Receptor-mediated Endocytosis of Asialoglycoproteins by Rat Hepatocytes: Receptor-positive and Receptor-negative Endosomes

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Abstract. We have used combinations of subcellular fractionation, specific cytochemical tracers, and quantitative immunoadsorption to determine when, where, and in which intracellular structure internalized asialoglycoproteins (ASGPs) are segregated from their receptor. All membrane vesicles containing the receptor (R+ vesicles) were quantitatively immunoadsorbed from crude microsomes with Staphylococcus aureus cells and affinity-purified anti-ASGP receptor. Using this assay, we varied the time and temperature of exposure of perfused livers to 125I-asialoorosomucoid (125I-ASOR) and followed the movement of ligand from R+ to R− vesicles. After 2.5 min at 37°C, 98% of the internalized ligand could be immunoadsorbed and thus was in R+ vesicles. Over the next 12 min of continuous 37°C perfusion with 125I-ASOR, an increasing fraction of the ligand was not immunoadsorbed and therefore was present in R− vesicles. A maximum of 30% of the ligand could be found in R− vesicles (14–44 min). When livers were maintained at 16°C, ligand was internalized but remained in R+ vesicles. Furthermore, ligand accumulating in R− vesicles at 37°C remained there when livers were cooled to 16°C. R− endosomes could be separated from R+ endosomes by flotation on sucrose density gradients and visualized by the presence of sequestered ASOR-horseradish peroxidase (ASOR-HRP). These structures resembled those labeled by ASOR-HRP in situ: R+ vesicles were relatively dense (1.12 g/cc), frequently tubular or spherical and small (100-nm diam), corresponding to the peripheral and internal tubular endosomes; R− structures were of lower density (1.09 g/cc), large (400-nm diam), and resembled internal multivesicular endosomes (MVEs). Endocytosed ASOR-HRP was found in both the peripheral and internal tubular endosomes in situ under conditions where 95% of the ligand was present in R+ vesicles by immunoadsorption, whereas MVEs containing ASOR-HRP were predominant in situ when ligand was found in R− vesicles and were often in continuity with the tubular internal endosomes. All of these results suggest that complete segregation of ligand and receptor occurs after arrival in the Golgi–lysosome region of the hepatocyte and that MVEs are R− and represent the final prelysosomal compartment.

Circulating asialoglycoproteins (ASGPs) are endocytosed by rat liver parenchymal cells (hepatocytes) and degraded in lysosomes. The ligand pathway has been mapped using electron microscopic (EM) tracers, and several prelysosomal compartments have been identified and biochemically characterized (11, 12, 18, 19, 52, 53). Mapping the receptor pathway has been more difficult, due, in part, to the existence of internal pools of ASGP receptors (ASGP-R) whose precise locations are still not resolved (12, 37, 52, 56). Each receptor is believed to cycle between the cell surface and the internal pools, mediating the entry of >200 ligand molecules in its lifetime (39, 47–49, 54). After internalization of the ligand–receptor complex (2, 55), a minimum of two steps would seem to be required for accomplishing both delivery of ligand to lysosomes and delivery of receptor back to the cell surface—dissociation of ligand from receptor and physical separation of the two molecules into distinct intracellular compartments. Much evidence implicates the complex, prelysosomal membrane system collectively termed endosomes as the site(s) for these two steps (e.g., 2, 12, 16, 17, 34, 50, 53, 55, 57). Recently, we identified and biochemically characterized three endosomal compartments of the ASGP pathway in rat hepatocytes (52). The internal (latent) ASGP receptors we detected were in a membrane compartment whose biochemical characteristics were strikingly similar to those of the peripheral and internal tubular endosomes. Results from immunoelectron microscopy have found intracellular ligand and the ASGP receptor together (12), but the precise location as well as the dynamics of the two components remain unclear.

In the present study, we have applied subcellular fraction-
ation and quantitative immunoadsorption, together with in situ and in vitro cytochemistry of a specific ASGP tracer, to address the questions of when, where, and in what structures segregation of ligand and receptor occurs. We report here that asialoorosomucoid (ASOR) internalized at 37°C is first found in receptor-positive (R+) endosomes but then appears in receptor-negative (R−) endosomes after entry into lysosomes. Several lines of evidence suggest that this step occurs after transport of ligand into the internal endosomal compartment. Finally, we have found that the R− endosomes are multivesicular.

**Materials and Methods**

**Materials**

Reagents were obtained from the following sources: [125I]methylene from Amersham Corp. (Arlington Heights, IL); NADH from Calbiochem-Behring Corp. (La Jolla, CA); Protosol from New England Nuclear (Boston, MA); and Ready-Solve EP from Beckman Instruments, Inc. (Palo Alto, CA). All other reagents were obtained from the same sources given in recent publications from this laboratory (43-45) or were reagent grade. CD Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA).

ASOR was prepared and iodinated using chloramine T as described (52). Anti-HA IgG, prepared as described (20), and anti-ASGP-R IgG, affinity-purified as described below, were iodinated in a similar manner (14). S. aureus was prepared according to Kessler (28), stored at -70°C in phosphate-buffered saline (PBS) with 0.02% NaN3, and washed three times in bovine serum albumin (BSA)-PBS immediately before use.

**Labeling the Contents of Endosome Compartments in the Isolated Perfused Liver**

Male rats (150-250 g) were used for all experiments and were starved 20-24 h before they were killed. The isolated liver perfusion system and temperature-shifft protocols have been described (9, 51). 125I-ASOR (1.1 x 10^7-6.6 x 10^6 cpm/µg) was perfused under conditions of time and temperature that are specified in the Results. A maximum of three 0.3-0.8 g biopsies were taken from major lobes of a liver in any experiment. They were rapidly chilled, homogenized, and processed as described below. Release of surface-bound ligand was accomplished by the addition of EGTA as described (51).

