AN ELECTRON MICROSCOPE AUTORADIOGRAPHIC STUDY OF THE CARBOHYDRATE RECOGNITION SYSTEMS IN RAT LIVER

I. Distribution of ^{125}I-Ligands among the Liver Cell Types

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ABSTRACT

Electron microscope autoradiography was used to study the cellular localization of seven glycoproteins rapidly cleared from the circulating plasma of rats and taken up by the liver. 1 and 15 min after intravenous administration of the ^{125}I-glycoproteins, livers were fixed *in situ* by perfusion and processed for autoradiography. Autoradiographic grains in the developed sections were found to represent the intact ^{125}I-ligand. A quantitative analysis of the distribution and concentration (density) of autoradiographic grains over the three major cell types of the liver was then performed. Three molecules, asialo-fetuin, asialo-orosomucoid, and lactosaminated RNase A dimer, the oligosaccharide chains of which terminate in galactose residues, were bound and internalized almost exclusively (>90%) by hepatocytes. Conversely, four molecules, the oligosaccharide chains of which terminate in either *N*-acetyl-glucosamine (agalacto-orosomucoid) or mannose (ahexosamino-orosomucoid, preputial β-glucuronidase, and mannobiosaminated RNase A dimer), were specifically bound and internalized by cells lining the blood sinusoids—that is, by Kupffer cells and endothelial cells. Endothelial cells were two to six times more active (on a cell volume basis) than were Kupffer cells in the internalization of these four ^{125}I-ligands. Mannose and *N*-acetylglucosamine-terminated glycoproteins competed with each other for uptake into either endothelial cells or Kupffer cells, indicating that a single system recognized mannose or *N*-acetyl-glucosamine residues. Finally, agalacto-orosomucoid and ahexosamino-orosomucoid were also associated with hepatocytes, but competition experiments utilizing excess asialo-orosomucoid demonstrated that residual galactosyl residues were responsible for this association.

KEY WORDS carbohydrate recognition · liver · autoradiography

The rapid removal of numerous glycoproteins from circulating blood plasma is well-documented, involves carbohydrate recognition, and occurs primarily in the liver.[1] Ashwell and colleagues have isolated and characterized protein complexes from rabbit and rat liver membranes that recognize exposed galactosyl residues on desialylated glycoproteins (24, 26, 27, 52; reviewed in reference 4). In addition, the presence of membrane receptors for exposed mannosyl/*N*-acetylglucosaminyl (2, 3, 5, 7, 29, 47, 48, 51) and fucosyl (39) residues has been postulated to explain the specific and rapid clearance of other glycoproteins by the liver.

Because the cell population of the liver is heterogeneous, consisting of ~70% hepatocytes and ~15% each Kupffer and endothelial cells (11, 16), it has been of interest to identify the cell type(s) responsible for the binding and removal of the various glycoproteins. Several different approaches have been used: (*a*) i.v. injection of the glycoprotein in vivo, followed by enzymatic digestion of the liver, separation of hepatocytes from both Kupffer, and endothelial cells, and subsequent assessment of the glycoprotein's distribution among the isolated cellular subpopulations (15, 39, 43, 54); (*b*) administration of the glycoprotein to previously separated cells (50, 54); and (*c*) injection of the glycoprotein together with a molecule (e.g., aggregated albumin) known to block the reticuloendothelial system (Kupffer cells primarily), followed by determination of the glycoprotein's clearance (17). Each approach has its limitations. The first suffers from incomplete recoveries of cells and injected ligand and may allow relocation of the ligand during the time required to separate the cells. In the second, damage to receptors from enzymatic digestion could influence subsequent binding measurements. Finally, the third assumes that, in a given cell type, the same receptor recognizes aggregated or particulate material and soluble ligands. This assumption appears to be invalid in that (*a*) most events in phagocytosis differ from those involved in adsorptive pinocytosis, and (*b*) the two processes can occur simultaneously in one cell (see reference 44 for review).

[1] Neufeld, E., and G. Ashwell. 1979. Carbohydrate recognition systems for receptor-mediated pinocytosis. *In* Biochemistry of Glycoproteins and Proteoglycans. W. Lennarz, editor. Plenum Press, New York. In press.

The uncertainties of these experimental designs directed us to a morphological approach—autoradiography—to assess the distribution in liver of seven different iodinated glycoproteins. Morell et al. (33) used light microscope autoradiography (LM-ARG)[2] to localize [^{3}H]asialo-ceruloplasmin to hepatocytes but did not present quantitative data. In addition, the resolution attained did not allow localization of the ligand to intracellular organelles. Achord et al. (1) used LM immunocytochemistry to localize β-glucuronidase to cells lining the sinusoid but could not distinguish between Kupffer and endothelial cells. To overcome these problems, we chose electron microscope autoradiography (EM-ARG). By fixing the liver *in situ* (by perfusion) and systematically sampling the tissue, we have been able to localize all of the liver-associated molecules detectable by ARG at precise times after injection. We have found that all three major cell types in the liver are involved in the clearance of circulating glycoproteins and that hepatocytes recognize a different set of glycoproteins than do the Kupffer or endothelial cells. Further, we have found that endothelial cells are more active than are Kupffer cells in the specific internalization of four glycoproteins used in this study. In an accompanying paper (23), we have followed the changes in the intracellular distribution of the ligands that occur as a function of time after their internalization. A brief report on part of this work has been presented elsewhere (22).

MATERIALS AND METHODS

Reagents were obtained from the following sources: fetuin, Grand Island Biological Co., Grand Island, N. Y.; acrylamide, Bio-Rad Laboratories, Richmond, Calif.; lactoperoxidase, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.; neuraminidase (*Clostridium perfringens*), Worthington Biochemical Corp., Freehold, N. J.; mono-iodotyrosine (MIT), glucose oxidase (Type V) and yeast mannan, Sigma Chemical Co., St. Louis, Mo.; Sepharose 4B, Sephadexes G-25 Fine, and G-150, Pharmacia Fine Chemicals, Div. of Phar-

[2] *Abbreviations used in this paper:* AGOR, agalacto-orosomucoid; AHOR, ahexosamino-orosomucoid; ASF, asialo-fetuin; ASGP, asialo-glycoproteins; ASOR, asialo-orosomucoid; BSA, bovine serum albumin; EM-ARG, electron microscope autoradiography; ER, endoplasmic reticulum; K phos, K-phosphate buffer; LM-ARG, light microscope autoradiography; L-RNase, lactosaminated RNase A dimer; MIT, mono-iodotyrosine; M-RNase, mannobiosaminated RNase A dimer; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

macia Inc., Piscataway, N. J.; Na^{125}I in 0.1 N NaOH, New England Nuclear, Boston, Mass.; Microdol-X, RP-X-Omat x-ray film, *N,N'*-methylenebisacrylamide, Eastman Kodak Co.; Hi-Speed FE x-ray intensifying screens, E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.; Triton-WR-1339, Ruger Chemical Co., Irvington on Hudson, N. Y.; glutaraldehyde, Electron Microscopy Sciences, Fort Washington, Pa.; paraformaldehyde, Matheson Gas Products, East Rutherford, N. J.; Ilford L-4 photographic gel emulsion, Polysciences, Inc., Warrington, Pa. All other chemicals were reagent grade.

Preparation of Glycoproteins

ASIALO-FETUIN (ASF): Fetuin at a concentration of 30 mg/ml was incubated in 0.1–0.15 M HCl[3] for 30 min at 80°C (or 0.5 M HCl for 10 min) to hydrolyze the terminal sialyl residues. The efficiency of removal was ~90% as measured by the thiobarbituric acid assay (58). The preparation was then dialyzed against 0.15 M NaCl-0.02 M K-phosphate buffer (K phos), pH 7.2, for 48 h with several changes and applied to a Sephadex G-150 column, 1.5 × 55 cm, equilibrated in the same buffer (flow rate, 12–13 ml/h, fraction vol 3 ml). Two protein peaks emerged: the first (~30–40% of the protein) eluted at the void volume, consisted of aggregated material and was discarded; the second eluted at a position corresponding to mol wt 48,000 (ASF monomer), was collected, concentrated by pressure dialysis (PM-10, Amicon Corp., Scientific Sys. Div., Lexington, Mass.), and rechromatographed under the same conditions. The fractions containing ASF monomers (the second peak) were again pooled, dialyzed against 0.5 M $(NH_4)_2CO_3$, lyophilized, and stored at −20°C for subsequent use.

