Hepatic Disposition Characteristics of ^{111}In-Labeled Lactosaminated Bovine Serum Albumin in Rats

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The hepatic disposition of lactosaminated bovine serum albumin (Lac-BSA) in rats was studied at the whole body, isolated liver, and isolated parenchymal cell levels. After intravenous injection, ^{111}In-Lac-BSA (1 mg/kg) was rapidly eliminated from the plasma due to extensive uptake by liver parenchymal cells; however, a significant decrease in hepatic clearance was observed at high dose (50 mg/kg). In a single-pass, constant infusion experiment in the isolated liver, ^{111}In-Lac-BSA was continuously extracted. The extraction ratio at steady state (E_{ss}) for ^{111}In-Lac-BSA was significantly decreased by coadministrating galactose, NH_4Cl, or chloroquine, and at low temperature, suggesting that hepatic uptake of Lac-BSA proceeds via receptor-mediated endocytosis for asialoglycoprotein. Kinetic analysis of ^{111}In-Lac-BSA binding with isolated parenchymal cells at 4°C yielded a dissociation constant (K_d) of 2.5×10^{-8} M and a value of 3.5×10^5 maximal binding sites/cell (B_{max}). The internalization rate constant (k_{int}) for ^{111}In-Lac-BSA was calculated to be 0.46 min^{-1} in liver perfusion experiments using the EDTA-wash method.

KEY WORDS: lactosaminated bovine serum albumin; liver targeting; receptor-mediated endocytosis; rat *in vivo* disposition; constant infusion of the liver; hepatocyte uptake.

INTRODUCTION

Asialoglycoprotein receptor-mediated drug targeting to the liver, first reported by Rogers and Kornfeld (1), has attracted great interest as a potential method to deliver drugs and macromolecules preferentially to specific cells in the liver. Natural desialylated glycoproteins such as asialoorosomucoid and asialofetuin have been employed for this purpose (2). Synthetic glycosylated protein (neoglycoprotein) has been utilized as an effective tool in elucidation of the characteristics of endocytosis via carbohydrate recognition in the cell. Neoglycoproteins have several advantages over natural desialylated glycoproteins in mass and uniform production, control of the number and structure of sugar residues, and probable reduction of antigenicity (3–5). Neoglycoproteins have been studied as a prototype of receptor-mediated endocytosis (6).

In the present study, lactosaminated bovine serum albumin (Lac-BSA) was selected as a model neoglycoprotein for examining the effect of chemical modification on systemic and hepatic disposition of proteins. Lac-BSA's binding to the cell surface and its subsequent internalization were investigated at the whole body, organ, and cellular levels. Finally, the hepatic disposition characteristics of Lac-BSA were compared with those of cationized protein (7,8).

MATERIALS AND METHODS

Animals

For *in vivo* and other animal experiments, male Wistar rats weighing, respectively, 240–250 and 190–210 g were used. Rats were maintained on standard rat foods and water ad libitum.

Chemicals

BSA (fraction V), type I collagenase, and chloroquine were obtained from Sigma Chemical Co. (St. Louis, MO). Diethylenetriaminopentaacetic acid (DTPA) anhydride was purchased from Dojindo Labs (Kumamoto, Japan). [^{111}In]Cl_3 (74 MBq/ml) was kindly supplied by Nihon Medi-physics Co. (Takarazuka, Japan). All other chemicals were of the finest grade available. Lac-BSA was synthesized by coupling of lactose to the ϵ-NH_2 of lysine residues of BSA by reductive amination with cyanoborohydride (9). Mannosylated BSA (Man-BSA) was synthesized by attaching 2-imino-2-methoxyethyl 1-thiomannoside to BSA (10). Cationized BSA (Cat-BSA) was synthesized by covalent coupling of hexamethylenediamine to BSA (11) and the product of $pI > 9$ was purified by chromatofocusing. The amounts of sugar conjugated to Lac-BSA and Man-BSA were calculated to be 18 and 16, respectively, by the phenol/sulfuric acid method (9) calibrated against galactose or mannose. The BSA derivatives have almost the same effective molecular size as original BSA by gel-filtration chromatography. BSA derivatives were labeled with ^{111}In using the bifunctional chelating agent DTPA anhydride (12), and the ^{111}In radioactivity was counted using a well-type NaI scintillation counter (Model ARC-500, Aloka, Tokyo, Japan).