**Subcellular Fractionation**

All manipulations were carried out at 4°C. Biopsies from livers containing sequestered 125I-ASOR were homogenized in 4 vol (wt/vol) of 0.25 M sucrose, 3 mM imidazole, pH 7.4, 0.02% NaN3 (S/I), diluted to 10% (wt/vol) with S/I and a microsomal fraction prepared as described (6). In early experiments, livers were homogenized as described for the isolation of plasma membrane sheets (22) and the supernate from a low-speed centrifugation step, which sediments plasma membrane sheets, subsequently centrifuged at 105,000 g for 90 min to obtain microsomes. Microsomes were fractionated on linear sucrose gradients (32 ml, 1.08-1.22 g/cc) as described previously (6) and the endothelial region pooled (~ 1.12 g/cc). Alternatively, microsomes were resuspended directly in 3.6 ml of 1.15 M sucrose and applied to the bottom of a discontinuous sucrose gradient as described previously for the isolation of Golgi fractions from ethanol-stocked rat liver (10). Fractions (0.5 ml) were collected automatically from the top of the gradient.

**Immunoprecipitation of Vesicles**

**Preparation of Anti-Receptor Antibody.** The ASGP receptor was purified according to the method of Hudgin et al. (24). Antibodies to the detergent-solubilized form of the receptor were generated in rabbits using multiple-site intradermal injections (100 µg for the primary immunization and 50 µg for the boost 2 wk later). Affinity-purified antibody was prepared by passing whole serum (1 ml) over a receptor-Sepharose column and eluting the bound antibody with 2 ml of 0.1 M glycine, 0.1 M NaCl, pH 3.0 (40). After neutralization with Tris base and dialysis against 150 mM NaCl, 0.02% NaN3, 20 mM Tris, pH 7.4 (TBS), the antibody (~0.05 mg/ml) was stored at 4°C in 1% BSA. Approximately 300 µg of IgG was obtained from 1 ml of antiserum.

**Immunoadsorption of Receptor-Positive (R+) Vesicles.** Two types of preparations were used for immunoadsorption of R+ vesicles. For the initial studies, we used vesicles obtained from sucrose gradients and enriched in endosomes, as determined by the presence of sequestered 125I-ASOR (see Fig. 2 in reference 52). In later experiments, we used microsomes, prepared either from livers perfused with 125I-ASOR (125I-ASOR-labeled microsomes) or from livers of rats injected intraperitoneally 48 h before they were killed with 125I-methionine (see below). [125I]Methionine or 125I-ASOR-labeled microsomes (50 µl at 6 µg of protein/µl) or gradient vesicles (100 µl at 1 µg of protein/µl) were added to 1 ml of 1% BSA in 20 mM NaP, pH 7.4, 150 mM NaCl, 0.02% NaN3 (BSA-PBS). 2-3 µg of affinity-purified antibody or preimmune IgG was then added and the suspension incubated on a rotary shaker for 1 h at 4°C. The incubation was continued for another hour at 4°C after addition of 125 µl of fixed S. aureus cells (10%, wt/vol). After incubation with S. aureus, the mixture was diluted to 10 ml with S/I and centrifuged at 1,600 g, for 17 min (Beckman TJ-6 centrifuge, Beckman Instruments, Inc.). The resuspended pellet was analyzed for receptor and 125I-ASOR content. 125I-ASOR remaining in the supernate was then measured by counting the radioactivity present in the top 1 ml and correcting to 10 ml. The amount of 125I-ASOR released from leaky vesicles generated during the immunoadsorption reaction and thus not present in R+ or R− vesicles was determined by centrifugation (90 min at 105,000 g) of supernates derived from the preimmune incubation mixtures and averaged 19±5% of the total (11 determinations). Therefore, the 81% 125I-ASOR that remained sedimentable was considered as 100%, and all values were normalized to it.

**[125I]Methionine Labeling of Liver Protein**

Endogenous proteins in the liver were labeled by injecting rats intraperitoneally with 0.5-0.75 mCi [125I]methionine 48 h before they were killed. The rats were starved 20-24 h before they were killed. The liver was perfused for 15 min and then processed as described above for the preparation of a microsomal fraction and linear sucrose gradient fractions. Samples (<100 µl) were solubilized in 0.5 ml Protosol and the 35S content measured by scintillation counting (Beckman model LS 7000, Beckman Instruments, Inc.) in 6 ml Ready-Solve EP.

**Other Procedures**

**SDS PAGE and Immunoblotting.** Pellets obtained by the immunoadsorption protocol described above, as well as the initial vesicles, and, in several experiments, the remaining (unadsorbed) vesicles after their sedimentation at 100,000 g for 60 min, were solubilized in final concentrations of 0.0625 M Tris, pH 6.8, 1% SDS, 10% sucrose, 0.005% bromophenol blue and heated to 50°C for 10 min. Electrophoresis, transfer to nitrocellulose, and immunoblot analysis were carried out as described (52).

**ASOR Binding Assay.** The 125I-ASOR binding activity present in fractions from sucrose gradients was determined as previously described (52), with the following modifications. Vesicles were permeabilized and the sequestered 125I-ligand (negligible relative to that used in the assay) released by dilution in 12 mM Hepes, pH 7.4, 187.5 mM NaCl, and 2 mM EGTA in the absence or presence of 0.04% (wt/vol) diisogen. The assay was started by addition of 125I-ASOR (1 x 10^6 cpm, 25 nm) and CaCl2 at a final concentration of 8 mM. The pH was brought to 7.4 by addition of 45 mM Hepes, pH 8.0, and the mixture incubated 40-60 min at 4°C. Background binding was measured in the presence of 100-fold excess of unlabeled ASOR.