ADDITIONAL GALACTOSE-TERMINATING GLYCOPROTEINS (ASGP): Orosomucoid (mol wt 44,000) was isolated from human plasma (56) and desialylated by exposure to neuraminidase (34) to yield asialo-orosomucoid (ASOR). The lactosaminated derivative of the cross-linked dimer of bovine pancreatic ribonuclease A (L-RNase) was prepared by reductive amination with cyanoborohydride (60). The preparation used in this study contained 8 N^{ϵ}-1-(1-deoxylactitolyl)-lysine residues/molecule of dimer (mol wt 30,000).[4]

OTHER GLYCOPROTEINS: Agalacto-orosomucoid (AGOR) was prepared by enzymatic hydrolysis of the galactosyl $\beta 1 \rightarrow 4$ *N*-acetylglucosaminyl sequences in ASOR using purified β-galactosidase from *Diplococcus pneumoniae* (28). Only 18 of the 20 galactose residues were removed, because the two sequences in which fucose is linked $\alpha 1 \rightarrow 3$ to the *N*-acetylglucosamine are not hydrolyzed by β-galactosidase (14, 38). Further hydrolysis of the oligosaccharide side chains of AGOR using β-*N*-acetylglucosaminidase, also from *D. pneumoniae*, exposed mannosyl residues and yielded ahexosamino-orosomucoid (AHOR). β-glucuronidase, from Dr. Phillip Stahl, Washington University, was isolated from the rat preputial gland as a tetramer of mol wt 280,000 (49, 55). Finally, the mannobiosaminated derivative of the cross-linked dimer of RNase (M-RNase) was prepared by coupling mannobiose to lysine residues using sodium cyanoborohydride.[5] The preparation contained eight disaccharide residues per dimer. Table I lists the natural, processed, or synthetic glycoproteins used in this study and the predominant terminal residues of their oligosaccharide chains.

Iodination of the Glycoproteins

All preparations were iodinated using the lactoperoxidase-glucose oxidase method (20). Lactoperoxidase (50–75 mU) and glucose oxidase (25–50 mU), each coupled to Sepharose 4 B (10), were added to a 0.5–1.0 ml mixture containing 0.1 M K phos, pH 7.0–7.2, 10 μmol glucose, 0.25–2.0 mg glycoprotein, 5–15 mCi Na ^{125}I in 0.1 N NaOH (neutralized with 2 vol of 0.5 M K phos, pH 7.0) and sufficient K ^{127}I to achieve a molar ratio of iodide (^{125}I + ^{127}I) to protein of 0.5–2.0/1. Two procedures were followed for iodination; they differed principally in the time and temperature of incubation. In the first (*a*), the reaction mixture was agitated for 1 h at room temperature and then the immobilized enzymes were sedimented away from the soluble proteins (3,000 rpm for 2 min). The supernate was removed, the beads resuspended in 0.5–1 ml 0.1 M K phos, pH 7.2, and centrifuged as above. The combined supernates were dialyzed, first against 1 liter of 10 mM KI, 0.02% NaN_3 in 0.1 M K phos, pH 7.2, for 1–3 h at room temperature, and then against 1 liter of 0.15 M NaCl, 0.02 M K phos, pH 7.2, overnight. The second (and more recent) labeling procedure (*b*) involved a shorter incubation (10 min), a lower temperature (4–10°C) and a shorter dialysis (2–3 h total at 4°C) using 2–3 liters of 0.15 M NaCl, 0.02 M K phos, pH 7.2. Equivalent levels of incorporation were achieved with both procedures (30–50% of the added isotope) and >90% of the radioactivity in each preparation was acid-precipitable. The specific radioactivities ranged from 40 to 480 mCi/μmol (1–12 mCi/mg). Radioactivity was quantitated in a gamma scintillation spectrometer having a 70% counting efficiency (Biogamma, Beckman Instruments, Inc., Fullerton, Calif.).

It was necessary to chromatograph ^{125}I-ASF, prepared by either procedure *a* or *b*, on Sephadex G-150 as described above, to remove aggregated material. The monomer peak was collected, concentrated, and used for all injections. Less than 10% of the radioactivity of ^{125}I-ASF prepared in this manner was in aggregates (material at the void volume in G-150). This filtration step was unnecessary for the other iodinated preparations, sug-

[3] It was found that incubation of fetuin in 0.025 M H_2SO_4 caused extensive aggregation.

[4] Although L-RNase is more properly a glycoconjugate, for simplicity it will be called a glycoprotein.

[5] G. Wilson, manuscript in preparation.

TABLE I

Terminal Residues of the Oligosaccharide Chains of the Glycoproteins Studied

Molecule	Predominant terminal residue	Reference*
Orosomucoid (mol wt = 44,000)	Sialic acid	(34)
Fetuin (mol wt = 48,000)	Sialic acid	(34)
ASOR	Galactose	(14)
ASF	Galactose	(34)
L-RNase (mol wt ≃ 30,000)	Galactose	(60)
AGOR	*N*-acetyl-glucosamine	(38)
AHOR	Mannose	(G. Ashwell, unpublished material)
β-Glucuronidase (mol wt = 280,000)	Mannose	(18, 55)
M-RNase (mol wt ≃ 30,300)	Mannose	(G. Wilson, manuscript in preparation)

* Study in which predominate terminal carbohydrate residue was determined.

gesting that iodination, per se, did not induce protein aggregation.

Assessment of the ^{125}I-Preparation in vivo

The kinetics of clearance from the blood and uptake into the liver, as well as the specificity of the clearance mechanisms, have been previously established by others for all molecules used in this study. However, because the methods of labeling and the amounts of protein administered in this study differed from those reported, the following set of experiments was performed for each ^{125}I-protein, and representative examples were selected for illustration.

CLEARANCE FROM THE BLOOD: Varying amounts of the ^{125}I-protein (up to the dose used for EM-ARG) in 0.15 M NaCl were injected into the saphenous vein of 140- to 250-g male Sprague-Dawley rats under ether anesthesia; 25-μl aliquots of blood were withdrawn from the tail into heparinized capillary tubes at timed intervals, and the total radioactivity of each aliquot was measured. The maximum radioactivity in the blood samples usually occurred 0.5–1 min after injection and was set to 100%. The 50% clearance time for a given ^{125}I-protein was defined as that time after injection at which 50% of the maximum was still found in the blood.

The blood clearance curves of three ^{125}I-glycoproteins given in Fig. 1 illustrate the rapidity with which each was removed from the circulation. For example, ^{125}I-ASF exhibited a 50% clearance time of 2–3 min (individual animals varied somewhat). In addition, 800 μg ^{125}I-ASF was cleared almost as rapidly (50% by 3 min) as was 18 μg (50% by 2.5 min). ^{125}I-AGOR and ^{125}I-β-glucuronidase also were removed very rapidly from the circulating blood (50% cleared by ~4 min, Fig. 1 *B* and *C*). However, this rapid clearance was not observed for ^{125}I-orosomucoid, >50% of which was still in the circulation 180 min after injection (data not shown). These kinetic results were in agreement with those previously reported (34, 39, 46, 51).[6]

APPEARANCE IN THE LIVER: Several rats were injected with the same amount of a given ^{125}I-protein, bled as described above, and decapitated at timed intervals. Blood (1 ml) was collected from the carotid and the liver was excised and weighed. In some cases, other organs were also removed. The total radioactivity of the collected samples was measured, as well as the radioactivity in their acid-soluble and -insoluble fractions after homogenization in 5 vol (wt/vol) 0.25 M sucrose, followed by precipitation with 10% TCA and centrifugation (>1,700 *g* for 15 min, IEC PR-6,000; Damon/IEC Div., Damon Corp., Needham Heights, Mass.). A blood vol of 8 ml/100 g body weight was used to calculate total radioactivity in the blood. The acid-insoluble radioactivity from the liver fractions was characterized by solubilization in SDS and electrophoresis in a 7–15% linear gradient of SDS-polyacrylamide (SDS-PAGE) as described previously (21, 32). Molecular weight markers and the intact ^{125}I-ligands were run in parallel with the samples from the liver. After electrophoresis, the gels were dried and autoradiographed.

Results of a typical experiment are illustrated in Fig. 2. The loss of ^{125}I-ASOR from the blood was accompanied by a concomitant increase of radioactivity in the liver. As early as 5 min, and up to at least 15 min, >90% of the recovered radioactivity was present in the liver. The bulk of the radioactivity was acid-insoluble (Fig. 2) and present as the intact ^{125}I-protein as detected by SDS-PAGE ARG (data not shown). Because the blood and liver contained ~85% of the injected radioactivity up to

[6] L-RNase was cleared more slowly than the ^{125}I-glycoproteins (50% clearance in 10 min).

15 min, it was clear that the liver was the primary site for removal of circulating ^{125}I-ASOR. Each of the other organs assayed (intestine, stomach, bladder, thyroid, kidney, and spleen) contained <2% of the injected radioactivity at these early times. Similar results with slightly different kinetics were obtained with the other ^{125}I-orosomucoids (AGOR and AHOR[7]), ^{125}I-ASF, and ^{125}I-β-glucuronidase. More than 50% of both modified RNase A dimers (^{125}I-L-RNase and ^{125}I-M-RNase) were found in the liver by 15 min, but significant amounts (~15%) were also found in the kidney (footnote 5 and reference 60).