In Vivo Disposition Experiment

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg) and saline solutions of ^{111}In-Lac-BSA (1 or 50 mg/kg) were injected into a femoral vein. The body temperature of rats was kept at 37°C by a heat lamp during the experiment. Blood samples (0.2 ml) were withdrawn from the jugular vein over 6 hr and centrifuged at 3000 rpm for 2 min. At 6 hr after injection, the liver was perfused with collagenase to isolate the liver cells, and parenchymal cells (PC) were separated from nonparenchymal cells (NPC) by centrifugation (7). In addition, the other organs were excised, rinsed with saline, and weighed, and their radioactivities were counted.

The plasma concentration–time curve of ^{111}In-Lac-BSA was analyzed on the basis of moment theory (13), and the area under the plasma concentration–time curves (AUC) and the mean residence time (MRT) were calculated by the trapezoidal method (13). Apparent total-body clearance (CL_{total}) and hepatic clearance (CL_{liver}) were calculated by dividing

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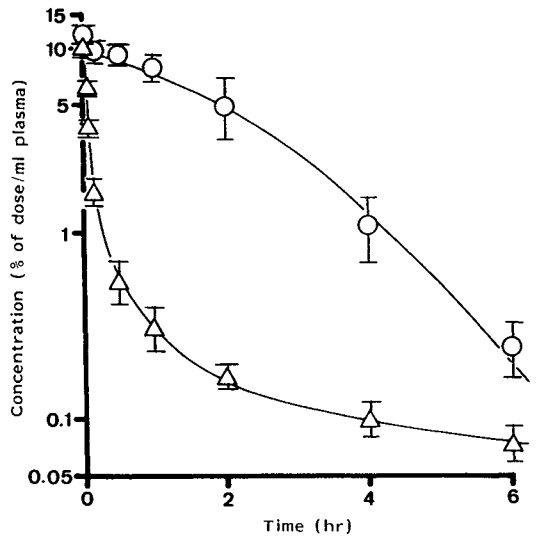

Fig. 1. Plasma concentration of ^{111}In-Lac-BSA after intravenous injection into rats at doses of 1 mg/kg (△) and 50 mg/kg (○). Values are means of three experiments, with the vertical bars indicating the SD.

the injection dose and the total uptake amount in the liver by AUC, respectively. The amount of ^{111}In-Lac-BSA in the liver was estimated from the total number of each cell type contained in 1 g liver (1.25×10^8 and 6.5×10^7 cells/g liver for PC and NPC, respectively) (14).

Isolation of PC

The method of isolation of PC has been previously described (7). The cell suspension was incubated for 4 hr in Williams E medium (Flow Labs, McClean, VA) containing 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.1% BSA in a 95% O_2–5% CO_2 atmosphere, in order to reexpose the asialoglycoprotein receptor which had existed in the cell surface before collagenase perfusion (15). Cell viability was assessed both by exclusion of 0.5% trypan blue and by release of lactate dehydrogenase into the culture medium (15) and estimated to be higher than 90% during the binding experiment.

