0026-895X/92/020404-08$03.00/0

MOLECULAR PHARMACOLOGY, 41:404-411

Lactosylated High Density Lipoprotein: A Potential Carrier for the Site-Specific Delivery of Drugs to Parenchymal Liver Cells

MARTIN K. BIJSTERBOSCH and THEO J. C. VAN BERKEL

Division of Biopharmaceutics, Center for Bio-Pharmaceutical Sciences, University of Leiden, 2300 RA Leiden, The Netherlands

Received July 16, 1991; Accepted November 3, 1991

SUMMARY

Mammalian liver contains two types of galactose receptors, specific for Kupffer or parenchymal cells. Because galactose-specific receptors are largely confined to the liver, galactose-bearing carriers are promising vehicles for the specific delivery of drugs to liver cells. In the present study, high density lipoprotein (HDL), a spherical particle with a diameter of 10 nm, in which a variety of lipophilic drugs can be incorporated, was provided with galactose residues by reductive lactosamination. After injection into rats, lactosylated ^{125}I-HDL was rapidly cleared from the plasma (half-life, <1 min). Ten minutes after injection, the liver contained about 95% of the dose, whereas only small amounts of radioactivity were found in other tissues. The hepatic uptake was inhibited by preinjection with *N*-acetylgalactosamine, which indicates that the hepatic recognition sites are galactose specific. Subcellular fractionation of the liver indicated that recognition of lactosylated HDL is followed by internalization and degradation of the apoprotein in the lysosomes. Liver cells were isolated at 10 min after injection of lactosylated ^{125}I-HDL, and it was found that uptake occurs almost exclusively by parenchymal cells. These cells contained about 98% of the hepatic radioactivity. The liver uptake of the lipid moiety of lactosylated HDL, labeled with [^{3}H]cholesteryl oleate, was identical to that of the ^{125}I-labeled apoproteins, which indicates that the particle is taken up as a unit. Thus, lactosylated HDL is taken up rapidly and selectively by parenchymal liver cells, and it appears that it might be a very effective vehicle for the specific delivery of lipophilic drugs to these cells.

Advances made in molecular and cell biology have increased the understanding of the mechanisms of uptake and subsequent handling of molecules by cells. This knowledge can be used to increase the therapeutic indices and efficacies of drugs, by development of methods for specific delivery to their desired sites of action. A number of studies have shown that circulating D-galactose-containing ligands are rapidly taken up by the liver by a galactose-specific mechanism (1–5). Two different types of galactose-specific hepatic receptors have been described, specific for parenchymal or Kupffer cells (2, 3). The receptor on parenchymal cells is the classical asialoglycoprotein receptor originally described by Ashwell and Morell (2). The receptor on Kupffer cells was characterized more recently (3–5). It specifically recognizes particles larger than 10–15 nm that have exposed galactose residues. Recent evidence suggests that this so-called galactose-particle receptor might be identical to a circulating lectin, C-reactive protein (6). Because galactose-specific receptors are largely confined to liver parenchymal and Kupffer cells, they can be considered as suitable targets for the specific delivery of drugs to these cells.

A variety of galactose-containing molecules and particles can be regarded as potential carriers for the site-specific delivery of drugs to liver parenchymal and Kupffer cells. Liposomes with galactose-containing structures on their surfaces are taken up via galactose-specific receptors on both liver cell types (7–10). Galactose-terminated (neo)glycoproteins have been shown to be taken up specifically by parenchymal liver cells (1, 2, 11–13). The amounts of drugs that can be transported by the (neo)glycoproteins are, however, relatively low, because only limited numbers of drug molecules can be coupled to them (14, 15). Furthermore, coupling of drugs to the surface of these carriers can disturb the recognition of the carrier by the galactose receptors on the target cells (14). In earlier studies, we modified lipoproteins to direct them to hepatic galactose receptors (16–18). Lipoproteins are spherical particles consisting of a oily core, composed of apolar lipids, surrounded by a phospholid monolayer in which cholesterol and apoproteins are embedded (19, 20). A variety of highly lipophilic compounds can be incorporated in the apolar core of lipoproteins (20, 21). Large quantities of lipophilic compounds can thus be transported, hidden inside the particle. Lipoproteins are, therefore, potentially attractive drug carriers, and the distribution of the incorporated drug is determined by the distribution of the lipoprotein carrier. The apoproteins remain available for mod-

This work was supported by the Dutch Heart Foundation (Grant 85.050).

ABBREVIATIONS: LDL, low density lipoprotein; HDL, high density lipoprotein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; apo E, apolipoprotein E; Tris-gal-chol, n-(tris(β-D-galactopyranosyloxymethyl) methyl)-n°-(4-(5-cholesten-3β-yloxy)succinyl) glycinamide.

ification. In a recent study, we provided the apoprotein of LDL with terminal galactose residues by means of reductive lactosamination. The lactosylated LDL was very rapidly and highly specifically taken up via the galactose receptors on Kupffer cells (18). In the present study, we have lactosylated HDL and studied the hepatic uptake of the particle. The results indicate that lactosylated HDL is internalized rapidly and specifically via the galactose-specific receptors on parenchymal liver cells. Lactosylated HDL may, therefore, be a suitable carrier for the specific delivery of lipohilic drugs to parenchymal liver cells.

Experimental Procedures

Reagents. Lactose was supplied by Merck (Darmstadt, FRG). Sodium cyanoborohydride was from Aldrich (Brussels, Belgium). [1α,2α(n)-^{3}H]Cholesteryl oleate, ^{125}I (carrier free), and [D-*glucose*-1-^{14}C]lactose were supplied by Amersham International (Amersham, Bucks., UK). Collagenase type I, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, bovine serum albumin, agarose-bound neuraminidase (from *Clostridium perfringens*; type VI-A), and fetuin (type IV) were obtained from Sigma (St. Louis, MO). Emulsifier Safe and Hionic Fluor scintillation cocktails and Soluene-350 were from Packard (Downers Grove, IL). All other chemicals were analytical grade.

Fetuin was desialyated enzymatically by incubation of the protein, dissolved in 0.1 M sodium acetate buffer, pH 5.5, with agarose-bound neuraminidase (20 milliunits/ml) for 4 hr at 37°. A minimum of 80% of the sialic acid residues, assayed as described earlier (22), was removed by this procedure.