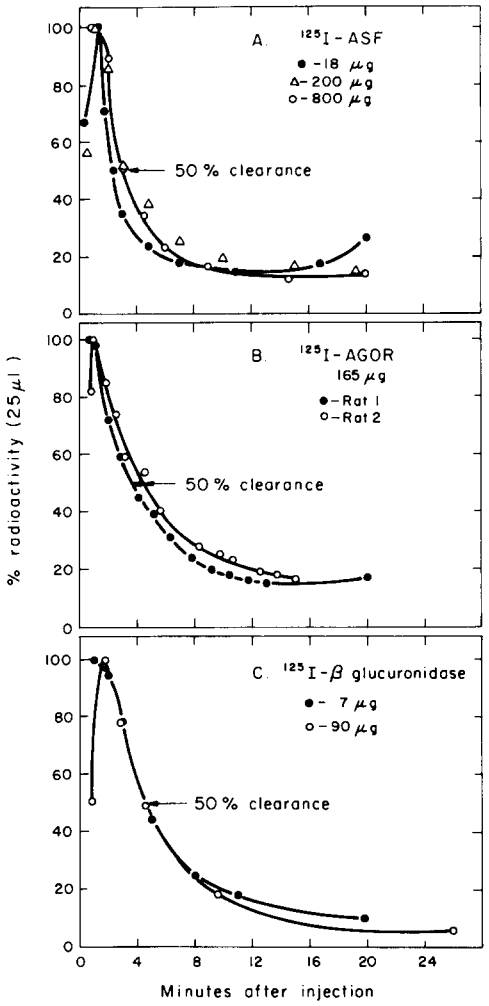

FIGURE 1 Kinetics of clearance from the circulation of three ^{125}I-glycoproteins. Blood samples (25 μl) were collected from anesthetized rats at the indicated times, after injection of a constant amount of ^{125}I-ligand and varying amounts of unlabeled ligand. The total radioactivity was measured as described in Materials and Methods. The sample containing the maximum radioactivity (which was usually at the 0.5- to 1.0-min time point) was normalized to 100% and all others expressed as a percentage of that sample. The maximum represented ~0.3–0.5% of the injected radioactivity. 50% clearance times are indicated by arrows. (*A*) ^{125}I-ASF (7 μCi); (*B*) ^{125}I-AGOR (17 μCi); (*C*) ^{125}I-β-glucuronidase (7 μCi).

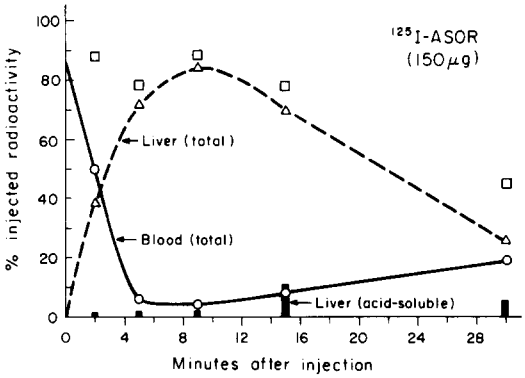

FIGURE 2 Kinetics of clearance of ^{125}I-ASOR from the circulation and its appearance in the liver. Rats were sacrificed at the indicated times after injection of 150 μg ^{125}I-ASOR (1 μCi), and blood and liver samples were analysed for total as well as acid-soluble radioactivity (described in Materials and Methods). (□) Percent of injected radioactivity recovered in the blood plus liver; (○) percent of injected radioactivity found in blood; (△) percent of injected radioactivity found in liver; solid vertical bars, percent of injected radioactivity found to be acid-soluble in the liver.

At later times (30 min, Fig. 2), the total amount of radioactivity in the liver declined, as did the radioactivity in the acid-insoluble fraction for all ^{125}I-proteins except β-glucuronidase. There was a small increase in radioactivity found in the blood but it was insufficient to account for the loss from liver. These data suggested that hydrolysis was occurring in the liver followed by loss of soluble components from the liver and blood. Our investigation of the metabolism of ligands cleared by the liver confirmed results previously reported for ^{125}I-ASF (30) and ^{125}I-RNase B (7) and will be presented in more detail in the accompanying paper (23). A finding of importance for the later ARG localization studies was that the acid-soluble pool in the liver was relatively small at all times (≤20% of the total liver radioactivity) and consisted predominantly of [^{125}I]iodide (≥70% of the acid-soluble radioactivity; see Material and Methods, and Results sections in the accompanying paper [23]).

SPECIFICITIES: To establish that each of the ^{125}I-

[7] When injected in μg amounts, 7–10% of the ^{125}I-AHOR was found in the kidney from the earliest time point (2 min) and did not change.

proteins used in this study was removed by a specific rather than a nonspecific mechanism, competition experiments were performed. The ^{125}I-protein (at a dose used for EM-ARG) was administered simultaneously with a 20- to 200-fold excess of a known competitor or noncompetitor. Blood samples were collected as described above, and the 50% clearance times were recorded. The results are summarized in Table II.

We found that the concomitant administration of structurally unrelated proteins such as bovine serum albumin (BSA) had no influence on the clearance rates of any of the ^{125}I-proteins. Further, native orosomucoid, which is identical to ASOR, AGOR, and AHOR in all but the terminal residues of its oligosaccharide chains, did not compete with any of these glycoproteins. These results indicated that the clearance mechanism was selective for terminal hexose residues, a conclusion confirmed by the competition we observed between glycoproteins having the same terminal residues on their oligosaccharide chains. For example, ASF prolonged the clearance of ^{125}I-ASOR (the oligosaccharide chains of both glycoproteins terminate in galactose). Similarly, a 33-fold excess of AGOR competed with ^{125}I-AGOR. AGOR and AHOR, although having different terminal hexose residues, did compete with each other for clearance (Table II). This finding is compatible with the report by Achord et al. (1) that α-methyl-D-mannoside inhibited the removal of ^{125}I-AGOR. We found that ASOR also competed to a limited extent with both AGOR and AHOR, perhaps indicating incomplete removal of terminal galactose residues from the latter (see Results). Finally, yeast mannans have been reported to retard the removal of circulating β-glucuronidase (1) and AGOR (3) but not of asialoglycoproteins (3). We have confirmed those observations (Table II).

The differences in 50% clearance times with and without competitor were of the same magnitude as those reported by others (e.g., 47, 51). The kidney cleared a large fraction of the injected ^{125}I-glycoprotein (e.g., 30% of ^{125}I-AHOR) in the presence of excess competitor (e.g., AHOR), thus explaining the continued disappearance of the particular ^{125}I-ligand from the blood yet its failure to appear in the liver under these circumstances. This phenomenon was not a nonspecific effect of excess protein, because clearance of ^{125}I-AHOR by kidney did not occur when large amounts of a noncompetitor (e.g., orosomucoid) were administered simultaneously with the ^{125}I-glycoprotein.

Localization of ^{125}I-Glycoproteins in situ

TREATMENT OF ANIMALS: The ^{125}I-glycoprotein, 0.5–1.7 mCi in 0.15 M NaCl–0.02 M K phos, pH 7.2, was administered to fasted rats as described above. (Specific details for each experiment are listed in the appropriate tables and figure legends.) At various times after injection, the livers were perfused *in situ* under a constant pressure of 80 mm Hg through the portal vein, first with normal saline until the liver blanched (~1 min) and then with 2.5% formaldehyde (freshly prepared from para-

TABLE II

*Specificity of Glycoprotein Clearance Mechanisms**

^{125}I-ligand (amount)	Predominant terminal carbohydrate	Additions (amount)	Predominant terminal carbohydrate	50% clearance time
				min
ASOR (100 μg)	Galactose	None	—	2
"	"	Orosomucoid (2.5 mg)	Sialic acid	2
"	"	Mannan (1 mg)	Mannose	2
"	"	ASF (2.5 mg)	Galactose	8
"	"	ASF (10 mg)	"	16
AGOR (140 μg)	*N*-acetyl-glucosamine	None	—	3
"	"	BSA (2 mg)	—	3
"	"	Orosomucoid (5 mg)	Sialic acid	3
"	"	ASOR (5 mg)	Galactose	5
"	"	AHOR (2.5 mg)	Mannose	12
"	"	AGOR (2.5 mg)	*N*-acetyl-glucosamine	12
AHOR (50 μg)	Mannose	None	—	1.75
"	"	Orosomucoid (5 mg)	Sialic acid	1.75
"	"	ASOR (5 mg)	Galactose	3.5
"	"	AHOR (2.5 mg)	Mannose	8
"	"	AGOR (2.8 mg)	*N*-acetyl-glucosamine	8
β-glucuronidase (20 μg)	Mannose	None	—	3
		Mannan (1 mg)	Mannose	17

* ^{125}I-ligand was administered together with the unlabeled molecule and rats were bled at timed intervals, over a period of 15 min in absence and 30–45 min in presence of competitor, as described in Materials and Methods. The 50% clearance times were obtained from the plotted data.

formaldehyde)-1.5% glutaraldehyde-2.5 mM $CaCl_2$ in 0.1 M Na cacodylate, pH 7.2 (25), until the liver hardened (2–3 min).