In Vitro Cellular Binding of ^{111}In-Lac-BSA

The binding experiment with PC was carried out in capped 17 × 120-mm polystyrene tubes (Falcon Plastics, Becton and Dickinson, Cockeysville, MD). PC (10^6 cells/ml) were incubated at 4°C in 10 m*M* HEPES-buffered Hanks' buffer (pH 7.2) containing ^{111}In-Lac-BSA (0.01–10 μg/ml). After a 60-min incubation, cells were washed twice with ice-cold Hanks' buffer (pH 7.2). Then, ice-cold Ca^{2+}- and Mg^{2+}-free Dulbecco phosphate-buffered saline (pH 5) containing 20 m*M* ethylenediaminetetraacetic acid (EDTA) (EDTA-wash buffer) was added and the radioactivity detached from the cell surface membrane was determined. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled Lac-BSA. The amount bound to the receptor was corrected for nonspecific binding.

Liver Perfusion Experiment

The method for *in situ* liver perfusion was reported previously (16). The liver was perfused with a single-pass mode at a flow rate of 13 ml/min with Krebs–Ringer bicarbonate buffer with 10 m*M* glucose (oxygenated with 95% O_2–5% CO_2 to pH 7.4 at 37°C). To avoid the effect of interactions with blood components, liver perfusions were carried out with blood component-free perfusate. After a stabilization period of 30 min, ^{111}In-Lac-BSA dissolved in the perfusate (0.7 μg/ml) was infused for 60 min. In the inhibition studies, galactose (20 m*M*), NH_4Cl (20 m*M*), or chloroquine (100 μ*M*) was also added to the perfusate. The venous outflow of the perfusate and bile were collected into weighed tubes at appropriate intervals. At the completion of infusion, the remaining radioactivity in the liver was measured. The viability of the liver was checked by both the bile flow and the glutamic oxaloacetic transaminase activity in the outflow. In all experiments, perfused livers remained viable during the course of the study.

From the outflow curves, the extraction ratio at steady state (E_{ss}) for ^{111}In-Lac-BSA is calculated as follows:

$$E_{ss} = (C_{in} - C_{out})/C_{in} \quad (1)$$

where C_{in} and C_{out} are the concentrations of ^{111}In-Lac-BSA in the perfusate before and after passing through the liver under steady-state conditions, and Q is the perfusion rate.

Determination of Surface-Bound and Internalized ^{111}In-Lac-BSA

Detachment of ^{111}In-Lac-BSA from the surface of the liver tissue was carried out by the EDTA-wash treatment (17) with slight modification of the perfused buffer. ^{111}In-Lac-BSA (0.7 μg/ml) was infused for 2–20 min and then the ice-cold EDTA-wash buffer was infused for 5 min. The amount of surface-bound ^{111}In-Lac-BSA was estimated from the radioactivity recovered in the outflow. The radioactivity remaining in the extracellular space and the catheter compartment at the end of the ^{111}In-Lac-BSA infusion was calculated by multiplying the log average concentration (17) in these compartments by their volume, and the real surface-

Table I. Pharmacokinetic Parameters for ^{111}In-Lac-BSA After Intravenous Injection into Rats[a]

Dose (mg/kg)	AUC (% dose · hr/ml)	MRT (hr)	CL_{total} (μl/hr)	Amount in the liver at 6 hr (% of dose)	CL_{liver} (μl/hr)
1	1.64 ± 0.09	0.22 ± 0.08	61,205 ± 3,464	97.3 ± 2.6	59,676 ± 1,595
50	20.41 ± 3.56	1.64 ± 0.04	5,067 ± 967	68.7 ± 5.7	3,503 ± 293

[a] Values are means ± SD of three experiments.

bound amount was determined by correcting for this value. After EDTA-wash treatment, the remaining radioactivity in the liver (internalized ligand) was determined.

Estimation of the Internalization Rate Constant

The internalization rate constant (k_{int}) may be calculated from the slope of the line described by the following equation:

$$[LR_i]_t = k_{int} \cdot \int_0^t [LR_s]dt \quad (2)$$

where $[LR_s]$ and $[LR_i]$ are the concentrations of the ligand–receptor complex at the cell surface and in the intracellular space, respectively, assuming first-order kinetics for internalization and no lysosomal degradation. $\int_0^t [LR_s]dt$ may be calculated by the trapezoidal method (13). The experimental period for the measurement of k_{int} was 20 min and degradation of ^{111}In-Lac-BSA detected by 5% trichloroacetic acid (TCA) precipitation was negligible (<3%), in good agreement with previous results (18,19).