**Other Assays.** Proteins were determined by a modification (30) of the procedure of Lowry (29) using BSA as standard. Degradation of 125I-ASOR was detected essentially as described (52).

NADH-ferricyanide reductase was assayed as described (42) with the following modifications: microsomes, vesicles immunoadsorbed to S. aureus cells, or vesicles sedimented from S. aureus supernates were solubilized at 4°C in 0.2 mM EDTA, 0.1 M NaCl, 24 mM Na deoxycholate, 0.02 M Tris-acetate, pH 8.1. After sedimentation of the unsolubilized material (5 min at 10,000 g, Eppendorf 5412 microfuge), 200-µl aliquots of the supernates were assayed in 1 mM EDTA, 4.8 mM Na deoxycholate, 0.1 M Tris-acetate, pH 8.1.

**Electron Microscopy**

Pellets from S. aureus immunoadsorptions were fixed overnight at 4°C in 0.5% glutaraldehyde, 1.5% formaldehyde, 0.1 M Na cacodylate, pH 7.4, 2.0 mM CaCl2, 2.0 mM MgCl2, and processed as described previously (23). For electron microscopic visualization of ASOR, 125I-ASOR-HRP was prepared as described (53) and mixed with 125I-ASOR. Yeast mannan (5 mg) was added to the perfusion medium 10 min before the addition of 125I-ASOR-HRP to reduce uptake of the conjugate via the HRP moiety (53). To determine the in situ distribution of ASOR-HRP, a small portion was taken from each biopsy to be used for subcellular fractionation, fixed by immersion overnight, and then 30-50-µm sections prepared using a Smith-Farquhar tissue chopper (46). Fractions of S. aureus pellets containing 125I-ASOR-HRP sequestered in vesicles were
fixed as above, embedded in agarose, and sections prepared as for the tissue. Samples were processed for visualization of HRP by a slight modification of the diaminobenzidine procedure of Rodewald (41), in which incubations were for 15–45 min at 24°C and 0.02 M 3-amino-1,2,4-triazole was included to inhibit catalase. After the diaminobenzidine reaction, samples were rinsed six times for 5 min each in 0.1 M Na cacodylate, pH 7.4, 2.5% sucrose, and postfixed 1 h in 1% OsO₄, 1% K₂Fe(CN)₆ in the same buffer (26). Further processing was as described (53). Unstained, ultrathin sections of material reacted for HRP were photographed using a Zeiss 10A electron microscope.

Quantitation of Vesicle Size and Distribution

The diameters of vesicles containing ASOR-HRP were measured on micrographs of unstained sections of sucrose gradient endosomes. The final magnifications ranged from 8,700 to 34,000.

The intracellular distribution of ASOR in hepatocytes from livers perfused with ASOR-HRP was determined on micrographs taken of sections overlying three randomly chosen grid holes. 12 micrographs at 8,600 final magnification were counted for each experiment. A vesicle profile containing HRP reaction product was identified as peripheral if it was located within 1.5 μm of the base of sinusoidal microvilli (53). All others were considered to be internal endosomes. Only micrographs containing peripheral and internal cytoplasm were used. The difference between means was tested using the Student's t-test.

The shapes and associations of various endosomal elements to one another were determined on serial sections of isolated vesicles containing ASOR-HRP and of hepatocytes exposed to the tracer in situ.

Results

Validation of the Immunoadsorption Approach

Initial experiments. We first established the specificity of our anti-ASGP-R antibody. Affinity-purified 125I-anti-ASGP-R recognized the major polypeptide (Mr ~ 42,000, reference 5) in a purified receptor preparation as well as a higher molecular weight polypeptide, which most likely represents an SDS-insoluble dimer (Fig. 1). The two additional polypeptide bands (bands 2 and 3, Fig. 1) that accounted for ~25% of the protein of the isolated receptor (5) were not efficiently labeled by the ASGP-R antibody. The same antibody recognized only a 42,000 M₀ polypeptide present in vesicles of density ~1.12 g/cc obtained from a sucrose density gradient separation of microsomes (Fig. 2, a–e), despite the presence of at least 60 other Coomassie Blue-staining polypeptides in these vesicles.

We next determined the ability of anti-ASGP-R to recognize the native receptor in membranes using the immunoadsorption protocol described in Materials and Methods. The 1.12 g/cc vesicle preparation, derived from sucrose gradient fractionation of microsomes and enriched in functional ASGP-R and endosomes (determined using 125I-ASOR as a marker and ASOR binding assays, reference 52), was selected for these initial experiments to facilitate receptor quantification. Immunoblot analysis was used to compare the amount of receptor in the starting vesicle fraction (Fig. 2, lanes a–e) to that found in S. aureus pellets after immunoadsorption of vesicles (Fig. 2, lanes f–k). As seen in Fig. 2, the immune antibody effectively recognized and bound vesicles containing the receptor (lanes g–k), while the preimmune IgG did not (lane f). Under optimal conditions of antibody and S. aureus

![Figure 1. Reactivity of the anti-ASGP-R with purified ASGP-R. Lane a, SDS PAGE of purified ASGP-R stained with Coomassie Blue (CB). Lane b, Immunoblot of purified ASGP-R labeled with 125I-anti-ASGP-R (Blot).](image)

![Figure 2. Immunoblot analysis of initial and immunoadsorbed vesicles. Lanes a–e, 38-, 30-, 23-, 15-, and 8-μg protein of an endosome-enriched fraction were separated by SDS PAGE, transferred to nitrocellulose, and labeled with affinity-purified 125I-anti-ASGP-R. Lanes f–k, sucrose gradient endosomes at the indicated protein inputs were immunoadsorbed to S. aureus cells with either 2–3 μg preimmune IgG (lane f) or 2–3 μg anti-ASGP-R (lanes g–k) and the polypeptides present in the final S. aureus pellets labeled on nitrocellulose blots with 125I-anti-ASGP-R.](image)
concentrations, recovery of the receptor in the immunoadsorbed fraction was >100% (Table I), perhaps due to more efficient solubilization of molecules from protein-depleted S. aureus pellets. The ASGP-R content of those vesicles not immunoadsorbed by the antibody was low (<5%). When vesicles prepared from [35S]methionine-labeled livers were incubated with specific and preimmune IgG, only 21% and 6–7% of the radioactivity was immunoadsorbed, respectively, indicating that the immune antibody did not induce extensive nonspecific aggregation of vesicles (Table I).