Isolation and radioiodination of HDL. Human HDL (1.063 < density < 1.210 g/ml) was isolated by two repetitive centrifugations, as described earlier (23). The lipoprotein was subsequently depleted of apo E-containing particles, using a Sepharose-heparin column (24). HDL was labeled with ^{125}I as described previously (17). Less than 1% of the labeled material was trichloroacetic acid soluble. For some experiments, the lipoprotein was labeled with ^{125}I-tyramine-cellobiose (TC). Synthesis and subsequent radioiodination of tyramine-cellobiose were carried out as described earlier (25). Coupling of ^{125}I-tyramine-cellobiose to HDL was done as follows. To 50 μl of 0.3 mM ^{125}I-TC were successively added 20 μl of 0.75 mM cyanuric chloride in acetone and 10 μl of 3.0 mM NaOH. After 20 sec, 20 μl of 2.25 mM acetic acid were added. The resulting activated ligand was added to 1–2 mg of HDL in 1 ml of 20 mM sodium tetraborate buffer, pH 9.0, containing 0.12 M NaCl and 1 mM EDTA. After 30 min at room temperature, the reaction was quenched by the addition of an equal volume of 0.2 M NH_4HCO_3. Unbound label was removed by exhaustive dialysis against phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 1 mM EDTA). Less than 1% of the labeled material was trichloroacetic acid soluble.

Incorporation of [^{3}H]cholesteryl oleate into HDL. The incorporation was done essentially as described by Pittman *et al.* (26). [^{3}H] Cholesteryl oleate was transferred from donor liposomal particles, consisting of phosphatidyl choline and cholesteryl oleate at a 50:1 mass ratio (27), using the cholesteryl oleate transfer protein present in lipoprotein-deficient human serum. The phospholipid transfer activity in the serum had been inactivated selectively by heating at 55° for 1 hr. The labeling was carried out by incubating a mixture of HDL and liposomes (apolipoprotein/liposome phospholipid mass ratio of 6:1) with an equal volume of lipoprotein-deficient serum, for 5 hr at 37°. The labeled HDL (specific radioactivity, 2–3 × 10^6 dpm/mg of apolipoprotein) was subsequently depleted of apo E-containing particles, using a Sepharose-heparin column (24).

Lactosylation of HDL. Aliquots of 0.2–1.0 mg of human HDL (2 mg/ml in 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA) were incubated sterilely at 37° with lactose and sodium cyanoborohydride, to final concentrations of 100 mg/ml and 50 mg/ml, respectively. After 8–168 hr, the reaction was stopped by the addition of 0.5 ml of 0.1 M NH_4HCO_3. Subsequently, sodium cyanoborohydride and unbound lactose were removed by exhaustive dialysis against phosphate-buffered saline.

Extraction of lactosylated HDL. [^{14}C]Lactosylated HDL, dissolved in borate-buffered saline (10 mM sodium borate buffer, pH 7.0, containing 150 mM NaCl and 1 mM EDTA), was freeze-dried. The lyophilized material was extracted with ethanol/diethyl ether (3:1) for 16 hr at 4° and subsequently centrifuged for 5 min at 2000 × g. The supernatant was aspirated, and the pellet was subjected to two similar extractions for 4 hr each. The final pellet was washed with ether, dried, and dissolved in 0.1 M sodium dodecyl sulfate. The dissolved pellet and the combined supernatants of the extractions were assayed for protein, phosphate (28), and radioactivity.

***In vivo* plasma clearance and liver association.** Male Wistar rats, weighing between 225 and 325 g, were used. The animals were anaesthesized by intraperitoneal injection of 15–20 mg of sodium pentobarbital, and the abdomen was opened. Radiolabeled lactosylated HDL was injected via the vena penis. At the indicated times, blood samples of 0.2–0.3 ml were taken from the inferior vena cava and collected in heparinized tubes. The samples were centrifuged for 2 min at 16,000 × g. Duplicate samples of plasma were assayed for ^{125}I radioactivity after precipitation of protein with 10% (w/v) trichloroacetic acid. Plasma samples containing ^{3}H radioactivity were not subjected to precipitation of protein. The total amount of radioactivity in plasma was calculated using the equation: plasma volume (ml) = [0.0219 × body weight (g)] + 2.66 (Ref. 18). At the indicated times, liver lobules were tied off and excised, and at the end of the experiment the remainder of the liver was removed. The amount of liver tissue tied off successively did not exceed 15% of the total liver mass. Radioactivity in liver at each time point was calculated from the radioactivities and weights of the liver samples and was corrected for radioactivity in plasma present in the tissue at the time of sampling (85 μl/g of fresh weight; Ref. 29).

Isolation of liver cells. Rats were anaesthesized and injected with radiolabeled lactosylated HDL, as described above. Ten minutes later, the vena porta was canulated and the liver was perfused with Ca^{2+}-free Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4 (8°), at a flow rate of 14 ml/min. After 8 min, a lobule was tied off for determination of the total liver uptake. Then, the liver was perfused with 0.05% (w/v) collagenase in Hanks' solution containing 10 mM HEPES, pH 7.4, and parenchymal and nonparenchymal cells were isolated as described previously (16). The nonparenchymal cell preparation was further fractionated into endothelial and Kupffer cells by centrifugal elutriation, as described in detail earlier (30). The contributions of the various cell types to the total liver uptake was calculated as described previously (30). As found earlier with other substrates (16, 30, 31), no significant amounts of radioactivity were lost from the cells during the isolation procedure. This was checked in each experiment by comparing the calculated liver uptake (i.e., the summation of the contributions of the various cell types) with the value actually measured in the liver lobule.

Determination of proteins. Protein concentrations in homogenates, subcellular fractions, cell suspensions, and solutions of HDL were determined by the method of Lowry *et al.* (32), with bovine serum albumin as the standard.

Determination of radioactivity. Samples containing ^{125}I were counted, without further processing, as γ-emitters in a Packard Auto-Gamma 5000 counter. Samples containing ^{3}H were counted in a Packard Tri-Carb 1500 liquid scintillation counter, using Emulsifier Safe or Hionic Fluor scintillation cocktails. Liver samples and cell suspensions containing ^{3}H were first digested with Soluene-350.