RESULTS

Elimination of ^{111}In-Lac-BSA in Plasma

The plasma concentrations of ^{111}In-Lac-BSA were determined for 6 hr after intravenous injection at doses of 1 and 50 mg/kg (Fig. 1). ^{111}In-Lac-BSA showed extremely rapid and biphasic elimination from plasma at a dose of 1 mg/kg. In contrast, the high dose gave prolonged plasma retention. The CL_{total} was significantly decreased at high doses (Table I), suggesting a saturable elimination process. The amount of ^{111}In-Lac-BSA recovered in the liver at the end of the experiment was 97 and 69% of dose at doses of 1 and 50 mg/kg, respectively; only small amounts of radioactivity were detected in other tissues.

Cellular Localization of BSA Derivatives in the Liver

Figure 2 shows the distribution (% of dose/10^8 cells) of ^{111}In-Lac-BSA and ^{111}In-Man-BSA between PC and NPC at 6 hr after intravenous injection. Results are compared with that of ^{111}In-Cat-BSA (8). ^{111}In-Man-BSA was predominantly taken up by NPC, which have the mannose receptor. On the other hand, ^{111}In-Lac-BSA and ^{111}In-Cat-BSA distributed mainly into PC, but the cellular uptake ratio between PC and NPC (PC/NPC) was much larger for ^{111}In-Lac-BSA (21.1) than for ^{111}In-Cat-BSA (2.8).

Hepatic Uptake During Constant Infusion of ^{111}In-Lac-BSA

During liver perfusion using a single-pass constant infusion mode, the recovery ratio (C_{out}/C_{in}) for ^{111}In-Lac-BSA reached plateau levels within 5 min and was continuously extracted by the liver. The E_{ss} and the recoveries in the liver tissue and bile are summarized in Table II. Hepatic uptake of ^{111}In-Lac-BSA was extremely large in the control condition. The E_{ss} for ^{111}In-Lac-BSA significantly decreased when 20 mM galactose was coadministered in the perfusate. In this case, the amount of ^{111}In-Lac-BSA bound to the cell surface was one-third of the control condition (data not shown). At low temperatures (4°C), internalization of ^{111}In-Lac-BSA was blocked without altering receptor binding because its retention was readily eliminated and the radioactivity of ^{111}In-Lac-BSA in the liver became nearly zero by the EDTA wash. Treatment with 20 mM NH_4Cl or 100 μM chloroquine, which inhibit the recycling of receptor–ligand complex by elevating the pH in endosomes and lysosomes (20,21), decreased E_{ss} for ^{111}In-Lac-BSA to 24 and 53% of the control values, respectively.

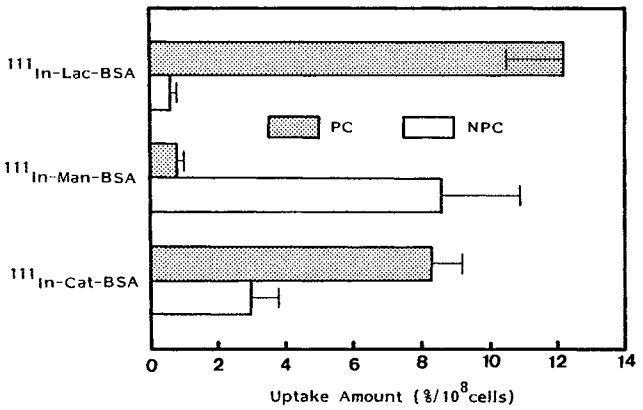

Fig. 2. Distribution of ^{111}In-labeled BSA derivatives in parenchymal (PC) and nonparenchymal cells (NPC) after intravenous injection into rats at a dose of 1 mg/kg. Values are means of three experiments, with the vertical bars indicating the SD.

