Sorting of Endocytosed Transferrin and Asialoglycoprotein Occurs Immediately after Internalization in HepG2 Cells

Willem Stoorvogel, Hans J. Geuze, and Ger J. Strous

Laboratory of Cell Biology, University of Utrecht Medical School, Utrecht, The Netherlands

Abstract. After receptor-mediated uptake, asialoglycoproteins are routed to lysosomes, while transferrin is returned to the medium as apotransferrin. This sorting process was analyzed using 3,3′-diaminobenzidine (DAB) cytochemistry, followed by Percoll density gradient cell fractionation. A conjugate of asialoorosomucoid (ASOR) and horseradish peroxidase (HRP) was used as a ligand for the asialoglycoprotein receptor. Cells were incubated at 0°C in the presence of both ^{131}I-transferrin and ^{125}I-ASOR/HRP. Endocytosis of prebound ^{125}I-ASOR/HRP and ^{131}I-transferrin was monitored by cell fractionation on Percoll density gradients. Incubation of the cell homogenate in the presence of DAB and H_2O_2 before cell fractionation gave rise to a density shift of ^{125}I-ASOR/HRP–containing vesicles due to HRP-catalyzed DAB polymerization. An identical change in density for ^{125}I-transferrin and ^{125}I-ASOR/HRP, induced by DAB cytochemistry, is taken as evidence for the concomitant presence of both ligands in the same compartment. At 37°C, sorting of the two ligands occurred with a half-time of ~2 min, and was nearly completed within 10 min. The ^{125}I-ASOR/HRP–induced shift of ^{131}I-transferrin was completely dependent on the receptor-mediated uptake of ^{125}I-ASOR/HRP in the same compartment. In the presence of a weak base (0.3 mM primaquine), the recycling of transferrin receptors was blocked. The cell surface transferrin receptor population was decreased within 6 min to 15% of its original size. DAB cytochemistry showed that sorting between endocytosed ^{131}I-transferrin and ^{125}I-ASOR/HRP was also blocked in the presence of primaquine. These results indicate that transferrin and asialoglycoprotein are taken up via the same compartments and that segregation of the transferrin–receptor complex and asialoglycoprotein occurs very efficiently soon after uptake.

MANY serum macromolecules can be bound to the cell surface by specific receptors, and subsequently internalized via receptor-mediated endocytosis. Depending on the final destination of the ligand, distinct intracellular transport routes can be followed. The first events in these differing pathways seem to be identical: binding of the ligand to the receptor at the plasma membrane; clustering of receptor–ligand complexes in coated pits; internalization of the complex via coated vesicles; and transport to the compartment of uncoupling receptor and ligand (CURL)[1] or endosomes. From there, different routes may be followed. Many ligands, such as asialoglycoproteins (ASGP) (reviewed in Breitfeld et al., 1985; Schwartz, 1984*b*) and low density lipoproteins (reviewed in Goldstein et al., 1985) are transported to the lysosomes where they are degraded, whereas their receptors recycle to the plasma membrane and can be re-utilized in a new endocytotic cycle. Other ligands, such as polymeric IgA, are taken up at the basolateral plasma membrane and are not uncoupled from their receptors, but directed as ligand–receptor complexes to the apical plasma membrane in a process called transcytosis (Limet et al., 1982; Courtoy et al., 1985; Solari and Kraehenbuhl, 1984). Transferrin, a serum glycoprotein that plays an important role in iron delivery to cells, releases its iron intracellularly at acidic pH and recycles bound to its receptor to the plasma membrane (Klausner et al., 1983; Dautry-Varsat et al., 1983).

If uptake of various types of receptor–ligand complexes occurs through the same entry route, sorting among and between different types of ligands and receptors must occur intracellularly. By using double-label immunoelectron microscopy, we found that sorting between ASGPs and their receptors in rat liver occurred in a system composed of tubules and vesicles, termed CURL (Geuze et al., 1983*a*). By using the same technique, polymeric IgA was shown to segregate from the mannose phosphate receptor and the ASGP receptor within CURL (Geuze et al., 1984). Furthermore, in rat hepatocytes, polymeric IgA and ASGP sorting was shown to occur in similar structures (Courtoy et al., 1985). The kinetics of this process has been studied in rat liver (Courtoy et al., 1985), using the 3,3′-diaminobenzidine (DAB) density shift method (Courtoy et al., 1984).

Transferrin is an ideal marker for the study of receptor recycling, since it probably follows the same route as its recep-

1. *Abbreviations used in this paper*: ASGP, asialoglycoprotein(s); ASOR, asialoorosomucoid; ASOR/HRP, asialoorosmucoid/horseradish peroxidase complex; CURL, compartment of uncoupling receptor and ligand; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase.

tor during the entire process of recycling. As the recycling parameters for the transferrin and the ASGP receptor are very much alike, it is assumed that both receptors follow the same route in HepG2 cells (Ciechanover et al., 1983*b*). In the present study we have used the DAB-induced density shift principle (Courtoy et al., 1984) to address the questions of when and where transferrin and ASGP segregate. We conclude that both ligands are taken up via the same compartments. In addition, we show that the two ligands segregate shortly after uptake.