Subcellular fractionation. Rats were anaesthesized and injected with radiolabeled lactosylated HDL as described above. Thirty minutes later, the liver was perfused with ice-cold 0.25 M sucrose, containing 10 mM Tris·HCl buffer, pH 7.4. Subsequently, the liver was divided into subcellular fractions as described previously (33). In brief, the liver was dispersed in 2 volumes of sucrose/Tris·HCl (see above), using a homogenizer of the Potter-Elvehjem type. Fractions enriched in nuclei,

mitochondria, lysosomes, and microsomes were obtained by collecting pellets obtained after subjecting the homogenate to consecutive centrifugation steps of 5 min at 1,200 × *g*, 5 min at 9,000 × *g*, 15 min at 22,000 × *g*, and 30 min at 210,000 × *g*, respectively (*g*-forces in the middle of the tubes). The final supernatant was the cytosol fraction. The fractions were assayed for radioactivity, protein, and the activity of several marker enzymes, as described in detail earlier (33).

Results

Coupling of lactose to HDL. Apo E-free HDL was provided with terminal D-galactosyl residues by incubation with 100 mg/ml lactose (D-galactosyl-D-glucose) and 50 mg/ml sodium cyanoborohydride. The latter reduces the Schiff's base between the glucose moiety of lactose and amino groups on HDL, which results in covalent attachment of lactose to HDL (34). The extent of coupling was studied by measuring radioactivity in the lipoprotein after incubation with [^{14}C]lactose and sodium cyanoborohydride. Fig. 1 shows that the association of lactose to HDL increases with time. After 48 hr of incubation, 0.24 ± 0.03 mg of lactose was associated per mg of apolipoprotein, which corresponds to approximately 100 lactose residues/HDL particle. During the next 120 hr, the association increased only slowly, to 0.31 ± 0.05 mg of lactose/mg of apolipoprotein (mean ± standard error of three experiments). All the following experiments were done with HDL that had been lactosylated for 48 hr.

HDL consists of apoproteins and a lipid moiety. To determine the site of incorporation of lactose, the protein and lipid moieties of [^{14}C]lactosylated HDL were separated by extraction with ethanol/ether. The results, shown in Table 1, clearly indicate that lactose associates predominantly with the apoproteins. Analysis by density gradient centrifugation indicated that the coupling of lactose to HDL caused a small change in the density of the particle (Fig. 2). The density of lactosylated HDL

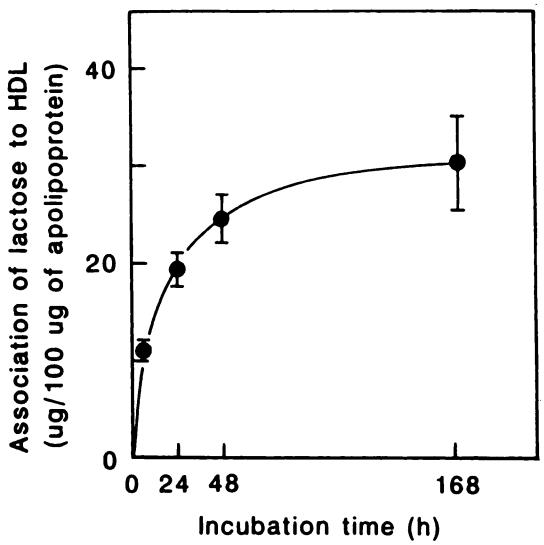

Fig. 1. Time course of coupling of [^{14}C]lactose to HDL. Aliquots of 0.2 mg of HDL were incubated with 20 mg of [^{14}C]lactose (specific radioactivity, 0.1 μCi/mg) and 10 mg of sodium cyanoborohydride, in 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA (final volume, 0.2 ml). At the indicated times, the reactions were stopped by the addition of 0.5 ml of 0.1 M NH_4HCO_3. Unbound lactose was removed by exhaustive dialysis against phosphate-buffered saline. The extent of coupling of lactose was calculated from the amounts of radioactivity and protein in the dialyzed sample. Values are means ± standard errors of three separate experiments.

TABLE 1

Distribution of lactose over the apolipoprotein and lipid moieties of lactosylated HDL

One milligram of HDL was lactosylated with [^{14}C]lactose (specific radioactivity, 0.1 μCi/mg) as described in Experimental Procedures. The apolipoprotein and lipid moieties were separated by extraction with ethanol/ether. The lipid and apolipoprotein fractions were assayed for protein, total phosphorus, and radioactivity. Values given, expressed as percentage of recovered amounts, are means ± standard errors of three separate experiments. Recoveries were phosphate, 95 ± 6%; protein, 108 ± 11%; and radioactivity, 93 ± 5%.

	Distribution	
	Protein fraction	Lipid fraction
	% of recovered amount	
Phosphate	14 ± 9	86 ± 9
Protein	93 ± 3	7 ± 3
Radioactivity	97 ± 1	3 ± 1

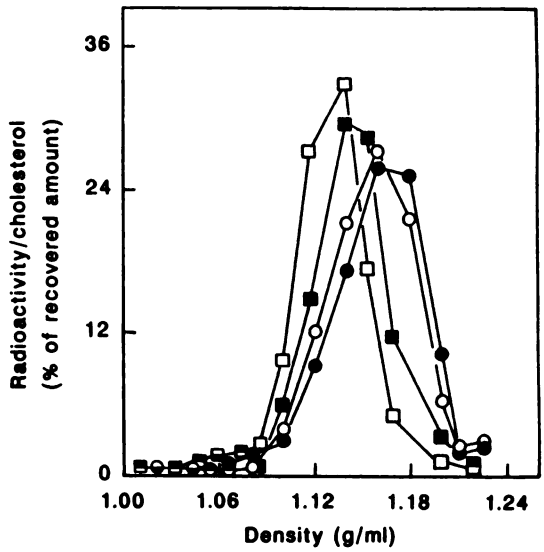

Fig. 2. Density gradient centrifugation of HDL and lactosylated HDL. Native ^{125}I-HDL and lactosylated ^{125}I-HDL (0.25 mg of apoprotein) were subjected to density gradient centrifugation, as described by Redgrave *et al.* (23). The gradients (12 ml) were fractionated into fractions of approximately 0.7 ml. The fractions were assayed for radioactivity and cholesterol, and their densities were measured. Recoveries were 82–94%. ■, Radioactivity of native HDL; □, cholesterol of native HDL; ●, radioactivity of lactosylated HDL; ○, cholesterol of lactosylated HDL.

(peak density, 1.157 g/ml) was found to be slightly higher than that of native HDL (peak density, 1.140 g/ml). Protein and cholesterol of lactosylated HDL were found at the same density, which indicates that lactosylation does not affect the integrity of the HDL particle.