In Vitro Binding of ^{111}In-Lac-BSA to PC

The binding of ^{111}In-Lac-BSA to PC at 4°C at different ligand concentrations is shown in Fig. 3A. The binding curve reached a plateau level at approximately 5 μg/10^6 cells. The Scatchard plot for ^{111}In-Lac-BSA binding is shown in Fig. 3B. The maximal number of binding sites (B_{max}) and the dissociation constant (K_d) were calculated to be 3.5×10^5 sites/cell and 2.5×10^{-8} M, respectively.

Estimation of Internalization Rate Constant

Figure 4A shows the time courses of the surface-bound and internalized amounts of ^{111}In-Lac-BSA. The surface-bound amount was almost constant after 5 min, while a linear increase was observed in the internalized amount over the 20-min period of measurement. Figure 4B shows a plot of

Table II. Extraction Ratio at Steady State (E_{ss}) and Recovery ^{111}In-Lac-BSA at an Inflow Concentration of 0.7 μg/ml Under Various Experimental Conditions in the Constant-Infusion Experiment[a]

Condition	Outflow, E_{ss} (%)	Recovery (% of dose)	
		Liver	Bile
Control (C_{in}: 0.7 μg/ml)	44.6 ± 4.0	35.6 ± 8.1	0.27 ± 0.09
+20 mM galactose	10.2 ± 3.2	5.6 ± 1.2	0.03 ± 0.01
+20 mM NH_4Cl	10.5 ± 0.2	9.8 ± 1.1	0.01 ± 0.01
+100 μM chloroquine	23.5 ± 1.0	26.2 ± 3.4	0
4°C	2.9 ± 1.7	9.8 ± 1.6	0

[a] Values are means ± SD of at least three experiments.

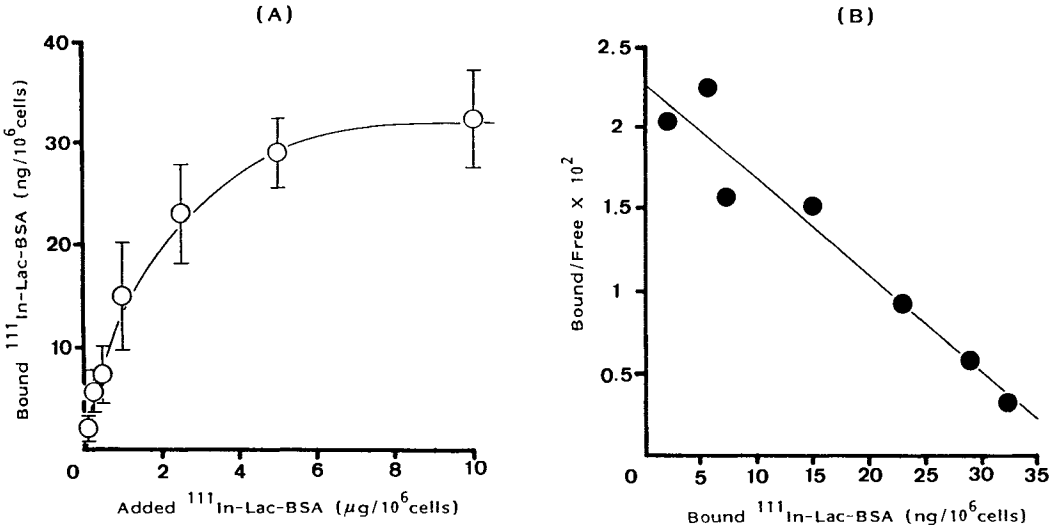

Fig. 3. Binding of ^{111}In-Lac-BSA to isolated rat parenchymal cells at 4°C (A) and Scatchard plots of data (r = 0.954) (B). Values are means of three experiments, with the vertical bars indicating the SD.

