL into intact control (△) or estradiol-treated (▲) rats, or perfusion of ^{125}I-LDL through isolated livers of estradiol-treated rats (●). These data were obtained from an analysis of approximately 6000 grains from 2000 electron micrographs. The number of grains analyzed was as follows: *in vitro* estradiol 3 min, 174; *in vitro* estradiol 3 min, 291; *in vivo* estradiol 3 min, 550; *in vivo* control 3 min, 131; *in vitro* estradiol 5 min, 494; *in vitro* estradiol 7 min, 338; *in vitro* estradiol 10 min, 614; *in vivo* estradiol 10 min, 188; *in vitro* estradiol 15 min, 787; *in vivo* estradiol 30 min, 586; *in vivo* estradiol 30 min, 1393; *in vivo* control 383.

By 15 min there was a remarkable redistribution of silver grains in similar or larger vesicles to the region of the GERL (Golgi complex–endoplasmic reticulum–lysosomes), which is usually located near the bile canaliculus (Fig. 4 *a* and *b*). These vesicles were packed with small vesicle-like structures as well as larger particles that also possessed lucent interiors. A unit membrane was seen to compose the wall of the larger particles in sections photographed without superimposition of the autoradiographic emulsion (Fig. 4*a*, *Inset*). The matrix surrounding the particles was clear. These multivesicular body-like structures (MVBLS) contained many more structures within them than do typical hepatic multivesicular bodies, and they were clearly distinguishable from the secretory vesicles of the Golgi complex that contain nascent VLDL (Fig. 4*b*). The secretory vesicles were rarely associated with silver grains. By 30 min the autoradiographic grains were concentrated over MVBLS that had a very dark matrix and often possessed an electron-dense "tail" (Fig. 4 *c–e*).

These qualitative observations of the grain distribution were documented by quantitative autoradiography (Fig. 3). The high concentration of silver grains over MVBLS at 15 and 30 min becomes more impressive when it is realized that these structures constitute less than 1% of the volume of the cytoplasm in hepatocytes from normal or estrogen-treated rats (unpublished observations). The distribution of grains among the other organelles was insignificant, with the exception of the smooth endoplasmic reticulum, in which there was a relatively large initial labeling (15–20%) that gradually increased to about 25% at 30 min. Many of the grains attributed to the endoplasmic reticulum were within two half-distances of the MVBLS near the cell surface or GERL. A small but systematic increase in labeling of secondary lysosomes was also observed at 30 min (Fig. 3). Some of these organelles appeared to be transitional between MVBLS and secondary lysosomes (Fig. 4*e*).

DISCUSSION

These autoradiographic studies show that the augmented catabolism of human LDL in the liver of estrogen-treated rats occurs in the parenchymal cells rather than in endothelial or Kupffer cells. This result was not readily predictable, because others have suggested that a substantial fraction of LDL in normal rats is catabolized in nonparenchymal cells of the liver (14). However, we found that the predominant early localization of silver grains in both control and estrogen-treated rats was at the microvillous surface of the parenchymal cells, consistent with an enhancement by estrogen of a normal mechanism by which LDL are taken up by the liver.

Estrogen treatment has been shown to stimulate a specific binding site for human LDL in a membrane preparation from rat liver (10). This site also binds rat lipoproteins containing apoproteins B or E with an affinity that is similar in normal and estrogen-treated rats (15). These as well as the present results are consistent with augmentation by estrogen of a receptor on the surface of hepatic parenchymal cells that participates in the normal catabolism of plasma lipoproteins containing these two apoproteins (chylomicron and VLDL remnants, LDL and apoprotein E-containing HDL).