Based on reports of ASGP uptake by isolated hepatocytes (2, 55) and our earlier morphological observations (53), we suspected that immediately after internalization, most of the ligand would be found in vesicles containing the receptor (R+ vesicles). A 2.5-min time point was chosen and 125I-ASOR–labeled vesicles from a sucrose gradient tested in the immunoadsorption reaction. We found that 100% of the ligand internalized and sequestered within vesicles at 2.5 min was immunoadsorbed by anti-ASGP-R antibody (Table I). (Since isolation of vesicles and immunoadsorptions were done in the absence of Ca++, any ligand associated with plasma membranes would have been released into the postmicrosomal supernate [36].)

**Validation of the Immunoadsorption Protocol Using a Crude Microsomal Fraction.** Our initial experiments established that the vesicles containing the ASGP-R could be quantitatively immunoadsorbed and that shortly after ligand internalization (2.5 min), 100% of the ligand was in R+ vesicles. However, the 125I-ASOR recovered in the sucrose gradient peak represented only 40% of the sedimentable (i.e., sequestered) 125I-ASOR in the homogenate. The crude microsomal pellet contained a more representative population of endosomes, ~60–80% of the total sedimentable 125I-ASOR. Therefore, crude microsomes were tested for immunoadsorption efficiency and specificity. Since quantitation of ASGP-R in the starting microsomes was variable, our criterion of complete immunoadsorption of R+ vesicles was quantitative immunoadsorption of 125I-ASOR in a microsomal fraction from livers that had internalized 125I-ASOR for 2.5 min. When examining later time points, equal amounts of protein were used for immunoadsorptions.

Using the microsomes described above, we found that 98% of the sequestered 125I-ASOR was present in the *S. aureus* pellet when immune antibody was used in the immunoadsorption reaction, but only 4% in the presence of preimmune IgG (Table II). Furthermore, only 9% of total microsomal protein ([35S]methionine-labeled) was immunoadsorbed with anti-ASGP-R versus 2% using preimmune IgG (Table II).

To further test the specificity of immunoadsorption from crude microsomes, we assayed both the starting and final vesicles for the presence of membranes expected to contain little or no ASGP-R (12, 20). Specifically, we determined whether vesicles derived from bile canalicular membrane or from the endoplasmic reticulum were adsorbed. A glycoprotein termed HA 4 has been shown by Hubbard et al. to be a specific marker of the bile canalicular domain (20). Nitrocellulose blots of microsomes and immunoadsorbed vesicles were incubated with 125I-anti-HA 4 (20), and the amount immunoadsorbed was quantified. Very little of either HA 4 antigen or NADH-ferricyanidase reductase, a marker for endoplasmic reticulum, was found in vesicles incubated with either anti-ASGP-R or preimmune IgG (Table II). Thus, the membrane vesicles immunoadsorbed using anti-ASGP-R antibody were not significantly contaminated by these other cellular membranes.

**Morphology of the Immunoadsorbed Vesicles.** Ultrastructural examination of the vesicles that were immunoadsorbed to *S. aureus* cells using anti-ASGP-R antibody revealed tubular and spherical profiles (Fig. 3b). Vesicle aggregates were sometimes seen attached to the *S. aureus* cells and probably arose from our assay conditions, since vesicles were first cross-linked by antibody and then bound to the *S. aureus* cells via the Fc moiety of the IgG. Very few vesicles were seen in preimmune *S. aureus* pellets (Fig. 3a).

**Table II. Validation of the Immunoadsorption Protocol for Microsomal Vesicles**

| Percentage immunoadsorbed | Immune | Preimmune |
|----------------------------|--------|-----------|
| **ASGP-receptor (blot)**   | 122    | 7         |
| Sequestered 125I-ASOR     | 101±2  | 6         |
| Protein ([35S]methionine-labeled) | 21±6   | 6         |

* Aliquots of an endosome-enriched fraction, prepared from livers exposed to 125I-ASOR at 4°C then warmed to 37°C for 2.5 min, were used for each immunoadsorption. The amount of receptor protein present in the immunoadsorbed vesicles was determined by immunoblot analysis as described in Materials and Methods and illustrated in Fig. 2. The percentage of 125I-ASOR sequestered within immunoadsorbed vesicles was adjusted for nonsedimentable 125I-ASOR, determined on preimmune supernates in this experiment to be 12%. A [35S]methionine-labeled liver was treated in a similar manner and the percentage of [35S]labeled protein in the immunoadsorbed vesicles determined.

* Represents ~40% of total sedimentable 125I-ASOR in homogenate.

* Represents <1.0% of total [35S]methionine in homogenate.