SPECIFICITY TESTS: To assess the extent of specific binding and internalization of the ^{125}I-ligands by different cell types in the liver, competition experiments similar to those described above were performed and followed by EM-ARG. The general protocol was to inject 100-fold excess unlabeled ligand 0.5–1 min before administration of the ^{125}I-ligand and to perfuse the liver *in situ* 4–5 min later as described above. Controls consisted of injecting saline instead of the putative competitor. Specific paired competitions were: (*a*) 160 μg ^{125}I-ASF ± 20 mg ASF; (*b*) 50 μg ^{125}I-AGOR ± 5 mg ASOR; (*c*) 50 μg ^{125}I-AHOR + 5 mg ASOR or + 5 mg AGOR; and (*d*) 80 μg ^{125}I-β-glucuronidase ± 1 mg yeast mannan (in heparinized saline).

ELECTRON MICROSCOPY: Slices (1 mm) were cut from the middle and peripheral regions of the right lobe of the fixed liver, further cut into 1-mm cubes, and fixed for an additional 0.5–1 h on ice in fresh aldehydes. The tissue blocks were rinsed twice in 0.1 M Na cacodylate, postfixed for 1–2 h in 1% OsO_4 in Veronal-acetate, rinsed, and stained en bloc with uranyl acetate (12) for 1–2 h at room temperature. The blocks were then dehydrated in graded ethanols and propylene oxide and embedded in Epon (31).

EM-ARG: The flat substrate technique of Salpeter and Bachman was followed for EM-ARG using Ilford L-4 emulsion (40, 41). Sections were 750–1,000 Å thick and the emulsion layer was ~1,000 Å. After an exposure time of 7–31 d, the preparations were developed with Microdol-X for 3 min at 20°C. After removal of the overlying collodion, the sections were stained with uranyl acetate and lead citrate (57) and finally examined in a Philips 301 electron microscope operated at 80 kV.

Analysis of Autoradiograms

CONTROLS: Before the analysis of the ARG results, it was necessary to examine the nature of the iodinated species being detected by ARG. Duplicate tissue samples (small cubes or homogenates) of saline-perfused livers from rats injected 15 min previously with an ^{125}I-ligand (β-glucuronidase) were either fixed with aldehydes or precipitated with TCA. The same percentage of total radioactivity was recovered in the tissue or precipitate. In addition, as mentioned earlier, the acid-insoluble radioactivity present in the liver from 4 to 30 min was indistinguishable from the native ^{125}I-protein when analyzed by SDS-PAGE. Finally, <5% of the aldehyde-fixed radioactivity was extracted during the postfixation and dehydration steps. It was concluded that >95% of the developed grains seen by ARG represented the intact ^{125}I-ligand.

CELLULAR DISTRIBUTION OF THE ^{125}I-GLYCOPROTEINS: Micrographs of liver cells present in ARG preparations were taken at random at a standard magnification of 3,400. Quantitative analysis was performed on prints at a final magnification of 9,200. Grains over hepatocytes, over cells lining the sinusoid (Kupffer, endothelial, and fat-storing), and over the lumen of the sinusoids were counted and the percent distribution was calculated. The area of each cell class was determined by using a calibrated grid (d = 2.4 cm, 80 points, 1 intersection point = 6.9 μm^2 at a magnification of 9,200) and recording the type of cell located at each intersection point. Simple grain density (grains/100 μm^2) was then calculated according to the Salpeter and McHenry (42). The percentage of total points located over a given cell class indicates the relative area occupied by that class.

Significant numbers of ARG grains were associated with cells lining the sinusoids after injection of ^{125}I-AGOR, ^{125}I-AHOR, ^{125}I-β-glucuronidase, and ^{125}I-M-RNase (see Results). However, no grains were found associated with fat-storing cells. Therefore, the remaining sinusoidal cells were categorized as endothelial or Kupffer cells, the grains overlying each cell type were counted, and the grain distribution and densities were calculated as above. Both morphological and cytochemical criteria were used to distinguish between Kupffer and endothelial cells. These criteria agree with those described in detail by others (11, 59, 61–64). Briefly, Kupffer cells were identified by the following features: a plasma membrane exhibiting large irregular folds (see Fig. 5*a* in accompanying paper [23]); veriform tubular invaginations in the peripheral cytoplasm (63, and Fig. 5*a* in [23]); a perikaryon that protruded into the sinusoidal lumen (Fig. 5*b*); a low frequency of cytoplasmic extensions emanating from the perikaryon; and the presence of partially digested material within secondary lysosomes (Fig. 5*b*, this study, and Figs. 5*a* and *b* in [23]). Cells having one or more of these features were confirmed as Kupffer cells when a cytochemical test for endogenous peroxidase activity, present only in Kupffer cells, was performed (see below and references 11 and 63). Endothelial cells were identified by the following features: a flat perikaryon (Figs. 4 and 5*b*); numerous fenestrations, called "sieve plates" (61, and Fig. 6*a* in [23]); attenuated cytoplasmic extensions that line the sinusoidal lumen and contain coated vesicles, macropinocytic vesicles, and dense bodies (Figs. 5*b* and 6*a* in [23]). On the basis of these criteria, when only the attenuated extensions of a cell lining the sinusoid were present in a given section, the cell was routinely identified as endothelial in nature (e.g., bottom of Fig. 4). However, if rough endoplasmic reticulum (ER) was present in the cytoplasmic extension, it was identified as originating from a Kupffer cell (from correlations with peroxidase). Finally, the grain distribution was not significantly affected (<10%) by exclusion of these attenuated cytoplasmic extensions from the analysis.

An accurate distributional analysis within a mixed cell population assumes that sampling is random and that cells belonging to the different cell types are uniformly distributed throughout a tissue. In the liver, Kupffer cells are present at a higher frequency in the periportal regions

(37). Therefore, in one ARG experiment, two distinct regions were selected for analysis: a centrolobular region; and a periportal region, identified by the presence of a portal triad. Grains overlying the two cell types in each region were counted, the cellular areas were calculated as described above, and the ARG grain densities were compared.

Cytochemistry

PEROXIDASE: Endogenous peroxidase activity has been reported to be present only in Kupffer cells of the liver (11, 59, 63). In an effort to distinguish between Kupffer and endothelial cells, we examined livers processed for detection of endogenous peroxidase. Rats were perfused first with saline in the manner described above; the liver was fixed by perfusion *in situ* for 2–3 min with 2% glutaraldehyde in 0.1 M Na cacodylate, pH 7.2, and subsequently washed (by perfusion) for 3–5 min with 0.15 M Na cacodylate buffer, all at room temperature. The liver was excised, cut into 1 × 2 × 10-mm blocks, embedded in 7% agar, and cut into 75-μm sections using the Smith-Farquhar tissue chopper (45). The sections were incubated for 30–45 min at room temperature in fresh incubation medium prepared as described by Fahimi (11). A control was performed in which H_2O_2 was omitted. The tissue was postfixed in OsO_4 and further processed for EM as described (11).

COMBINED ARG-CYTOCHEMICAL LOCALIZATION EXPERIMENTS: In order to confirm the EM-ARG localization of several of the ^{125}I-ligands to a specific type of sinusoidal cell, as identified by peroxidase cytochemistry, we performed both experiments on the same liver. That is, first an iodinated ligand was injected, then the liver was fixed and processed for cytochemistry. The sections were subsequently processed for EM-ARG. Finally, the distribution of ARG grains in the developed sections was analyzed and compared to the distribution of peroxidase.

RESULTS

Cellular Distribution of the Injected Glycoproteins

We have found that each major cell type in the liver specifically binds and internalizes at least one of the glycoproteins used in this study. Figs. 3–5 illustrate qualitatively the different distributions of four glycoproteins 15 min after their i.v. injection. By this time, ≥90% of the injected dose of each glycoprotein had been cleared from the circulation and was present in the liver (see Materials and Methods for data). In addition, ~90% of the radioactivity associated with the liver was acid-precipitable and thus macromolecular. Three of the molecules examined in this study, ^{125}I-ASF, ^{125}I-ASOR, and ^{125}I-L-RNase, which all bear terminal galactosyl residues, were associated almost exclusively with hepatocytes, as demonstrated by the presence of ARG grains representing ^{125}I-ASOR[8] overlying primarily hepatocytes (Fig. 3). The cellular distribution of ^{125}I-AGOR was different from that of the asialo-glycoproteins. This molecule was associated with sinusoidal cells and hepatocytes 15 min after injection (Fig. 4). ^{125}I-AHOR exhibited a cellular distribution qualitatively similar to that of ^{125}I-AGOR (not shown). Finally, ^{125}I-β-glucuronidase and ^{125}I-M-RNase demonstrated yet a different distribution; they were present almost exclusively in cells lining the sinusoids (^{125}I-β-glucuronidase, Fig. 5 *a*). These last four molecules, ^{125}I-AGOR, ^{125}I-AHOR, ^{125}I-β-glucuronidase, and ^{125}I-M-RNase, were found in both endothelial cells and Kupffer cells, often within the same sinusoid, as illustrated for ^{125}I-β-glucuronidase in Fig. 5 *b*. This view was reinforced by a dual localization experiment in which ^{125}I-AGOR was found overlying cells positive (Kupffer) and negative (endothelial) for endogenous peroxidase activity (Fig. 6).