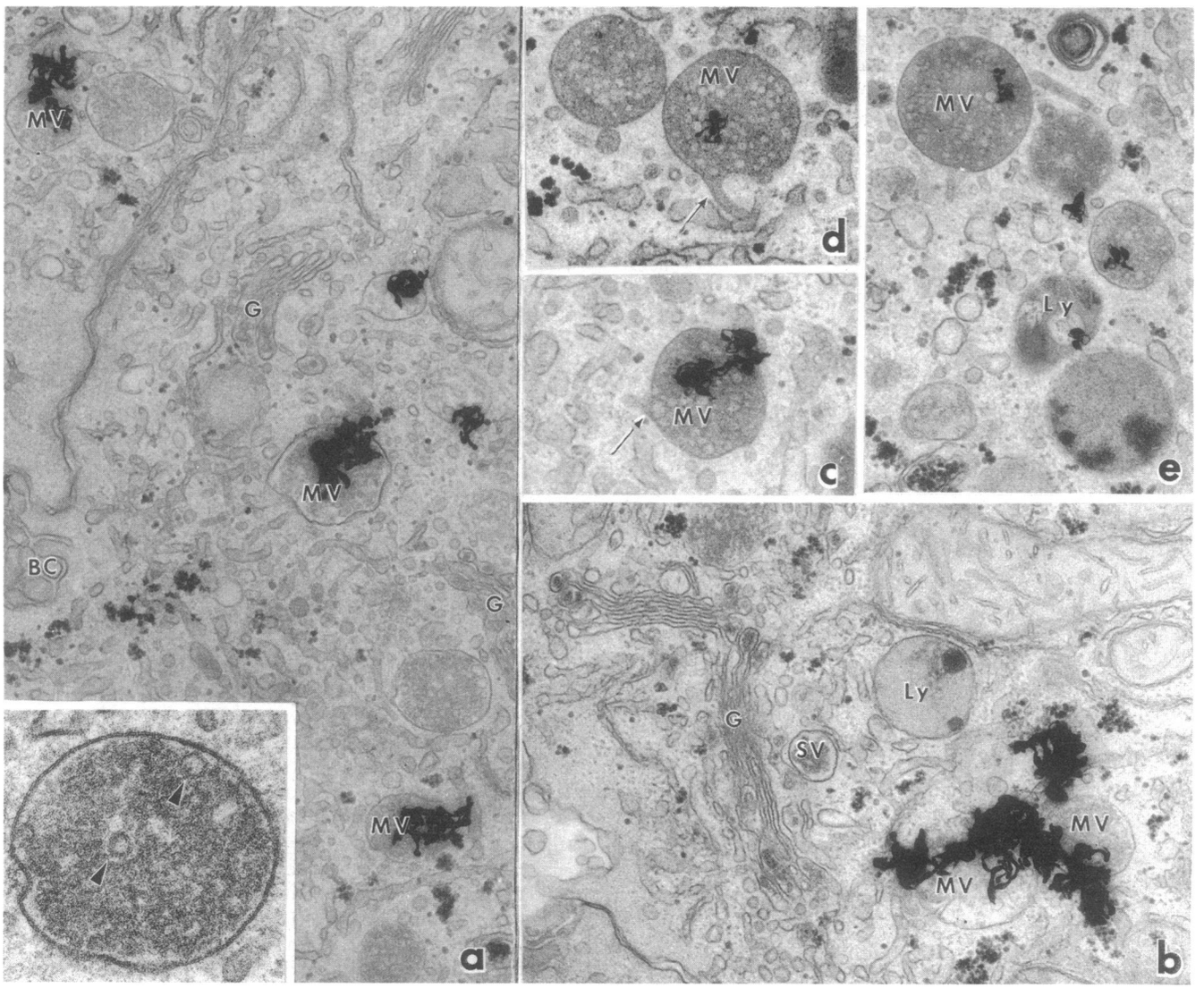

FIG. 4. Electron microscopic autoradiograms of liver from estradiol-treated animals. After 15 min (*a* and *b*) and 30 min (*c–e*) labeled B apoprotein is noted over MVBLS (MV) (*a–e*). These vesicles are clearly different from the secretory vesicles (SV) near Golgi complex (G) containing electron-dense nascent VLDL (*b*). After 30 min some grains are also found over secondary lysosomes (Ly) (*e*). Particles with lucent interiors are prominent within the larger vesicles. In sections examined without a photographic emulsion overlay (*Inset* of *a*), some of the larger particles are seen to have a typical unit membrane. Note the relatively clear matrix of the MVBLS at 15 min (*a* and *b*) compared to the more electron-opaque matrix after 30 min (*c–e*). Many of the MVBLS possess an electron-dense "tail" (*c* and *d*, arrows). BC, bile canaliculi. (*a*, ×22,000; *Inset* of *a*, ×63,000; *b–e*, ×28,000.)