Livers were perfused continuously at 37°C with 2–48 μg 125I-ASOR/g wet weight liver and R+ vesicles isolated from biopsies taken 2.5–44 min after the addition of 125I-ASOR. In these experiments, acid-soluble radioactivity indicative of 125I-ASOR degradation was first detected 18 ± 3 min after exposure of livers to the ligand (data not shown). Thus, from 2.5 to 15 min, ligand was accumulating progressively through...
Figure 3. Electron micrographs of immunoadsorbed vesicles. The *S. aureus* pellets from incubation of a crude microsomal fraction with preimmune (a) or immune (b) antibody were fixed and processed for EM. Vesicles and tubules were commonly observed in close association with the *S. aureus* cells (large arrowheads). Aggregates of vesicles and tubules, most likely formed during the primary incubation with anti-ASGP-R with vesicles, were observed adjacent to or sometimes a small distance from the *S. aureus* cell, depending upon the plane of section (lower right). A large number of *S. aureus* cells relative to vesicles were required for quantitative immunoadsorption. Occasional rough microsomes were present (small arrowhead). On average the immunoadsorbed vesicles were 88 ± 65-nm wide. Bar, 0.5 μm.

Figure 4. The time course of transfer of 125I-ASOR from receptor-positive to receptor-negative vesicles during continuous uptake at 37°C. Perfused livers were exposed continuously to 125I-ASOR at 37°C. Biopsies were taken at the indicated times and microsomes containing 67 ± 11% (average of 26 experiments) of the sedimentable 125I-ASOR prepared as described in Materials and Methods. Vesicles were immunoadsorbed, the amount of 125I-ASOR in the *S. aureus* pellet and supernate determined, and the percent distribution calculated, after adjusting for the presence of, on average, 19 ± 3% nonsedimentable 125I-ASOR (average of 11 experiments). Preimmune binding was 3–7%. Separate experiments (i.e., livers) are represented by the different symbols. (*, ▲, +, ◆, △, x, ◇, ○ represent, respectively, 2, 8, 13, 16, 38, 41, 42, 48 μg 125I-ASOR input/g wet wt liver.)
Electron microscopy of livers exposed continuously to the cytochemical ligand, ASOR-HRP, at 37°C showed that hepatocytes were the only cell type accumulating the conjugate. This analysis also confirmed that at 2.5 min the ligand was restricted to structures near the sinusoidal surface, but by 7.5 min labeled vesicles had reached internal sites (52).

**Effects of Temperature on the Transfer of Internalized 125I-ASOR**

125I-ASOR is internalized at 16°C, but it is not transferred to lysosomes (7). Having identified an endosome at 37°C that contained sequestered ligand but lacked the ASGP-R, we used immunoadsorption to determine first if ligand was found in these R− vesicles at 16°C and then if ligand already present in R− vesicles (from uptake at 37°C) was transferred out of this population at 16°C.

**16°C → 37°C Experiments.** Biopsies were taken from livers exposed to 125I-ASOR at 16°C for 90 min and then warmed to 37°C, and crude microsomes were prepared and used for immunoadsorption. The results are presented in Table III (experiment A), where it can be seen that virtually all (94%) 125I-ASOR internalized at 16°C was present in R+ vesicles. Subsequent warming of these livers to 37°C for 14 min resulted in the appearance of ligand in R− vesicles.

Cytochemistry of tissue from livers that had endocytosed 125I-ASOR-HRP was performed to determine the in situ distribution and morphology of structures containing ligand at 16°C and after warming to 37°C. At 16°C, when all ligand was in R+ vesicles biochemically, 34% of the labeled structures were found in internal regions of the cell. After warming to 37°C, significantly more labeled structures (46%) were

**Table III. Effects of Temperature on the Transfer of Sequestered 125I-ASOR into and out of R-Vesicles**

| Experiment | Condition | First | Second | Immune Percentage of 125I-ASOR in S. aureus pellet | Preimmune |
|------------|-----------|-------|--------|-----------------------------------------------|-----------|
| A          | 16°C, 90 min | —     | 37°C, 14 min | 94 ± 17 6 ± 4 | 72 ± 15 5 ± 1 |
| B          | 37°C, 2.5 min | —     | 16°C, 14 min | 83 ± 3 7 ± 3 | 70 ± 8 7 ± 3 |
|            | 37°C, 14 min | —     | 16°C, 44 min | 74 ± 13 7 ± 1 |

* Microsomes from homogenates of livers treated as indicated were immunoadsorbed and the distribution of sequestered 125I-ASOR determined as described in Materials and Methods.
* Mean of six experiments. 125I-ASOR in microsomes represented 81% ± 7% of the sedimentable 125I-ASOR in the homogenate.
* Mean of three experiments. 125I-ASOR in microsomes represented 68% ± 10% of the sedimentable 125I-ASOR in the homogenate.

**Figure 5.** Electron micrographs of hepatocytes labeled with 125I-ASOR-HRP at 16°C followed by warming to 37°C. Biopsies were taken after perfusion of a liver with 125I-ASOR-HRP at 16°C for 90 min, and then again after warming the liver to 37°C. Tissue was fixed, and processed for thin section visualization of HRP. (a) At 16°C, 34% of labeled structures were internal, comprising small tubules and vesicles grouped adjacent to the nucleus (N). After warming to 37°C, 46% of labeled structures were internal, comprising prominent MVEs (large arrowheads) and small vesicles and tubules (small arrowhead). Bar, 0.5 μm.
found to be internal \((P < 0.05)\). More dramatic than the quantitative increase in labeled internal endosomes was the appearance of large multivesicular structures in these regions at 37°C (Fig. 5). Analysis of serial sections revealed that these multivesicular endosomes \((MVEs)\) were not simple spheres but possessed tubular projections that were as long as 0.7–0.9 \(\mu\)m (not shown).

\[ 37^\circ C \rightarrow 16^\circ C \text{ Experiments.} \] To examine the effect of lowered temperature on the transfer of \(^{125}\)I-ASOR out of \(R^-\) vesicles, \(^{125}\)I-ASOR previously sequestered within \(R^-\) vesicles at 37°C was followed after chilling the liver to 16°C. Ligand was internalized into \(R^-\) endosomes by continuously perfusing livers with \(^{125}\)I-ASOR at 37°C for 12–14 min, then livers were chilled to 16°C for an additional 44 min. Biopsies were taken both at 37° and 16°C and immunoadsorptions carried out using crude microsomes. The results are presented in Table III (experiment B). Preloading at 37°C resulted in a transfer of \(~13\%\) sequestered \(^{125}\)I-ASOR from \(R^+\) to \(R^-\) vesicles between 2.5 and 14 min \((83–70\% \text{ in } R^+ \text{ vesicles})\).