Our qualitative observations were confirmed by a quantitative analysis of ARG grain distribution of specimens fixed 1 and 15 min after i.v. injection of the seven ^{125}I-glycoproteins. Livers were examined at 1 min to identify the cells initially involved in the removal of the ^{125}I-ligand from the circulating plasma, and at 15 min for the reasons already given. The first three molecules in Table III (ASF, ASOR, and L-RNase) exhibited the same cellular distribution. Over 90% of the total ARG grains were found in hepatocytes at 1 and 15 min after injection, a distribution that remained unchanged up to 60 min, the latest time point examined. L-RNase exhibited the most striking localization in this class, with 99% of the ARG grains present in hepatocytes after 15 min (Table III, C). Generally, small (≤10%) and variable amounts of ARG grains were associated with cells lining the sinusoids after injection of ^{125}I-ASGP (Table III, A-C). ^{125}I-ASF consistently (in seven experiments) exhibited relatively more grains over these cells than did the other two ASGPs. Although both Kupffer and endothelial cells contained grains, reliable data on the distribution of ASGP in each cell type were not obtained due to the small number of grains counted.

[8] The terms "^{125}I-ASOR", "ARG grains" and "^{125}I-glycoprotein" will be used interchangeably and are understood to mean "ARG grains representing ^{125}I-ASOR (or AGOR, etc.)."

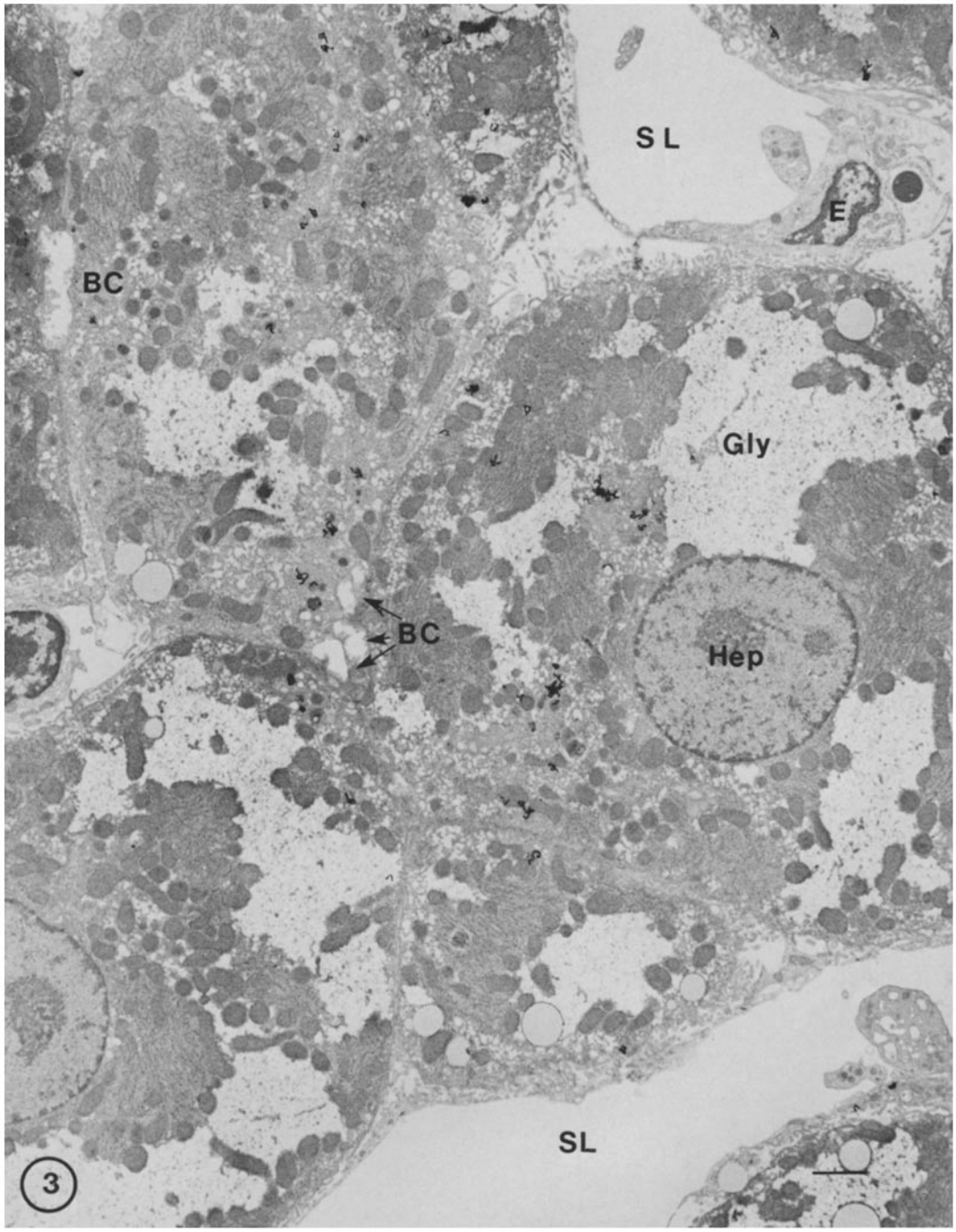

FIGURE 3 EM-autoradiograph of liver perfused 15 min after injection of ^{125}I-ASOR (400 μg, 1.7 mCi). ARG grains are found predominately overlying hepatocytes (*Hep*) near bile canaliculi (*BC*). Endothelial cells (*E*) lining the sinusoidal lumen (*SL*) are not labeled. This rat was not starved before the experiment; thus pools of glycogen (*Gly*) are present. 8 d exposure; bar, 2 μm; × 3,800.

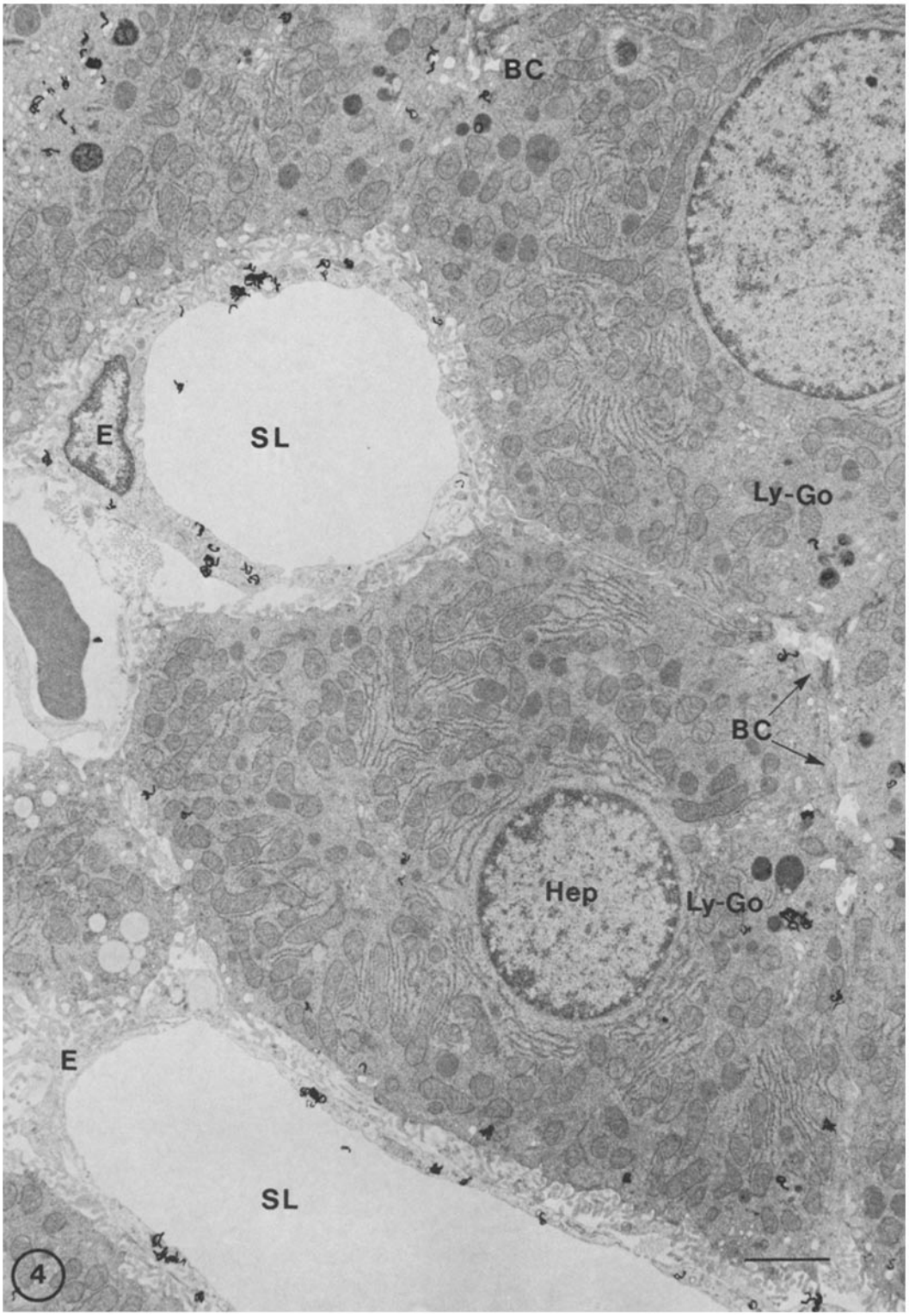

FIGURE 4 EM-autoradiograph of a liver perfused 15 min after injection of ^{125}I-ASOR (400 μg, 2.9 mCi). ARG grains are found overlying hepatocytes (*Hep*) in lysosome-Golgi (*Ly-Go*) regions near bile canaliculi (*BC*). In addition, ARG grains are associated with endothelial cells (*E*) lining the sinusoidal lumen (*SL*). 8 d exposure; bar, 2 μm; × 6,300.