Plasma clearance and liver uptake of lactosylated HDL. Native HDL disappears very slowly from the circulation of the rat (half-life, >4 hr), and only 3.3 ± 0.8% (mean ± standard error of three animals) of the injected dose is found in the liver at 10 min after injection of radioiodinated HDL. In sharp contrast, lactosylated ^{125}I-HDL is cleared from plasma very rapidly after intravenous injection into rats (Fig. 3). Virtually all the injected material was cleared from the circulation within 10 min. The decrease in plasma radioactivity coincided with an increase in radioactivity associated with the liver. Radioactivity in liver was maximal at 10 min after injection and subsequently declined. The decrease in liver radioactivity was accompanied by an increase of the trichloroacetic acid-soluble radioactivities in liver and plasma (see Fig. 3, *insets*).

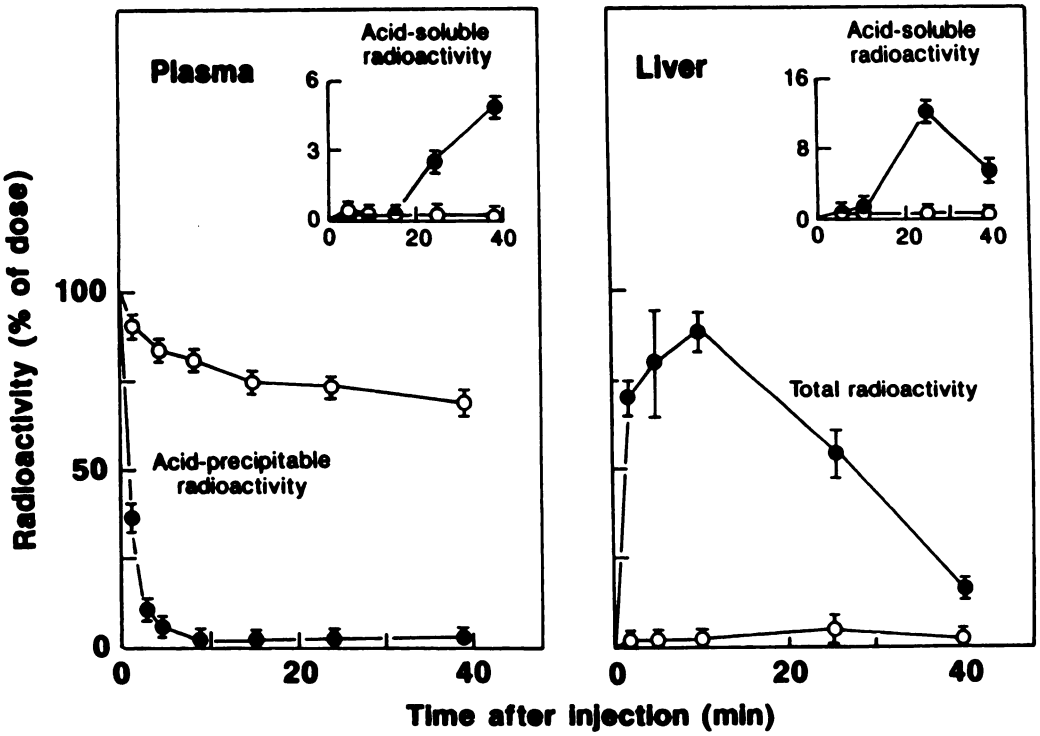

Fig. 3. Plasma clearance and liver uptake of intravenously injected native ^{125}I-HDL and lactosylated ^{125}I-HDL. Rats were injected with lactosylated ^{125}I-HDL (●) or native ^{125}I-HDL (○) at a dose of 50 μg of apolipoprotein/kg of body weight. At the indicated times, the amounts of radioactivity in plasma and liver were determined. *Left*, trichloroacetic acid-precipitable (*inset*, acid-soluble) radioactivities in plasma. *Right*, total (*inset*, trichloroacetic acid-soluble) radioactivities in liver. Values are means ± standard errors of three rats.

These findings indicate that lactosylated HDL, once associated with the liver, is degraded to acid-soluble products that diffuse into the blood. Because the degradation products have a low molecular weight, they do not accumulate in the circulation but are rapidly excreted into the urine by the kidneys. The lag periods in the increases in acid-soluble radioactivities in plasma and liver suggest that lactosylated HDL has to be internalized before degradation takes place.

To investigate the intracellular processing of lactosylated HDL, the liver was subjected to a subcellular fractionation (33). For these experiments, HDL was labeled with ^{125}I-tyramine-cellobiose, a label that, after degradation of the protein, is retained in cells (25). The distribution pattern of radioactivity closely resembled that of the lysosomal marker acid phosphatase (Fig. 4), whereas the microsomal marker glucose-6-phosphatase and the cytoplasmic marker lactate dehydrogenase showed clearly different distributions.

The mechanism of liver association was determined by preinjecting the animals with either *N*-acetylgalactosamine or *N*-acetylglucosamine. Preinjection of rats with *N*-acetylgalactosamine (400 mg/kg of body weight) inhibited the liver uptake of lactosylated HDL considerably, whereas preinjection with the same dose of *N*-acetylglucosamine had no effect at all (see Fig. 5). This indicates that galactose-specific recognition sites in the liver are responsible for uptake.

Cellular distribution of lactosylated LDL in the liver. To identify the cell type(s) responsible for uptake in the liver, rats were injected with lactosylated ^{125}I-HDL, and parenchymal, Kupffer, and endothelial cells were isolated from the liver 10 min later. The cell-isolation procedure was performed at a