Chilling these livers to 16°C for an additional 44 min resulted in little change in the distribution of \(^{125}\)I-ASOR between \(R^+\) and \(R^-\) vesicles. The small increase in \(^{125}\)I-ASOR found in \(R^+\) vesicles \((70–74\% \text{ in } R^+)\) was most probably due to continued uptake of \(^{125}\)I-ASOR at 16°C into \(R^+\) vesicles, since excess ligand was not removed before reducing the temperature in these experiments. We confirmed this by repeating the 37°C → 16°C experiment, but interjecting a 4°C EGTA step to remove both surface-bound and circulating ligand. The distribution of \(^{125}\)I-ASOR in \(R^-\) vesicles was the same \((75\% \text{ and } 77\%)\) before and after cooling to 16°C.

**Separation of Endosomes into Receptor-depleted and Receptor-enriched Fractions by Flotation on Sucrose Gradients**

**Kinetics.** In a recent paper, we reported a difference on continuous sucrose gradients in the distribution of sequestered \(^{125}\)I-ASOR from livers exposed to ligand 2.5 min or 10 min \((52)\). A lighter density endosome fraction was present at 10 min. The absence of any difference in the distribution of ASOR binding activity led us to suggest that the lighter endosomes might be depleted of receptor. Our in situ morphological observation of the appearance of MVEs in the 16°C → 37°C shift experiments further suggested that these structures might be the \(R^-\) endosomes. Therefore, we turned to discontinuous sucrose gradients and flotation in an effort to separate \(R^+\) and \(R^-\) vesicles, since sedimentation in continuous gradients gave variable results. Microsomes prepared from livers perfused with \(^{125}\)I-ASOR for 2.5 or 14 min at 37°C were applied to gradients and the distributions of sequestered \(^{125}\)I-ASOR and ligand binding activity determined. The results are presented in Fig. 6 and Table IV. A. At 2.5 min, virtually all \((97\%)\) of the sequestered \(^{125}\)I-ASOR was found in vesicles at the 0.86 M/1.15 M interface \((-1.12 \text{ g/cc})\), with very little to none at the lower densities \((3\% \text{ at } 0.60 \text{ M/0.86 M, } -1.09 \text{ g/cc})\). By 14 min, the 1.09 g/cc peak accounted for \(19\%\) of the \(^{125}\)I-ASOR sequestered within vesicles, the \(-1.05 \text{ g/cc} \text{ peak} \sim 77\%\), and the \(-1.05 \text{ g/cc} \text{ peak} \sim 2\%\). ASOR binding

\(^3\) This relatively low average value for the 2.5-min time point was caused by a new preparation of apparently less dense \(S. aureus\) cells. Longer centrifugation of the immunoadsorption reaction mixture \((25 \text{ min})\) and aspiration of all but the last 100 \(\mu\)l of supernate above the pellet increased the 2.5 min value to \(~100\%\).

![Figure 6. Separation of receptor-depleted from receptor-enriched vesicles by flotation on discontinuous sucrose gradients. Microsomes prepared from a liver continuously perfused at 37°C with \(^{125}\)I-ASOR for 2.5 (top) or 14 min (bottom) were resuspended in 1.15 M sucrose and centrifuged on discontinuous sucrose gradients as described in Materials and Methods. Sequestered \(^{125}\)I-ASOR \((\bigcirc)\), total \(^{125}\)I-ASOR binding activity \((\triangle)\), and ASGP-R determined by immunoblotting \((\Delta)\) were measured on the fractions as described in Materials and Methods. The solid bar in the lower panel indicates the position of the load. Assays and immunoblot analysis across gradients indicated that the 1.09 g/cc peak contained very little binding activity or receptor protein \(<3\%)\) either 2.5 min or 14 min after exposure of livers to ligand. Furthermore, the ratio of sequestered \(^{125}\)I-ASOR to ASOR binding sites was seven times greater for the lighter endosomes \((-1.09 \text{ g/cc})\) than for the heavier endosomes \((-1.12 \text{ g/cc})\) at 14 min.

Microsomes from biopsies of livers exposed to \(^{125}\)I-ASOR for 90 min at 16°C were fractionated on discontinuous sucrose gradients, and only 5% of the \(^{125}\)I-ASOR was found in the 1.09 g/cc peak, with 95% present in the 1.12 g/cc peak \((\text{Table IV, B})\). Warming to 37°C resulted in an increase in the percentage of sequestered \(^{125}\)I-ASOR found in the 1.09 g/cc peak \((17\%)\). Detection of the ASGP-R by immunoblot analysis or ligand binding again revealed no concomitant shift in the receptor distribution.