$[LR_i]$ versus $\int_0^t [LR_s]dt$ values according to Eq. (2). The k_{int} for ^{111}In-Lac-BSA was calculated to be 0.46 min^{-1}.

DISCUSSION

In the *in vivo* disposition study, more than 90% of ^{111}In-Lac-BSA was recovered from PC; this is consistent with the supposition that this BSA derivative was taken up by PC via asialoglycoprotein receptor-mediated endocytosis (6). ^{111}In-Cat-BSA also distributed largely into PC (8), but its cellular uptake ratio (PC/NPC) was small and approximately corresponded to the surface area ratio of PC to NPC (22). It may be speculated that this is a nonspecific interaction based on electrostatic forces. On the other hand, ^{111}In-Man-BSA distributed primarily into NPC which possess the mannose receptor (6,18). These characteristics of glycosylated proteins should facilitate drug targeting to different types of liver cells.

In the liver perfusion experiment, high hepatic clearance of ^{111}In-Lac-BSA was observed and the hepatic uptake of ^{111}In-Lac-BSA was almost completely inhibited by coadministration of 20 m*M* galactose, suggesting participation of the galactose receptor in cellular uptake of ^{111}In-Lac-BSA. Furthermore, the hepatic uptake of ^{111}In-Lac-BSA at steady state was blocked at low temperatures (4°C) without altering the binding of ^{111}In-Lac-BSA to the cell surface, suggesting that the internalization process was energy dependent. This result corresponded well with the previous finding that ^{125}I-asialofetuin was not internalized into the hepatocyte below 20°C (23). Chloroquine and NH_4Cl have been demonstrated

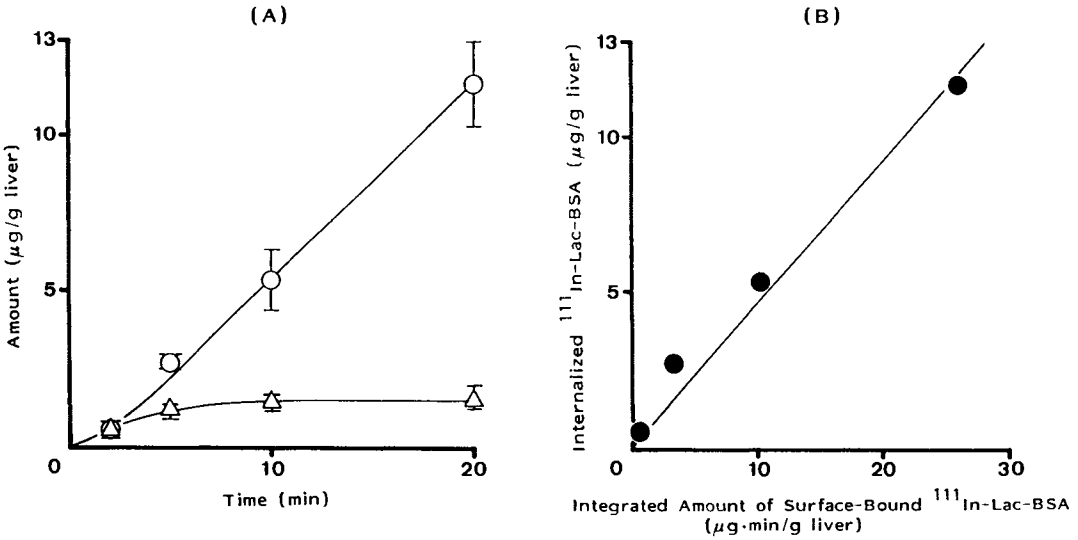

Fig. 4. Time course of the amount of surface-bound (△) and internalized (○) ^{111}In-Lac-BSA in the isolated rat liver perfusion system (A) and plot of ^{111}In-Lac-BSA internalized in the liver versus integrated amount of surface-bound ^{111}In-Lac-BSA (B). Values are means of three experiments, with the vertical bars indicating the SD.

to interfere with receptor-mediated uptake and degradation of asialoglycoprotein by raising the pH of endosomes and lysosomes (20,21). In the present study, NH_4Cl (20 m*M*) and chloroquine (100 μ*M*) inhibited hepatic uptake of ^{111}In-Lac-BSA, suggesting that the uptake of ^{111}In-Lac-BSA would proceed by this pathway.