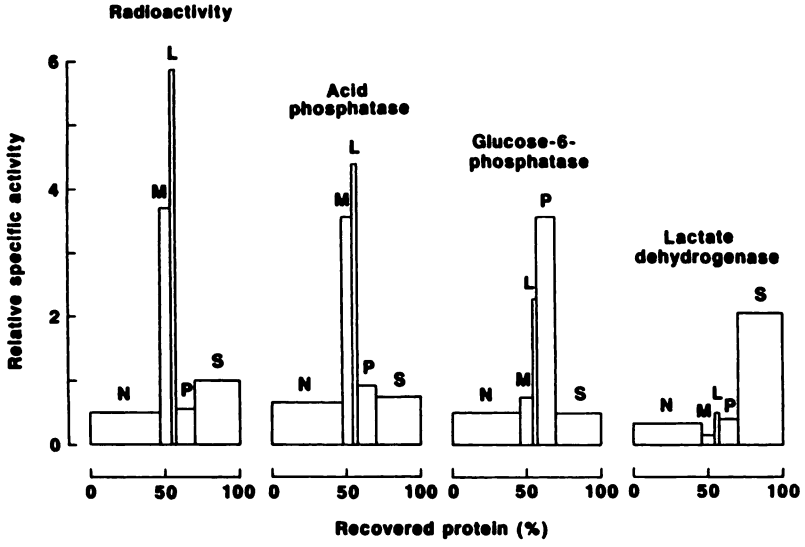

Fig. 4. Distribution patterns of radioactivity and marker enzymes over subcellular fractions of the liver after intravenous injection of radiolabeled lactosylated HDL. Rats were injected with 50 μg of lactosylated HDL (labeled with ^{125}I-tyramine-cellobiose)/kg of body weight. Thirty minutes after injection, the liver was perfused with ice-cold 0.25 M sucrose, containing 10 mM Tris·HCl buffer, pH 7.5, and was divided into subcellular fractions by differential centrifugation, as described earlier (33). The fractions were assayed for radioactivity, protein, and the activity of several marker enzymes (33); recoveries were >80%. Blocks (*left* to *right*) represent nuclear (*N*), mitochondrial (*M*), lysosomal (*L*), microsomal (*P*), and supernatant (cytosol) (*S*) fractions. *Abscissa*, relative protein concentration. *Ordinate*, relative specific activity (percentage of total recovered activity divided by percentage of total recovered protein).

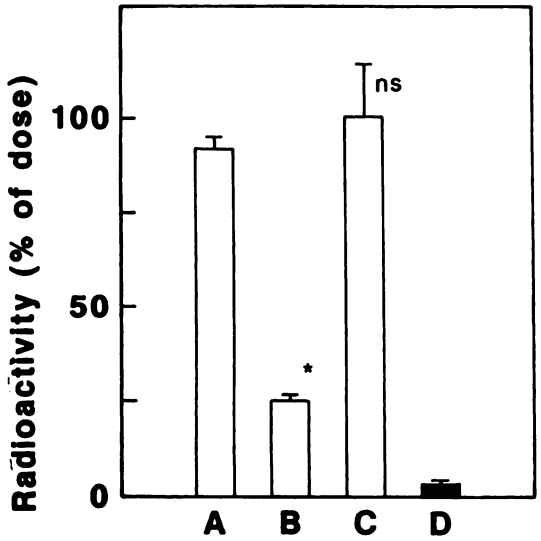

Fig. 5. Effects of *N*-acetylgalactosamine and *N*-acetylglucosamine on the liver association of lactosylated ^{125}I-HDL. Rats were injected with lactosylated ^{125}I-HDL at a dose of 50 μg of apolipoprotein/kg of body weight. One minute before injection, the animals received *N*-acetylgalactosamine (B) or *N*-acetylglucosamine (C), both at a dose of 400 mg/kg of body weight. Controls (A) were preinjected with solvent (phosphate-buffered saline). Ten minutes after injection, the total radioactivity in the liver was determined. Differences with respect to the controls were tested for significance by Wilcoxon's two-sample test (38). D, For comparison, the total amount of radioactivity present in the liver at 10 min after injection of native ^{125}I-HDL is given. Values are means ± standard errors of three to six rats. *, $p < 0.05$; *ns*, not significant.

TABLE 2

Uptake of intravenously injected lactosylated ^{125}I-HDL by liver cell types

Rats were injected with lactosylated ^{125}I-HDL at a dose of 50 μg of apolipoprotein/kg of body weight. Ten minutes later, parenchymal, endothelial, and Kupffer cells were isolated, and the association of radioactivity to each cell type was determined. Uptake by each cell type is expressed as ng of lactosylated HDL associated/mg of cell protein and as the relative contribution to the total liver uptake. The latter values were calculated from the uptake/mg of cell protein and the contribution of each cell type to the total liver protein (30). Values are means ± standard errors of three rats.

Cell type	Uptake of lactosylated HDL	
	ng/mg of cell protein	*% of total liver uptake*
Parenchymal cells	6.0 ± 1.5	98.1 ± 0.6
Kupffer cells	2.4 ± 1.5	1.0 ± 0.5
Endothelial cells	1.5 ± 0.5	0.9 ± 0.2

low temperature (8°) to prevent degradation of the internalized ligand. The results are shown in Table 2. The parenchymal cells were found to be the most active liver cells in uptake of lactosylated HDL. The uptake by these cells, expressed per milligram of cell protein, was 2.5 and 4 times higher than uptake by Kupffer and endothelial cells, respectively. From the contributions of each cell type to the total liver protein, it can be calculated that the liver uptake occurs almost exclusively by parenchymal cells.

Asialofetuin blocks uptake via the galactose-specific receptors on parenchymal liver cells, but not galactose-mediated uptake by Kupffer cells (35). In animals preinjected with asialofetuin (50 mg/kg of body weight), only 9.7 ± 1.7% of the dose was found in the liver at 10 min after injection of lactosylated ^{125}I-HDL (mean ± standard error of three rats). This finding confirms the major role of the parenchymal cells in the hepatic uptake of lactosylated HDL.

Tissue distribution of lactosylated HDL. The results shown in the previous section point to a prominent role of the liver in the removal of lactosylated HDL from the circulation. To investigate a possible specific uptake by other organs and tissues, the distribution of lactosylated ^{125}I-HDL over a large number of tissues was determined at 10 min after injection. Table 3 shows that only 1.8 ± 0.2% of the recovered dose was present in plasma as acid-precipitable radioactivity. More than 95% of the recovered dose was found in the liver, with the remaining 3% evenly distributed throughout the body. The specific uptake by liver was found to be at least 2 orders of magnitude higher than that in any other tissue.

Plasma clearance and liver uptake of [^{3}H]cholesteryl oleate-labeled lactosylated HDL. In the previous sections, the fate of lactosylated HDL was followed by monitoring the radioactivity of the ^{125}I-labeled apolipoproteins. As outlined in the introduction, the lipid moiety of lactosylated HDL is crucial for its putative role as drug carrier. In order to follow the lipid core of the particle, we incorporated [^{3}H]cholesteryl oleate into HDL, in a separate series of experiments. The labeled HDL was lactosylated and subsequently injected into rats. Fig. 6 shows that lactosylated [^{3}H]cholesteryl oleate-labeled HDL is rapidly cleared from plasma by the liver. Preinjection with *N*-acetylgalactosamine or asialofetuin (400 and 50 mg/kg of body weight, respectively) inhibited the plasma clearance and the hepatic association of radioactivity, whereas preinjection with *N*-acetylglucosamine had no effect. The intrahepatic distribution of [^{3}H]cholesteryl oleate-labeled lactosylated HDL was determined by isolating liver cells at 10 min after injection. The results, shown in Table 4, clearly indicate that the parenchymal cells are the main site of the hepatic uptake. This distribution is similar to the distribution found after injection of ^{125}I-labeled lactosylated HDL.