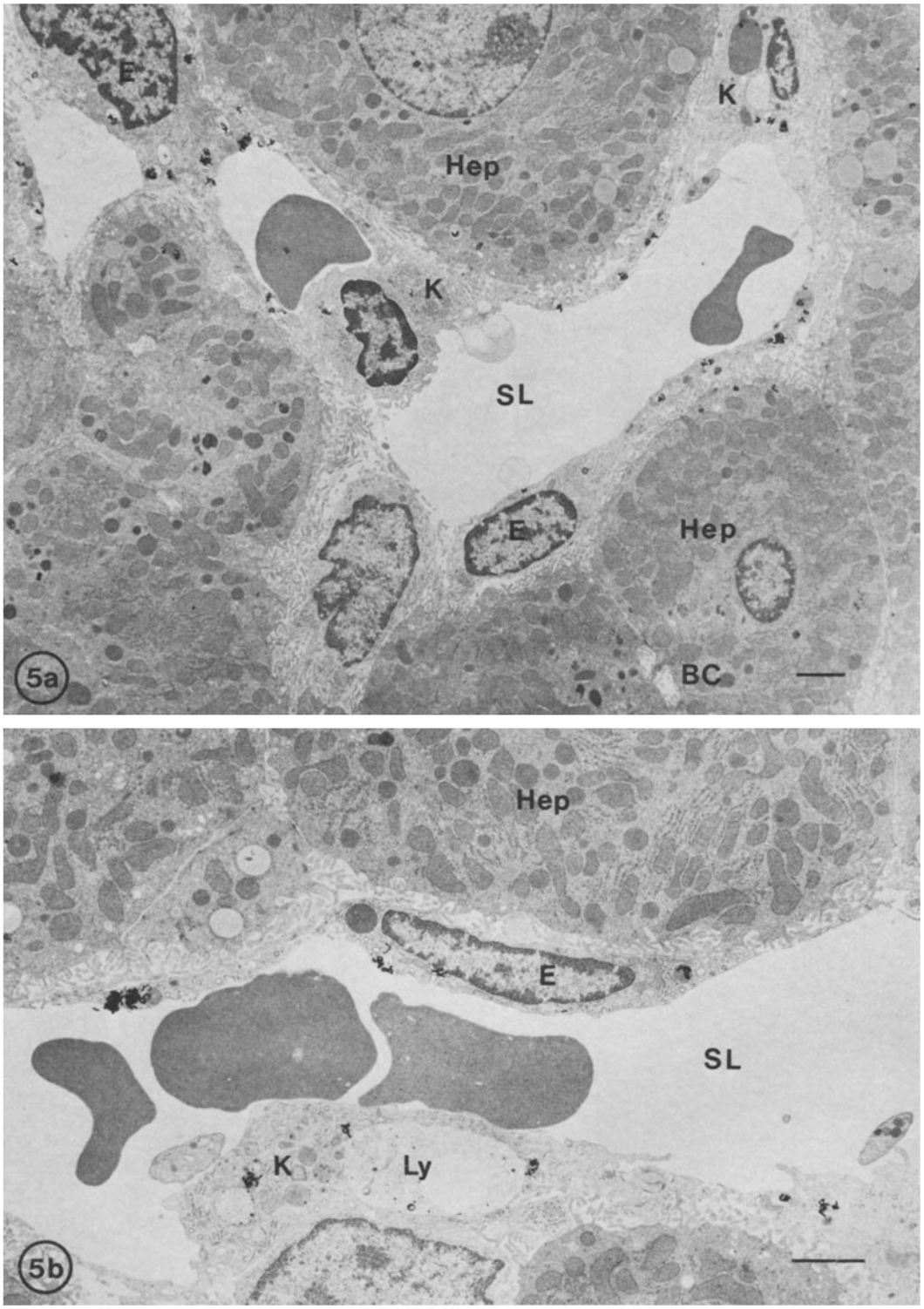

FIGURE 5 EM-autoradiographs of a liver perfused 15 min after injection of ^{125}I-β-glucuronidase (160 μg, 1 mCi). (*a*) ARG grains are found overlying endothelial (*E*) and Kupffer (*K*) cells lining the sinusoidal lumen (*SL*), but not hepatocytes (*Hep*). 18 d exposure; bar, 2 μm; × 4,000. (*b*) An endothelial cell (*E*) and a Kupffer cell (*K*) lining a sinusoidal lumen (*SL*) have internalized the ^{125}I-ligand. The presence of structures resembling secondary lysosomes (*Ly*) with heterogenous contents identify Kupffer cells. See Materials and Methods for other criteria used to identify each cell type. 18 d exposure; bar, 2 μm; × 5,400.

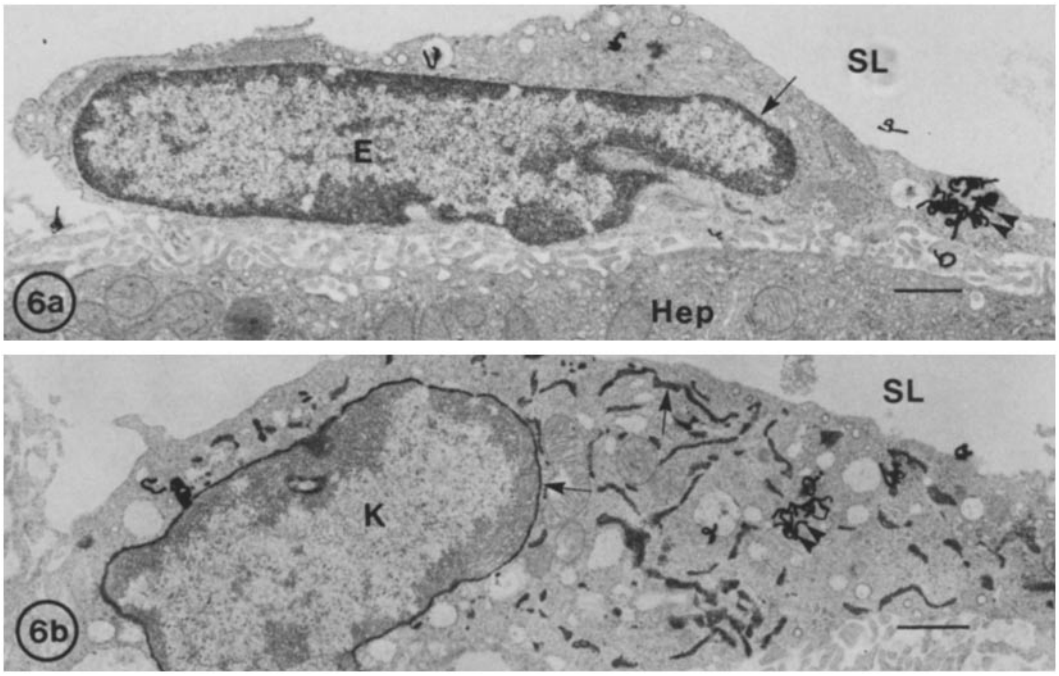

FIGURE 6 EMs from a combined ARG-cytochemical localization experiment. The liver was perfused 10 min after i.v. injection of 30 μg ^{125}I-β-glucuronidase (0.3 mCi), excised and 75-μm sections incubated 45 min at 23°C for detection of endogenous peroxidase activity. The sections were then processed for ARG. (*a*) Endothelial cell (*E*) contains ARG grains (double arrowheads) but no peroxidase reaction product in either the ER or the perinuclear cisternae (arrow). 19 d exposure; bar, 1 μm; × 8,750. (*b*) A Kupffer cell (*K*) contains both ARG grains (double arrowheads) and peroxidase reaction product in both ER near the sinusoidal lumen (*SL*) and in the perinuclear cisternae (arrows). 19 d exposure; bar, 1 μm; × 9,000.