**Morphological Identification of \(R^-\) and \(R^+\) Endosomes.** To identify the types of vesicles that contained ASOR but were depleted of the ASGP-R, we separated endosomes containing the specific cytochemical tracer, \(^{125}\)I-ASOR-HRP, into receptor-depleted and receptor-enriched fractions on discontinuous sucrose gradients as described above. Endosomes were
ASGP-R in Discontinuous Sucrose Gradients*  

Many recent studies in the field of receptor-mediated endocytosis have focused on the morphological, biochemical, and functional characteristics of a series of complex prelysosomal compartments, collectively termed endosomes (1, 18, 35). Endosome compartments have been most extensively characterized in the ASGP system of rat hepatocytes, where ligand-receptor dissociation and subsequent separation have been analyzed by both biochemical (2, 17, 55, 57) and morphological (12) approaches. Results of these studies have suggested that ASGPs destined for lysosomes dissociate and segregate from their receptor somewhere in the endosome compartment before entering lysosomes. In the present study, we have focused on the second (segregation) step and found that endocytosed ASGPs are transferred from tubular and vesicular endosomes containing ASGP receptors to MVEs lacking receptors, most likely after transport into the internal regions of the cell. Our approach combined in situ and in vitro cytochemistry of specific EM tracers, subcellular fractionation, and quantitative immunoadsorption.

### Immunoadsorption of Membranes Containing the ASGP-R

Immunoadsorption has been used previously to isolate coated vesicles (15, 32), vesicles containing NADPH-cytochrome P-450 reductase (25, 27), and plasma membrane domains (33, 45), usually from partially purified fractions. We chose to start with crude microsomes rather than a more purified vesicle preparation, since 60–80% of the total sedimentable (sequestered) 125I-ASOR was recovered in this fraction and, thus, a representative population of endosomes would be studied in our kinetic experiments. Our immunoadsorption procedure was fast (~5 h), quantitative (immunoadsorption of 100% of the ASGP-R), and specific for those membranes containing the ASGP-R. We demonstrated that little membrane derived from endoplasmic reticulum or from plasma membrane domains lacking the ASGP-R was immunoadsorbed. However, the vesicles that were immunoadsorbed by the ASGP-R antibody were not exclusively endosomal, since our polyclonal antibody would recognize ASGP-R present in any membrane compartment, without regard to the molecule’s orientation. Furthermore, numerous lines of evidence suggest that most of the ASGP-R resides in intracellular membrane compartments irrespective of the endocytic load on the cell (reviewed in reference 34). Thus, newly synthesized or recycling ASGP-R in Golgi elements (12, 13), in the lateral and sinusoidal plasma membrane domains (20), as well as in endosomes (occupied or unoccupied by ligand), would be present in the S. aureus pellet. Nonetheless, we achieved 45–60-fold enrichments of sequestered 125I-ASOR in the immunoadsorbed (R+) fraction relative to homogenate when crude microsomes were used and ~135-fold when sucrose gradient vesicles were used. These values compare favorably with those reported for endosomal subfractions prepared using different separation techniques (1, 4, 38).

### R+ and R− Endosomes

In this study we have identified two kinetically, biochemically, and morphologically distinguishable populations of ASGP-containing endosomes: one that is receptor-positive and the other, receptor-negative. By the immunoadsorption approach, we find ligand first solely in R+ endosomes, but ~7 min after the start of endocytosis, 10–20% appears in R− vesicles. By 14 min, before degradation can be detected, a maximum of 30% of the internalized 125I-ASOR is sequestered within R− endosomes. Thus, the half-time of ligand accumulation in the R− subcompartment is ~7 min. Since ligand is degraded rapidly once in lysosomes (8), but degradation is not detected until at least 15 min after the first molecules are internalized, ligand must reside in the R− compartment a minimum of ~8 min. Thus, the transfer of ligand out of R− endosomes appears to be a slow step in its transport to the lysosomes.

### Table IV. Distribution of Internalized 125I-ASOR and ASGP-R in Discontinuous Sucrose Gradients

| Condition   | Sequestered 125I-ASOR | Sequestered ASGP-R |
|-------------|------------------------|--------------------|
| A Continuous uptake at 37°C |                       |                    |
| **2.5 min** | 3 97                  | 1 99               |
| **14 min**  | 8 91                  | 1 99               |
| **14 min**  | 19 78                 | 3 99               |

| B 16°C → 37°C | Sequestered 125I-ASOR | Sequestered ASGP-R |
|---------------|------------------------|--------------------|
| **90 min, 16°C** | 5 95                  | <5* >95           |
| **then 14 min, 37°C** | 17 83                 | 1 99               |

* Microsomes (containing 71–90% of total sedimentable 125I-ASOR) were resuspended in 1.15 M sucrose and floated into discontinuous sucrose gradients, fractions collected automatically, and analyzed for presence of 125I-ASOR, ligand binding activity and ASGP-R (see Materials and Methods).

** Brackets indicate that data was obtained from the same liver.

** The ASGP-R detected by immunoblot analysis in LDVs was below the standard curve of purified ASGP-R run in parallel and so represents an absolute maximum in the LDVs of the experiment.

* ASGP receptor gave identical distributions when measured by immunoblot or ligand binding activity.

† The amount of sequestered 125I-ASOR (ng) and binding activity (ng 125I-ASOR) were determined for the peak fractions only (see Fig. 6).

‡ LDV, low density vesicles from the 0.6/0.86 M interface (fractions 12–15 in Fig. 6, A and B).