PC binding parameters (B_{max} and K_d) for ^{111}In-Lac-BSA were in good agreement with those of asialoorosomucoid (15,24). In comparison with ^{111}In-Cat-BSA (7), affinity of ^{111}In-Lac-BSA to PC was higher, whereas PC binding capacity (B_{max}) was reduced approximately 1000-fold. Similarly, the k_{int} for ^{111}In-Lac-BSA (0.46 min^{-1}) in the liver perfusion system was similar to those for ^{125}I-asialoorosomucoid (0.48 min^{-1}) (25) and ^{125}I-galactosylated BSA (0.26 min^{-1}) (26) observed in the *in vitro* experiment with PC. The k_{int} for ^{111}In-Cat-BSA was estimated to be 0.015 min^{-1} in the liver perfusion system (8) using the surface-bound amount calculated from the *in vitro* data.

Based on these results, it appears that introduction of a galactose residue onto the protein results in preferential delivery to PC; mannose introduction appears to lead to specific uptake by NPC. While cationization of proteins also affords targeting to the liver, there is no specificity in their cellular distribution.

REFERENCES

1. J. C. Rogers and S. Kornfeld. Hepatic uptake of proteins coupled to fetuin glycopeptide. *Biochem. Biophys. Res. Commun.* 45:622–629 (1971).
2. D. K. F. Meijer and P. V. D. Sluijs. Covalent and noncovalent protein binding of drugs: Implications for hepatic clearance, storage, and cell-specific drug delivery. *Pharm. Res.* 6:105–118 (1989).
3. L. Fiume, C. Busi, and A. Mattioli. Lactosaminated human serum albumin as hepatotropic drug carrier rate of uptake by mouse liver. *FEBS Lett.* 146:42–46 (1982).
4. L. Fiume, A. Mattioli, C. Busi, G. Spinosa, and Th. Wieland. Conjugates of adenine 9-β-D-arabinofuranoside monophosphate (ara-AMP) with lactosaminated homologous albumin are not immunogenic in the mouse. *Experientia* 38:1087–1089 (1982).
5. L. Fiume, B. Bassi, C. Busi, A. Mattioli, and G. Spinosa. Drug targeting in antiviral chemotherapy: A chemically stable conjugate of 9-β-D-arabinofuranosyl-adenine-5′-monophosphate with lactosaminated albumin accomplishes a selective delivery of the drug to liver cells. *Biochem. Pharmacol.* 35:967–972 (1986).
6. G. Ashwell and J. Harford. Carbohydrate-specific receptors of the liver. *Annu. Rev. Biochem.* 51:531–554 (1982).
7. K. Nishida, C. Tonegawa, S. Nakane, Y. Takakura, M. Hashida, and H. Sezaki. Effect of electric charge on the hepatic uptake of macromolecules in the rat liver. *Int. J. Pharm.* 65:7–17 (1990).
8. K. Nishida, K. Mihara, T. Takino, S. Nakane, Y. Takakura, M. Hashida, and H. Sezaki. Hepatic disposition characteristics of electrically charged macromolecules in rat in vivo and in the perfused liver. *Pharm. Res.* 8:437–444 (1991).
9. G. R. Gray. The direct coupling of oligosaccharides to proteins and derivatized gels. *Arch. Biochem. Biophys.* 163:426–428 (1974).
10. Y. C. Lee, C. P. Stowell, and M. J. Krantz. 2-Imino-2-methoxyethyl 1-thioglycosides: New reagents for attaching sugars to proteins. *Biochemistry* 15:3956–3963 (1976).
11. W. M. Pardridge, A. K. Kumagai, and J. B. Eisenberg. Chimeric peptides as a vehicle for peptide pharmaceutical delivery through the blood-brain barrier. *Biochem. Biophys. Res. Commun.* 146:307–313 (1987).