Discussion

We show in the present study that incubation of HDL with lactose and sodium cyanoborohydride results in the reproducible, time-dependent, covalent coupling of lactose to the apolipoproteins. Intravenously injected lactosylated ^{125}I-HDL (0.24 ± 0.03 mg of lactose/mg of apolipoprotein) is cleared extremely

TABLE 3

Tissue distribution of intravenously injected lactosylated ^{125}I-HDL

Rats were injected with lactosylated ^{125}I-HDL (50 μg of apolipoprotein/kg of body weight). At 10 min after injection, radioactivities in the indicated tissues and organs were determined (bone, skin, and muscle were dissolved in 10 M NaOH at 95°). The results are expressed as percentage of the recovered amount of radioactivity and as relative specific activity (percentage of recovered radioactivity divided by percentage of recovered weight). Recoveries of radioactivity and tissues were 96.6 ± 2.4% and 97.4 ± 0.2%, respectively. Values are means ± standard errors of three rats.

Tissue/organ	Radioactivity	
	% of recovered dose	
Liver	95.7 ± 0.6	23.9 ± 0.6
Plasma, acid-soluble	0.2 ± 0.1	<0.1
Plasma, acid-precipitable	1.8 ± 0.2	0.5 ± 0.1
Bone	0.5 ± 0.1	<0.1
Small intestine	0.2 ± 0.0	<0.1
Muscles	0.6 ± 0.1	<0.1
Skin	0.5 ± 0.1	<0.1
Relative specific radioactivity	0.5 ± 0.2	<0.1

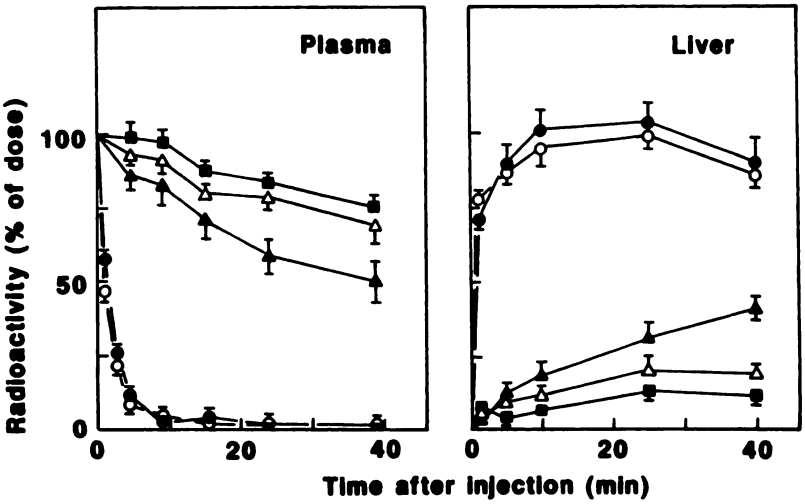

Fig. 6. Plasma clearance and liver uptake of [^{3}H]cholesteryl oleate-labeled lactosylated HDL and effects of *N*-acetylgalactosamine, *N*-acetylglucosamine, and asialofetuin. Rats were injected with [^{3}H]cholesteryl oleate-labeled lactosylated HDL at a dose of 200 μg of apolipoprotein/kg of body weight. One minute before injection of the label, the animals were preinjected with 400 mg of *N*-acetylgalactosamine/kg of body weight (▲), 400 mg of *N*-acetylglucosamine/kg of body weight (●), 50 mg of asialofetuin/kg of body weight (△), or solvent (phosphate-buffered saline) (○). At the indicated times, radioactivities in plasma and liver were determined. ■, Plasma clearance and liver association of native [^{3}H]cholesteryl oleate-labeled HDL. Values are means ± standard errors of three or four rats.

TABLE 4

Uptake of intravenously injected [^{3}H]cholesteryl oleate-labeled lactosylated HDL by liver cell types

Rats were injected with [^{3}H]cholesteryl oleate-labeled lactosylated HDL at a dose of 200 μg of apolipoprotein/kg of body weight. Ten minutes later, parenchymal, endothelial, and Kupffer cells were isolated, and the association of radioactivity to each cell type was determined. The contribution of each cell type to the total hepatic uptake was calculated as described in the legend to Table 2. Values are means ± standard errors of three rats.

Cell type	Uptake of lactosylated HDL
	% of total liver uptake
Parenchymal cells	96.7 ± 1.0
Kupffer cells	1.8 ± 1.0
Endothelial cells	1.5 ± 0.1

rapidly from the circulation of the rat. The rapid plasma clearance of lactosylated HDL was found to be due to galactose-specific uptake by the liver. This organ contained, at 10 min after injection, approximately 95% of the injected dose. The specific uptake by the liver was at least 100 times higher than that by any other tissue. In the liver, parenchymal cells were found to be responsible for the uptake of lactosylated HDL. These cells contributed about 98% to the total liver uptake, whereas Kupffer and endothelial cells contained only small amounts of lactosylated HDL. The uptake of lactosylated HDL via the galactose receptor on parenchymal cells is even more specific than the uptake of asialofetuin, because after injection of ^{125}I-asialofetuin only 80–85% of the hepatic radioactivity was found in parenchymal cells (35).

The protein and cholesterol of HDL show a similar shift in density on lactosylation. Also, the plasma clearance and hepatic uptake of the lipid moiety of lactosylated HDL, labeled with [^{3}H]cholesteryl oleate, were identical to those of the ^{125}I-labeled apoproteins. Thus, the integrity of the HDL particle is not affected by lactosylation, and it is taken up as a unit. This indicates that effective transport of core-associated drugs can be assured.

We have shown previously that galactose-specific uptake of HDL by parenchymal liver cells can also be induced by the incorporation of a galactose-containing cholesterol derivative (tris-gal-chol) into the lipid moiety of the lipoprotein (17). The complex of HDL with tris-gal-chol seems, nonetheless, less suitable as a drug carrier. Circulating tris-gal-chol-loaded HDL loses its galactose recognition marker due to exchange of tris-gal-chol from HDL to other lipoproteins and cell membranes. With tris-gal-chol-loaded HDL, about 80% of the injected dose can be directed to the parenchymal cells, whereas 20% remains in the circulation. In the case of lactosylated HDL, the galactose residues are covalently coupled to the apolipoproteins, and complete removal of the particle from the circulation is achieved. Furthermore, the incorporation of tris-gal-chol into HDL might hamper the capacity of the lipid moiety to accomodate lipophilic drugs. The galactose residues of lactosylated HDL, on the other hand, are attached to the apolipoproteins, which leaves unaffected the capacity of the core to incorporate drugs.