§ HDV, high density vesicles from the 0.86/1.15 M interface (fractions 16–25 in Fig. 6, A and B).

\[ * \text{Bracket indicates data was obtained from the same liver.} \]

\[ ** \text{Not determined.} \]

\[ \text{* The ASGP-R detected by immunoblot analysis in LDVs was below the standard curve of purified ASGP-R run in parallel and so represents an absolute maximum in the LDVs of the experiment.} \]

\[ \text{** Brackets indicate that data was obtained from the same liver.} \]

\[ \text{† The ASGP-R detected by immunoblot analysis in LDVs was below the standard curve of purified ASGP-R run in parallel and so represents an absolute maximum in the LDVs of the experiment.} \]
The R+ and R- endosomes were identified morphologically using ASOR-HRP as a specific EM tracer for ASGP-containing endosomes on sucrose gradients and on appropriate fractions after immunoadsorption using anti-ASGP-R. The R- vesicles identified in vitro resemble the MVEs that we have identified previously in situ as being predominantly in the internal regions of the hepatocyte (52, 53). They are large and contain lipoprotein particles and/or small vesicles in their lumen. The R+ vesicles resemble both peripheral endosomes and the more internal endosomes that are often found in continuity with the multivesicular structures in situ (21 and Fig. 5).

Although R+ and R- endosomes are clearly distinct components in liver homogenates, R- endosomes may not exist as discrete entities in situ. The serial section analyses reported here and previously (e.g., 21, 53) indicate that most MVEs in situ have projecting arms. However, the labeled MVEs in our R- fractions did not have these extensions when analyzed by serial sections, suggesting that the arms were pinched off during homogenization and that they most likely are part of the internal R+ compartment in situ.

The localization of MVEs predominantly to the Golgi/lysosome region of hepatocytes in situ and their identification as the R- endosomes, suggests that ligand and receptor segregate in the internal regions of the cell. This view is reinforced by several lines of evidence indicating that R+ endosomes are in both internal and peripheral locations. At 16°C, ASOR was present only in R+ vesicles, yet structures containing ASOR-HRP were located in situ in both peripheral (66%) and internal (34%) sites. Similarly, after ASOR-HRP has been internalized at 37°C for 14 min after prebinding at 4°C, the majority (83%) of labeled structures in situ were located in the internal cytoplasm when immunoadsorption indicated ~70–90% of the sequestered ligand was in R+ vesicles (data not shown). Thus, the transfer of ASOR into R- vesicles most likely occurs after transport of ligand to the internal sites. Of course, removal of some but not all of the ASGP receptors could begin in peripheral endosomes and be completed in the internal endosomal compartment.

**Comparison to Other Studies**

Several of our findings confirm and significantly extend those reported by others. For example, the existence of prelysosomal vesicles lacking the ASGP-R has been proposed on the basis...
of experiments in which the ionophore, monensin, and precipitation of ligand–receptor complexes were used to monitor movement of ASOR from R+ to R− compartments in cultured rat hepatocytes (17). However, these kinetic compartments were not correlated with any morphological structures in the isolated cell. Unfortunately, progression of ligand through the endocytic pathway in primary rat hepatocytes in culture is both slower and less efficient than that in the perfused liver, precluding meaningful comparisons to the results reported here. Nonetheless, the identification of R+ and R− populations in the perfused liver, which retains the normal in vivo epithelial architecture and polarity of cellular organization, suggests that intracellular events in ligand transport are similar in both systems. Whether lack of normal hepatocyte polarity is related to the slow and inefficient transport in isolated cells remains to be determined.

Our finding that ASGPs in the perfused liver remain in the R− endosomal subcompartment for ~8 min before entry into and degradation within lysosomes, confirms the prediction by Bridges et al. that ASGPs in isolated hepatocytes undergo an obligate maturation process (2). These investigators speculated that transport of ligand to intracellular regions rich in lysosomes might account for the obligate delay. Our results strongly indicate that the R− compartment corresponds in situ to internal MVEs. Therefore, intracellular transport of ligand is clearly not the limiting event in delivery of ligand to lysosomes. Rather, the ligand, once in an MVE, and/or the MVEs themselves, must change in some way before they are competent to fuse with lysosomes.

Since low temperature was first reported to block endosome–lysosome fusion in the perfused liver (7), this perturbation has been exploited in many studies of endocytosis (31, 55, 57). Although there is general agreement that the final step in the pathway is blocked, acidification (dissociation) seems to be variously affected at low temperatures (see references 50 and 57). Using immunoadsorption, we found that both transfer of ligand from R+ to R− endosomes and from R− endosomes to lysosomes were inhibited at 16°C. However, more molecules of ASOR were internalized into the R+ endosomes at this temperature than the number of cell surface binding sites (1 × 10⁶ molecules endocytosed at 16°C vs 250,000 molecules bound at 4°C). This result suggests either that unoccupied receptors from the internal pool can move to the cell surface and internalize ligand and/or that dissociation and recycling of receptors continues at 16°C, with excess unoccupied receptors (recycling or not) residing in endosomes.

Geuze et al. (12) have observed an endocytic compartment in hepatocytes (termed CURL) where ligand and receptor appear to be segregated into morphologically distinct regions. Large vesicular profiles with ASGPs scattered in the lumen and little ASGP-R, as detected by antibody and protein A-gold, are connected to tubular structures lacking the ligand but containing the ASGP-R. These investigators have proposed that the tubular elements represent receptors leaving CURL and that the vesicular elements represent immediate precursors of multivesicular bodies, which are apparently discrete structures. It is difficult at present to reconcile our results with this view. The MVEs we observe in situ are in continuity with tubular elements, and the latter contain ligand (albeit a cytochemical tracer, whose reaction product could diffuse). Further work will hopefully clarify these issues.

Final Comments

The results we have presented in this and a previous article (52) demonstrate the biochemical and functional heterogeneity of the endosomal compartment in rat hepatocytes. It is clear that this complex organelle plays a central role in the endocytosis and sorting of ASGPs. We and others also have good evidence that ligands and receptors with different fates from those of ASGP and its receptor share all or part of the same endocytic pathway (e.g., 3, 21). The combined approach we have used here should facilitate study of roles the several endosomal subcompartments play in correctly and simultaneously sorting these various molecules.

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