^{125}I-AGOR and ^{125}I-AHOR, at 1 and 15 min after injection, were found in all three major cell types of the liver (Table III, D and E). 40–50% of the ^{125}I-AGOR-generated grains were present in hepatocytes at both times examined. The fraction of ^{125}I-AHOR in hepatocytes was smaller than that of ^{125}I-AGOR at 1 or 15 min.

40–50% of the ARG grains representing ^{125}I-AGOR were found over both Kupffer and endothelial cells at the two times examined. Acknowledging the difficulties inherent in identifying and accurately sampling these cell populations (see Materials and Methods), we found more ^{125}I-AGOR in endothelial cells than in Kupffer cells at both times examined (Table III, D). 40% of the total ^{125}I-AGOR was associated with endothelial cells and the remainder (8%) with Kupffer cells. ^{125}I-AHOR exhibited a similar distribution (Table III, E).

Finally, both ^{125}I-β-glucuronidase and ^{125}I-M-RNase demonstrated distributions complementary to that of ASGP (Table III, F and G). That is, 93% of the ARG grains representing ^{125}I-β-glucuronidase were associated with both types of cells lining the sinusoids and only 6% with hepatocytes. Again, endothelial cells contained more of these two molecules than did Kupffer cells.

Cellular Density or Concentration of the Injected Glycoproteins

Table IV presents the ARG grain densities of four ^{125}I-glycoproteins characterized by different cellular distributions 15 min after injection. Grain density is a measure of the concentration of a molecule in a given cell and takes into account differences in fractional volumes among different cell populations in the liver. Thus, although 98% of the liver-associated ^{125}I-ASOR was present in hepatocytes, the relative grain density of this molecule in hepatocytes was approximately the same as that over the total liver, because hepatocytes accounted for 89% of the total area (volume) of cells in the sections examined. Further, the relative

TABLE III

Distribution of Autoradiographic Grains among Liver Cell Types after i.v. Administration of Seven Different ^{125}I-Glycoproteins

Molecule	Specific radioactivity	Amount injected	Time of sacrifice	Total grains counted	Distribution Over: Hepatocytes	Sinusoidal cells (Kupffer and endothelial)	Kupffer	Endothelial	Sinusoidal lumen
	mCi/mg	*μg protein*	*min*				%		
A. ASF	1.2	600	2	220	91	9	ND*	ND	0.5
	3.0	800	15	155	94	6	ND	ND	0
B. ASOR	6.8	94	1	382	96	4	ND	ND	1
	4.2	400	15	386	98	2	ND	ND	0
C. L-RNase	11.7	60	15	279	99	1	ND	ND	0
D. AGOR	11.8	80	1	242	53	45	10	35	2
	7.2	400	15	285	50	49	3‡	46	1
	6.25	160	15	380	41	58	11‡	47	1
E. AHOR	6.8	90	1	94	39	62	17	45	0
	6.8	90	15	443	17	80	12	68	3
F. β-Glucuronidase	9.1	30	10	421	7	92	16	76	2
	5.8	160	15	162	6	93	27	66	1
G. M-RNase	5	70	15	175	5	93	3	90	3

* ND, not determined.

‡ The differences in ARG grain distribution over Kupffer cells in the two experiments presented reflect the problem of accurately sampling the cells lining the sinusoid. Few Kupffer cells were present in the ARG sections examined in the first experiment. When ARG grain densities were calculated, the apparent discrepancy was eliminated (see Table IV: AGOR, 1 and 2).

TABLE IV

Density Distribution of ARG-Grains in Liver 15 min following Injection of ^{125}I-Glycoproteins

Molecule	Grain density over: Total liver	Hepatocytes	Sinusoidal cells (Kupffer and endothelial)	Kupffer	Endothelial
			grains/100 μm²		
A. ASOR	3.0 (1)*	3.5 (1.2)	1.1 (0.4)	ND‡	ND
B. 1. AGOR	2.0 (1)	1 (0.5)	18.8 (9.4)	3.6 (1.8)	27.5 (13.8)
2. Repeat AGOR	4.2 (1)	2.5 (0.6)	20 (4.8)	6.7 (1.6)	39.1 (9.3)
C. AHOR	5.4 (1)	1.2 (0.2)	40 (7.4)	18.5 (3.4)	51.2 (9.5)
D. β-Glucuronidase	2.0 (1)	0.2 (0.1)	16.9 (8.5)	6.9 (3.4)	32.8 (16.4)

The general protocol for the above experiments and grain density calculations is given in Materials and Methods, and specific details for each molecule (specific radioactivities, amounts injected, etc.) are given in Table I (15 min data).

* Values in parentheses are relative grain densities, with those over the total liver normalized to 1.

‡ ND, not determined (due to the low number of ARG grains).

density of ^{125}I-ASOR in cells lining the sinusoids was rather high, 0.3, because the fractional volume of these cells was very small (4.4%). However, when half of the grains were over cells lining the sinusoids, as with ^{125}I-AGOR, the grain density over these cells was >18 times higher than that over hepatocytes (Table IV, B). That is, cells lining the sinusoids accumulated 18.8 times more ^{125}I-AGOR than did hepatocytes per unit cell volume. In a second experiment with ^{125}I-AGOR, the cor-

responding ratio was 7.5/1 (Table IV, B, 2). In both ^{125}I-AGOR experiments, endothelial cells accumulated more molecules per cell volume than did either hepatocytes or Kupffer cells (Table IV, B).

For ^{125}I-β-glucuronidase, the grain density over the cells lining the sinusoids was dramatically higher (85 times) than that over hepatocytes, and, again, the highest values were found over endothelial cells (165 and 5 times higher than over hepatocytes and Kupffer cells, respectively). When periportal regions, which are rich in Kupffer cells, were selected for quantitative EM-ARG analysis, a pattern of grain densities similar to that illustrated in Table IV was obtained. That is, the relative grain density over endothelial cells was approximately fivefold higher than that over Kupffer cells.

Specificity of the Cellular Associations Observed

The results of blood clearance experiments using competitors and noncompetitors of the ^{125}I-glycoproteins confirmed that the clearance mechanisms depended specifically on the nature of the terminal or subterminal hexose residues of the oligosaccharides in the glycoproteins injected (see Materials and Methods). An additional type of competition experiment was performed to examine by morphology the specificities of the cellular associations observed. Identical amounts of the ^{125}I-glycoprotein were injected into animals of equal weight, with or without excess amounts of a known competitor. The livers were processed in parallel for EM-ARG and the resulting grain distributions compared. The data are summarized in Table V. In the first experiment (Table V, 1) the retention of ^{125}I-ASF in liver was reduced 92% by the prior injection of a 100-fold excess of unlabeled ASF and the number of ARG grains associated with hepatocytes was reduced by 99%, reflecting the specificity of ASGP recognition and internalization by these cells. Under the same conditions, ^{125}I-ASF-generated grains were reduced by only 24% over the cells lining the sinusoids, suggesting a lack of specificity in the uptake of ASGP by sinusoidal cells.

Our finding that simultaneous administration of ^{125}I-AGOR with excess ASOR retarded somewhat the blood clearance of the ^{125}I-ligand (Table II) prompted a study of ^{125}I-AGOR localization by EM-ARG 5 min after its injection with or without excess ASOR. As can be seen in Table V, 2, there was a significant reduction of ARG grains overlying hepatocytes in the presence of ASOR (90%) but no change over either cell type lining the sinusoid. These results suggest very strongly that the two residual, terminal galactosyl residues on ^{125}I-AGOR, not the exposed *N*-acetylglucosaminyl

TABLE V

*Effect of Competitors on ARG Grain Distribution of ^{125}I-Proteins in the Liver**

Experiment		^{125}I-protein	Competitor	Total grains counted	Grains‡ over		
					Hepatocytes	Kupffer cells	Endothelial cells
1	a.	^{125}I-ASF (160 μg)	None	564	521	42	
	b.	^{125}I-ASF (160 μg)	ASF (20 mg)	41	9	32	
		Change§		↓92%	↓99%	↓24%	
2	a.	^{125}I-AGOR (50 μg)	None	467	196	33	238
	b.	^{125}I-AGOR (50 μg)	ASOR (5 mg)	303	20	36	246
		Change		↓35%	↓90%	0	0
3	a.	^{125}I-AHOR (50 μg)	ASOR (5 mg)	307	22	25	260
	b.	^{125}I-AHOR (50 μg)	AGOR (5 mg)	81	10	3	69
		Change		↓74%	↓55%	↓88%	↓74%
4	a.	^{125}I-β-glucuronidase (80 μg)	None	324	11	53	260
	b.	^{125}I-β-glucuronidase (80 μg)	Mannans (1 mg)	91	14	12	65
		Change		↓72%	0	↓78%	↓75%

* Competitor was injected 0.5–1 min before administration of the ^{125}I-ligand. Rat livers were perfused *in situ* 4 min later for 1 min with normal saline, then 1–2 min with aldehydes, or until the liver hardened. Tissue was processed for EM-ARG as described in Materials and Methods.