12. D. J. Hnatowich, W. W. Layne, and R. L. Childs. The preparation and labeling of DTPA-coupled albumin. *Int. J. Appl. Radiat. Isot.* 33:327–332 (1982).
13. K. Yamaoka, T. Nakagawa, and T. Uno. Statistical moments in pharmacokinetics. *J. Pharmacokinet. Biopharm.* 6:547–558 (1978).
14. R. Blomhoff, H. K. Blomhoff, H. Tolleshaug, T. B. Christensen, and T. Berg. Uptake and degradation of bovine testes β-galactosidase by parenchymal and nonparenchymal rat liver cells. *Int. J. Biochem.* 17:1321–1328 (1985).
15. P. L. Zeitlin and L. Hubbard. Cell surface distribution and intracellular fate of asialoglycoproteins: A morphological and biochemical study of isolated rat hepatocytes and monolayer cultures. *J. Cell Biol.* 92:634–647 (1982).
16. K. Nishida, C. Tonegawa, T. Kakutani, M. Hashida, and H. Sezaki. Statistical moment analysis of hepatobiliary transport of phenol red in the perfused rat liver. *Pharm. Res.* 6:140–146 (1989).
17. H. Sato, Y. Sugiyama, Y. Sawada, T. Iga, T. Fuwa, and M. Hanano. Internalization of EGF in perfused rat liver is independent of the degree of receptor occupancy. *Am. J. Physiol.* 258:G682–G689 (1990).
18. A. L. Hubbard, G. Wilson, G. Ashwell, and H. Stukenbrok. A microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of ^{125}I-ligands among the liver cell types. *J. Cell Biol.* 83:47–64 (1979).
19. P. H. Weigel and J. A. Oka. Endocytosis and degradation mediated by the asialoglycoprotein receptor in isolated rat hepatocytes. *J. Biol. Chem.* 257:1201–1207 (1982).
20. H. Tolleshaug, T. Berg, M. Nilsson, and K. R. Norum. Uptake and degradation of ^{125}I-labelled asialofetuin by isolated rat hepatocytes. *Biochim. Biophys. Acta* 499:73–84 (1977).
21. A. L. Schwartz, A. Bolognesi, and S. E. Fridovich. Recycling of the asialoglycoprotein receptor and the effect of lysosomotropic amines in hepatoma cells. *J. Cell Biol.* 98:732–738 (1984).
22. E. R. Weibel, W. Staubli, H. R. Gnagi, and F. A. Hess. Correlated morphometric and biochemical studies on the cell. I. Morphometric model, stereologic methods, and normal morphometric data for rat liver. *J. Cell Biol.* 42:68–91 (1969).
23. W. A. Dunn, A. L. Hubbard, and N. N. Aronson, Jr. Low temperature selectively inhibits fusion between pinocytic vesicles and lysosomes during heterophagy of ^{125}I-asialofetuin by the perfused rat liver. *J. Biol. Chem.* 255:5971–5978 (1980).
24. P. H. Weigel and J. A. Oka. The large intracellular pool of asialoglycoprotein receptors functions during the endocytosis of asialoglycoproteins by isolated rat hepatocytes. *J. Biol. Chem.* 258:5095–5102 (1983).
25. A. L. Schwartz, S. E. Fridovich, and H. F. Lodish. Kinetics of internalization and recycling of the asialoglycoprotein receptor in a hepatoma cell line. *J. Biol. Chem.* 257:4230–4237 (1982).
26. R. J. Sharma and D. A. W. Grant. A differential effect between the acute and chronic administration of ethanol on the endocytotic rate constant, k_e, for the internalization of asialoglycoproteins by hepatocytes. *Biochim. Biophys. Acta* 862:199–204 (1986).