The binding site for LDL in liver membranes that is increased by estrogen resembles in a number of its characteristics those of the well-characterized "LDL receptor" in cultured fibroblasts and other cells. These include requirement for Ca^{2+} and sensitivity to proteolysis or modification of lysyl or arginyl residues on apo-LDL (10). LDL bound to the LDL receptor in cultured fibroblasts have been shown to be localized primarily in clathrin-associated "coated" pits (16), and it is therefore logical to expect that similar localization occurs on the surface of hepatic parenchymal cells. At least two factors may have prevented such a demonstration in the present research. First, localization by ^{125}I autoradiography is less precise than certain other techniques, such as ferritin labeling. Second, in most studies with cultured cells, incubations have been performed at 4°C to inhibit endocytosis. When such studies are performed at 37°C, as in the present study, the number of coated regions on the plasma membrane is reduced and fewer of these regions are indented to form pits (17).

A major observation in the present study is the demonstration of probable intermediate stages in the pathway of intracellular transport and degradation of LDL. As observed in cultured cells, endocytosis rapidly followed binding to the plasma membrane. However, the heavily labeled vesicles that we observed just beneath the plasma membrane, which usually contained particles resembling LDL, were larger and more irregular than the endocytic pits at the cell surface, consistent with an origin from fusion of several endocytic vesicles. The subsequent predominant localization of grains in even larger structures resembling multivesicular bodies in the region of GERL suggests that fusion of endocytic vesicles proceeds rapidly as the forming organelle migrates to this region of the cell.

When silver grains were first heavily localized in the MVBLS 15 min after the start of perfusion of ^{125}I-LDL, the matrix of the structures was electron-lucent. By 30 min, after injection of labeled LDL, the matrix of most of these structures, still heavily labeled, became electron dense and the structures frequently had a dense tail-like appendage. In all likelihood the changed appearance of the MVBLS represents the onset of proteolysis (7, 9) and perhaps lipolysis, secondary to the acquisition of lysosomal enzymes delivered by primary lysosomes, represented

perhaps by the tail-like structure. This interpretation is further supported by the accumulation of silver grains in typical secondary lysosomes at this time.

The MVBLS observed here resemble the "lipoprotein-containing" structures observed by Novikoff and Yam in livers of rats treated with orotic acid and subsequently with clofibrate as well (18). They considered these bodies to be derived from the Golgi complex, but in the present studies of livers from normal and estrogen-treated rats, it is clear that these organs contain lipoproteins acquired from the sinusoids. The heavily labeled MVBLS could be clearly distinguished from the poorly labeled secretory vesicles of the Golgi complex. In rats, more than 90% of the nascent VLDL that are secreted from the liver by way of these secretory vesicles are rapidly returned to the liver as VLDL remnants after the bulk of the contained triglycerides has been removed in extrahepatic tissues (19, 20). In addition, hepatic parenchymal cells readily take up similar remnants derived from chylomicrons (1, 2, 5, 6). Therefore, the traffic of triglyceride-rich lipoprotein remnant particles into the liver under most conditions probably exceeds the rate of secretion of nascent VLDL particles. In preliminary studies, localization of rat VLDL remnants in the MVBLS of hepatic parenchymal cells has been found to resemble closely that observed here for human LDL (unpublished observations). It is not surprising, therefore, that, even in normal livers, we observed MVBLS that contained particles of various sizes in the region of GERL, together with secretory vesicles containing nascent VLDL. However, in electron microscopic morphometric studies, the number of these bodies has been found to increase rapidly when livers from estrogen-treated rats are exposed to large amounts of human LDL, as in the present study (21).

The only other significant site of localization of silver grains, observed in livers of normal and estrogen-treated rats alike, was in the region of membranes classified as smooth endoplasmic reticulum. Much of this apparent localization may reflect the proximity of nascent MVBLS to smooth endoplasmic reticulum.

The evidence presently available indicates that the pathway described in this research may be involved in the hepatic catabolism of a number of plasma lipoproteins in the rat, but its quantitative role in normal lipoprotein catabolism remains to be established. Similar ultrastructural studies with homologous lipoproteins in the rat and other species should be useful in the pursuit of the question.

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