‡ Equal areas of each cell type within each experiment were examined for grains.

§ Percent change was calculated as follows: ([grains without competitor − grains with competitor] ÷ grains without competitor) × 100.

TABLE VI

Summary of Cellular Associations Observed by EM-ARG of ^{125}I-Glycoproteins

Molecule	Predominant terminal residues	Cellular association of molecule: Hepatocyte	Kupffer cell	Endothelial cell
ASOR	Galactose	+	–	–
ASF	Galactose	+	–*	–
L-RNase	Galactose	+	–	–
AGOR	*N*-acetylglucosamine	+‡	+	+
AHOR	Mannose	+‡	+	+
β-Glucuronidase	Mannose	–	+	+
M-RNase	Mannose	–	+	+

* The small and variable amounts of ^{125}I-ASF localized to cells lining the sinusoids were found to be nonspecific by competitor experiments (see Results).

‡ The ^{125}I-ligand associated with hepatocytes was 90% diminished by concomitant administration of a 100-fold excess of ASOR and is interpreted as representing galactose-specific binding and uptake.

groups, were recognized by hepatocytes.

In another experiment, the cross competition observed by AGOR and AHOR in the blood clearance studies (see Table II) was examined by EM-ARG (Table V, 3). Administration of a 100-fold excess of AGOR together with ^{125}I-AHOR resulted in a comparable reduction of grains over both Kupffer and endothelial cells (88% and 74%) and suggested that biologically similar carbohydrate recognition systems were present in both cell types.

Finally, the overall reduction in ^{125}I-β-glucuronidase-generated ARG grains after administration of yeast mannans was only 70%, possibly because of the small dose of competitor used (Table V, 4). However, the specificity of this molecule's association with both endothelial and Kupffer cells was strongly suggested by a parallel reduction in grains over these two cell types. Hepatocytes exhibited no such reduction suggesting a lack of specificity in their binding and uptake of β-glucuronidase.

DISCUSSION

Validity of the EM-ARG Approach

We have used EM-ARG to elucidate the role played by each cell type of the liver in the clearance of circulating glycoproteins. The approach involved administration of an ^{125}I-ligand in vivo, followed by *in situ* fixation of the liver, processing of tissue for EM-ARG, and finally analysis of the grain distribution throughout the entire liver. Such an approach allowed us to visualize the ^{125}I-ligand (as represented by developed ARG grains) in all cell types at precise times without the necessity of isolating cell subpopulations. The controls performed ruled out cross linkage of diffusible molecules (MIT or I^-) or extraction of ^{125}I-macromolecules during fixation, and established that ~95% of the radioactivity in the liver was the intact ^{125}I-protein at each time analyzed. Thus, the ARG grains visualized represent the ^{125}I-glycoprotein administered and not degradation products. The sensitivity of ^{125}I under the conditions of this study averaged 0.40 (13), which means that ~40% of the isotopic decays were detected. However, it is reasonable to expect that the ARG grains visualized were representative of the distribution of all the ^{125}I-ligands present in the liver. Thus, *in situ* localization by EM-ARG is not affected by the variety of artifacts likely to occur in localization studies involving cell separation. In such studies, the low recovery of ^{125}I-ligand usually obtained and the possibilities of selective cell loss or damage to cell surface receptors raise questions about the representative character of the distributions reported.

Cellular Localization of ^{125}I-Ligands

Hepatocytes, Kupffer cells, and endothelial cells all participated in the specific removal of at least three of the seven ligands studied. In each case, the clearance was rapid and appeared to depend on the presence of a particular terminal or subterminal hexose residue in the oligosaccharide chains of the protein. A summary of our findings is presented in Table VI.

^{125}I-ASIALO-GLYCOPROTEINS LOCALIZED TO HEPATOCYTES: We have obtained direct evidence that hepatocytes preferentially and specifically bind and internalize three proteins whose oligosaccharide chains terminate with galactose (ASGP). The participation of endothelial and Kupffer cells in the clearance of ^{125}I-ASGP is small and probably nonspecific, notwithstanding the more favorable exposure of these cells to the circulating ^{125}I-ASGP. Thus, the original hypothesis of Morell et al. (33) that only hepatocytes in the liver clear galactose-terminating glycoproteins has been confirmed by quantitative EM-ARG.

Transport of ASGP across endothelial cells is evidently not a prerequisite to hepatocyte binding

and entry, because we find from the earliest times studies (0.75-1 min, ^{125}I-ASOR, Table III) that >90% of ^{125}I-ASGP in the liver is associated with hepatocytes. In addition, the endothelium is highly fenestrated, allowing direct passage of large particles (e.g., chylomicra) from the circulating plasma to the hepatocyte (35).

It is clear from our EM-ARG results that receptors for ASGP are present on the hepatocyte plasmalemma at the sinusoidal front. However, their exact localization within this domain and their presence in other plasmalemmal domains (e.g., at the bile front or along the lateral surface) are not yet known (see Discussion in accompanying paper [23]). Bergeron et al. (6), using EM-ARG, have reported that receptors for [^{125}I]insulin are located in both the sinusoidal and lateral domains of the hepatocyte plasmalemma.

Recently, colloidal gold to which desialylated ceruloplasmin was absorbed was used as a marker to localize ASGP receptors on isolated hepatocytes (19). Those receptors visualized were uniformly distributed along the microvilli (with or without prior fixation of cells), but no quantitation was given as to the fraction being detected. Receptor localization *in situ* still remains to be determined, because it is not known whether tissue dissociation perturbs this distribution.

N-ACETYLGLUCOSAMINE- AND MANNOSE-TERMINATING GLYCOPROTEINS LOCALIZED TO CELLS LINING THE SINUSOIDS: In this study, we found that ^{125}I-AGOR and ^{125}I-AHOR were internalized by the three major cell types of the liver—hepatocytes, Kupffer cells and endothelial cells—whereas ^{125}I-β-glucuronidase and ^{125}I-M-RNase were taken up by only Kupffer and endothelial cells (Table VI). Our ARG competition experiments indicated that the association of the first two ligands (AGOR and AHOR) with hepatocytes was galactose-specific, because ASOR competed for their uptake. Thus, our results are clear: within the limits of the parameters so far tested, cells lining the sinusoids specifically bound and internalized ligands whose oligosaccharide chains terminated in either *N*-acetylglucosamine (AGOR) or mannose (AHOR, M-RNase, and β-glucuronidase). Furthermore, from the results of our competition experiments and those of others (1, 3), it is apparent that the same receptor recognized all of the ligands.

Our finding that both endothelial cells and Kupffer cells take up mannose- and/or *N*-acetylglucosamine-terminating glycoproteins is based on a correct identification of the two cell types. We have listed our morphological and cytochemical criteria, which are in agreement with those used in other studies, and have provided examples (see Materials and Methods). Other workers have reported the involvement of Kupffer cells in the clearance of preputial β-glucuronidase (43), but ours is the first report in which endothelial cell involvement has been documented. The finding that endothelial cells internalized more of a given ^{125}I-ligand than did Kupffer cells might be explained by a greater number of receptors per cell and/or more rapid endocytosis of the bound ligand.

It is generally accepted that proteins which have lost their native conformation are taken up by cells of the reticuloendothelial system (53), Kupffer as well as endothelial cells of the liver being major constituents of this system. Apparently, the state of aggregation is not the sole structural characteristic signaling removal, because formaldehyde-treated albumin monomers are also rapidly cleared by this system in the liver (8, 9, 36). Therefore, we must consider whether the localization of molecules such as ^{125}I-β-glucuronidase to cells lining the liver sinusoids (particularly endothelial cells) reflects uptake via the mannose/*N*-acetyl-glucosamine recognition or the less specific "colloidal uptake" mechanism. We believe that the uptake is not via this latter system for the following reasons: (*a*) the clearance of ^{125}I-orosomucoid was not rapid and should have been if our iodination or handling methods were nonspecifically altering the ligands; (*b*) gel filtration of the ^{125}I-ligands did not reveal the presence of aggregates; (*c*) yeast mannans competed for binding sites to the same degree on both endothelial and Kupffer cells; and (*d*) the clearance of each of the three ^{125}I-ligands was faster, more complete, and more specific to the liver than that reported for formaldehyde-treated albumin (8) or colloidal particles (53). Of course, definitive evidence for a specific receptor will come only after its isolation from a pure population of liver endothelial cells.

We would like to thank Ms. A. Ma for her technical assistance, Ms. P. Brodie, L. Mazzacane, and L. Wootton for their collaboration in preparing the manuscript, Ms. P. Stenard-Ossorio for her photographic work, Dr. M. G. Farquhar for valuable discussions of EM-ARG, and Dr. G. E. Palade for his critical reading of the manuscript, continued interest in the study, and support for early aspects of the work (GM 21714).

Much of this research was supported by a grant to A. L. Hubbard (GM 23669); the preparation of the glycosaminated derivatives of the RNase A dimer by G. Wilson was supported by GM 07256.

Received for publication 5 March 1979, and in revised form 11 June 1979.

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