We have demonstrated recently that extensive lactosylation of LDL results in a rapid, highly specific, galactose-mediated uptake by the liver (18). However, in contrast to lactosylated HDL, uptake of lactosylated LDL occurs predominantly by Kupffer cells. Why does the lactosylation of LDL and HDL lead to such a differential targeting to hepatic galactose receptors? Results from earlier studies suggest that the uptake of galactose-containing ligands by Kupffer cells and parenchymal cells depends largely on the size of the ligand (5). Unlike parenchymal cells, Kupffer cells are unable to internalize small molecules like asialofetuin (diameter, 4.5 nm) via their galactose-specific receptors. Asialofetuin-coated gold particles with an overall diameter of >10–15 nm are, however, taken up readily by Kupffer cells (5). Recently, the binding of a wide range of galactose-terminated ligands to isolated Kupffer and parenchymal cells was studied in our laboratory. It was found that, for an efficient uptake by Kupffer cells, a minimum size of 12 nm is required, whereas parenchymal cells internalize ligands of all sizes.[1] HDL has a mean size of 10 nm (36) and appears to be too small for uptake via the galactose receptors on Kupffer cells. It can, however, be taken up by parenchymal cells. LDL, on the other hand (mean diameter, 23 nm) (36), is sufficiently large to be taken up via the galactose receptors on Kupffer cells. The difference in size may, therefore, explain the different fates of the lactosylated lipoproteins, although identical recognition markers are present on both particles.

Particular types of liposomes have been proposed as carriers of drugs to liver parenchymal liver cells. Scherphof *et al.* (37) demonstrated that preferential uptake of liposomes by paren-

[1]Biessen, E.A.L., D.M. Beuting, H.F.Bakkeren, J. Kuiper and Th. J.C. van Berkel. Evaluation of the particle size as a determinant of affinity for the hepatic galactose-particle and asialoglycoprotein receptor.

chymal liver cells can be achieved if the vesicles are prepared using specific combinations of lipids. The uptake of liposomes by liver parenchymal cells can also be enhanced by incorporating lactosylceramide or by coupling asialofetuin to the vesicles, which induces uptake via the galactose receptors on these cells (7, 8). However, these various types of liposomes are cleared much more slowly (half-lives, >10 min) from the circulation than is lactosylated HDL. The short plasma half-life of lactosylated HDL is probably advantageous, because it forestalls possible leakage of incorporated drugs from the vehicle. Furthermore, the uptake of the liposomes was not restricted to parenchymal cells. Other cell types, in particular Kupffer cells, showed considerable uptake. Uptake by Kupffer cells appears difficult to avoid, because the minimum size of liposomes (25 nm) is larger than the maximum size of 10–15 nm that is required for galactose-containing particles to escape uptake by Kupffer cells (5).

A number of galactose-terminated (neo)glycoproteins are, like lactosylated HDL, rapidly internalized via the galactose receptors on liver parenchymal cells (1, 2, 11–13). They have, therefore, also been proposed as carriers of drugs to these cells. The drugs to be transported by these putative carriers have to be coupled to the surface of the proteins. It was found, however, that new structures on these proteins may disturb recognition by the galactose receptor on the target cell and induce uptake by other cells. It was even questioned whether the glycoprotein targeted a particular drug or whether the drug was targeting the glycoprotein (14). Furthermore, the capacity of these carriers is relatively low, because only limited amounts of drugs can be coupled. Lactosylated HDL, however, can accomodate relatively large amounts of lipophilic drugs in its lipid moiety. Moreover, it is less likely that the drugs present in the lipid moiety interfere with the recognition of the lactose residues coupled to the surface of the apoproteins.

In conclusion, it appears that lactosylated HDL is a promising transport vehicle for the specific delivery of drugs to parenchymal liver cells. Although no drug delivery experiments using HDL have been reported, it has been found that some lipophilic drugs incorporate spontaneously into lipoproteins, including HDL (20). Hydrophilic drugs can be rendered lipohilic, for instance by coupling of fatty acyl chains, and subsequently incorporated (21). The specific delivery of drugs to parenchymal liver cells may be relevant for the treatment of metabolic diseases or viral infections like hepatitis B.

Acknowledgments

We thank Ms. M. Wieriks for typing the manuscript and Ms. M. N. Pieters and Mr. D. Schouten for preparing HDL.

References

1. Ashwell, G., and J. Harford. Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.* **51**:531–554 (1982).
2. Ashwell, G., and A. G. Morell. The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. *Adv. Enzymol.* **41**:99–128 (1974).
3. Kolb-Bachofen, V., J. Schlepper-Schäfer, and W. Vogell. Electron microscopic evidence for an asialoglycoprotein receptor on Kupffer cells: localization of lectin-mediated endocytosis. *Cell* **29**:859–866 (1982).
4. Roos, P. H., H.-J. Hartman, J. Schlepper-Schäfer, H. Kolb, and V. Kolb-Bachofen. Galactose-specific receptors on liver cells. II. Characterization of the purified receptor from macrophages reveals no structural relationship to the hepatocyte receptor. *Biochim. Biophys. Acta* **847**:115–121 (1985).
5. Schlepper-Schäfer, J., D. Hülsmann, A. Djovkar, H. E. Meyer, L. Herbertz, H. Kolb, and V. Kolb-Bachofen. Endocytosis via galactose receptors *in vivo*: ligand size directs uptake by hepatocytes and/or liver macrophages. *Exp. Cell Res.* **165**:494–506 (1986).
6. Kempka, G., P. H. Roos, and V. Kolb-Bachofen. A membrane-associated form of C-reactive protein is the galactose-specific particle receptor on rat liver macrophages. *J. Immunol.* **144**:1004–1009 (1990).
7. Gregoriadis, G., and E. D. Neerunjun. Homing of liposomes to target cells. *Biochem. Biophys. Res. Commun.* **65**:537–544 (1975).
8. Spanjer, H. H., and G. L. Sherphof. Targeting of lactosylceramide-containing liposomes to hepatocytes *in vivo*. *Biochim. Biophys. Acta* **734**:40–47 (1983).
9. Spanjer, H. H., H. Morselt, and G. L. Scherphof. Lactosylceramide-induced stimulation of liposome uptake by Kupffer cells *in vivo*. *Biochim. Biophys. Acta* **774**:49–55 (1984).
10. Spanjer, H. H., T. J. C. van Berkel, G. L. Scherphof, and H. J. M. Kempen. The effect of a water-soluble tris-galactoside terminated cholesterol derivative on the *in vivo* fate of small unilamellar vesicles in rats. *Biochim. Biophys. Acta* **816**:396–402 (1985).
11. Wilson, G. Effect of reductive lactosamination on the hepatic uptake of bovine pancreatic ribonuclease A dimer. *J. Biol. Chem.* **253**:2070–2072 (1978).
12. Hubbard, A. L., G. Wilson, G. Ashwell, and H. Stukenbrok. An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. *J. Cell Biol.* **83**:47–64 (1979).
13. Wall, D. A., G. Wilson, and A. L. Hubbard. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. *Cell* **21**:79–93 (1980).
14. Van der Sluys, P., H. P. Bootsma, B. Postema, F. Moolenaar, and D. K. F. Meijer. Drug targeting to the liver with lactosylated albumins: does the glycoprotein target the drug or is the drug targeting the glycoprotein? *Hepatology* **6**:723–728 (1986).
15. Fiumi, L., C. Busi, and A. Mattioli. Lactosaminated human serum albumin as hepatotropic drug carrier. *FEBS Lett.* **146**:42–46 (1982).
16. Van Berkel, T. J. C., J. K. Kruijt, H. H. Spanjer, J. F. Nagelkerke, L. Harkes, and H. J. M. Kempen. The effect of a water-soluble tris-galactoside-terminated cholesterol derivative on the fate of low density lipoproteins and liposomes. *J. Biol. Chem.* **260**:2694–2699 (1985).
17. Van Berkel, T. J. C., J. K. Kruijt, and H. J. M. Kempen. Specific targeting of high density lipoproteins to liver hepatocytes by incorporation of a tris-galactoside-terminated cholesterol derivative. *J. Biol. Chem.* **260**:12203–12207 (1985).
18. Bijsterbosch, M. K., G. J. Ziere, and T. J. C. van Berkel. Lactosylated low density lipoprotein: a potential carrier for the site-specific delivery of drugs to Kupffer cells. *Mol. Pharmacol.* **36**:484–489 (1989).
19. Brown, M. S., and J. L. Goldstein. A receptor-mediated pathway for cholesterol homeostasis. *Science (Washington D. C.)* **232**:34–47 (1986).
20. Counsell, R. E., and R. C. Pohland. Lipoproteins as potential site-specific delivery systems for diagnostic and therapeutic agents. *J. Med. Chem.* **25**:1115–1120 (1982).
21. Shaw, J. M., K. V. Shaw, S. Yanovich, M. Iwanik, W. S. Futch, A. Rosowsky, and L. B. Schook. Delivery of lipophilic drugs using lipoproteins. *Ann. N. Y. Acad. Sci.* **507**:252–271 (1987).
22. Spiro, R. G. Analysis of sugars found in glycoproteins. *Methods Enzymol.* **8**:3–26 (1966).
23. Redgrave, T. G., D. C. K. Roberts, and C. E. West. Separation of plasma lipoproteins by density-gradient ultracentrifugation. *Anal. Biochem.* **65**:42–49 (1975).
24. Weisgraber, K. H., and R. W. Mahley. Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* **21**:316–325 (1980).
25. Pittman, R. C., T. E. Carew, C. K. Glass, S. R. Green, C. A. Taylor, and A. D. Attie. A radioiodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation *in vivo*. *Biochem. J.* **212**:791–800 (1983).
26. Pittman, R. C., T. P. Knecht, M. S. Rosenbaum, and C. A. Taylor, Jr. A nonendocytotic mechanism for the selective uptake of high density lipoprotein-associated cholesterol esters. *J. Biol. Chem.* **262**:2443–2450 (1987).
27. Hough, J. L., and D. B. Zilversmit. Comparison of various methods for *in vitro* cholesteryl ester labeling of lipoproteins from hypercholesterolemic rabbits. *Biochim. Biophys. Acta* **792**:338–347 (1984).
28. Bartlett, G. R. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**:466–468 (1959).
29. Caster, W. O., A. B. Simon, and W. D. Armstrong. Evans blue space in tissues of the rat. *Am. J. Physiol.* **183**:317–321 (1955).
30. Nagelkerke, J. F., K. P. Barto, and T. J. C. van Berkel. *In vivo* and *in vitro* uptake and degradation of acetylated low density lipoprotein by rat liver endothelial, Kupffer and parenchymal cells. *J. Biol. Chem.* **263**:12221–12227 (1983).
31. Kuiper, J., M. Otter, D. C. Rijken, and T. J. C. van Berkel. Characterization of the *in vivo* interaction of tissue-type plasminogen activator with liver cells. *J. Biol. Chem.* **263**:18220–18224 (1988).
32. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
33. De Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux, and F. Appelmans. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat liver tissue. *Biochem. J.* **60**:604–617 (1955).
34. Gray, G. R. The direct coupling of oligosaccharides to proteins and derivatized gels. *Arch. Biochem. Biophys.* **163**:426–428 (1974).
35. Van Berkel, T. J. C., C. J. Dekker, J. K. Kruijt, and H. G. van Eijk. The

interaction *in vivo* of transferrin and asialofetuin with liver cells. *Biochem. J.* **243**:715–722 (1987).
36. Forte, T., and A. V. Nichols. Application of electron microscopy to the study of plasma lipoprotein structure. *Adv. Lipid Res.* **10**:1–41 (1972).
37. Scherphof, G. J., H. H. Spanjer, J. T. P. Derksen, F. Kuipers, R. J. Vonk, T. Daemen, and F. Roerdink. Delivery of liposome-associated drugs to liver cells. *Biochem. Soc. Trans.* **15**:345–348 (1987).
38. Wilcoxon, F. Individual comparisons by ranking methods. *Biom. Bull.* **1**:80–83 (1945).

Send reprint requests to: Dr. M. K. Bijsterbosch, Division of Biopharmaceutics, Center for Bio-pharmaceutical Sciences, University of Leiden, P.O. Box 9503, 2300 RA Leiden, The Netherlands.