Primaquine, like other amines, neutralizes acidic compartments (Maxfield, 1982; Ohkuma and Poole, 1978; Poole and Ohkuma, 1981; Tycko et al., 1983), and interferes both with the recycling of the ASGP receptor (Strous et al., 1985) and the degradation of endocytosed ASGP (Schwartz et al., 1984*a*). In this paper we show that in the presence of 0.3 mM primaquine the transferrin receptor recycling is blocked and that complete inhibition of sorting between endocytosed transferrin and the asialoorosomucoid/horseradish peroxidase complex (ASOR/HRP) is achieved.

Materials and Methods

Cells

The human hepatoma cell line HepG2 (Knowles et al., 1980) was cultured in monolayer in MEM supplemented with 10% decomplemented fetal bovine serum, and antibiotics (Schwartz et al., 1981). For experiments, 80% confluent cultures were used. The medium was refreshed 1 d before the experiment.

Preparation of the ASOR/HRP Conjugate

Orosomucoid was a gift from Dr. A. L. Schwartz (Children's Hospital, St. Louis, MO) and desialylated by incubating at 80°C for 60 min in 25 mM H_2SO_4 (Schwartz et al., 1980). The extent of desialylation was determined according to the procedure of Warren (1959), and exceeded 95%. ASOR was conjugated to HRP (type VI; Sigma Chemical Co., St. Louis, MO) essentially according to the method of Nakane and Kowaoi (1974), using equimolar quantities of ASOR and HRP. The conjugate was separated from free ASOR and HRP by Sephadex G-100 column chromatography. Fractions were analyzed by SDS-PAGE and by determining the ratio of adsorbances at 280 and 403 nm. 90% of the conjugation products from the pooled peak fractions consisted of one molecule ASOR and one molecule HRP ($ASOR_1/HRP_1$). The remaining 10% consisted of two ASOR and one HRP molecules ($ASOR_2/HRP_1$). The peroxidase activity of ASOR/HRP was measured as described by the HRP manufacturer. The molar activities of the preparations used varied between 70 and 96%, compared with nonconjugated HRP.

Iron Saturation of Transferrin

Human transferrin (98% pure; Sigma Chemical Co.) was iron saturated using a modified method of Klausner and co-workers (1983): 6 mg transferrin was dissolved in 1 ml freshly prepared 0.25 M Tris/HCl, pH 8.0, 10 μM $NaHCO_3$, 2 mM sodium nitrillotriacetate, 0.25 mM $FeCl_3$, and incubated for 30 min at room temperature, followed by dialysis against 3 × 1 liter 0.15 M NaCl, 20 mM Hepes, pH 7.2 at 4°C.

Ligand Iodination

Portions of 200–500 μg ASOR/HRP were iodinated in 500 μl PBS containing 1 mCi ^{125}I (essentially carrier free; Amersham Corp., Arlington Heights, IL) and six iodobeads (Pierce Chemical Co., Rockford, IL). ASOR (100 μg) was iodinated in 500 μl PBS containing 0.5 mCi ^{125}I and six iodobeads. Diferric-transferrin (500 μg) was iodinated in 500 μl 0.15 M NaCl, 20 mM Tris/HCl, pH 7.4, containing 1 mCi ^{131}I (10 mCi/μg; New England Nuclear, Boston, MA) and six iodobeads, or alternatively 200 μg with 0.5 mCi ^{125}I and three iodobeads. All iodinations were performed for 30 min at room temperature. Free ^{125}I or ^{131}I were removed by chromatography on a Sephadex G-25 column equilibrated in PBS. The specific activity and concentration of the iodinated proteins were determined by TCA precipitation before and after gel filtration. The specific activities were: 0.8 × 10^6–3.4 × 10^6 cpm/μg ^{125}I-ASOR/HRP, 4.6 × 10^6 cpm/μg ^{125}I-ASOR, 0.7 × 10^6 cpm ^{131}I/μg transferrin, and 2.5 × 10^6 cpm ^{125}I/μg transferrin.

Ligand Binding

Semiconfluent cultures were washed three times with binding medium (MEM, 0.85 g/liter $NaHCO_3$, 20 mM Hepes/NaOH, pH 7.2), before a 30-min incubation period in binding medium at 37°C to deplete ASGP and transferrin receptors from their ligands. Cells were incubated for 1 h on a rocker in ice-cold binding medium supplemented with iodinated ligand(s), followed by two quick and two 5-min washes with binding medium at 0°C to remove excess ligand. Further processing is described in the text.

Gradient Centrifugation

Cells were scraped in homogenization buffer (0.25 M sucrose, 2 mM $CaCl_2$, 10 mM Hepes/NaOH, pH 7.2), and homogenized with 50 strokes, using a dounce with a tight fitting pestle. Nuclei were removed by centrifugation for 10 min at 300 *g* in a minifuge (Heraeus Christ GmbH, Osterode, Federal Republic of Germany). 500 μl of the postnuclear supernatant was layered on top of 13 ml (25%) Percoll solution (Pharmacia Fine Chemicals, Piscataway, NJ) in 0.25 M sucrose, 2 mM $CaCl_2$, 10 mM Hepes/NaOH, pH 7.2. A density gradient was formed during 49.300 g_{max} centrifugation for 60 min in a superspeed centrifuge (Sorvall RC-5B; Dupont Co., Sorvall Instruments Div., Newtown, CT) using an SM 24 rotor, and fractionated by downward displacement in 0.5-ml fractions. ^{125}I and ^{131}I were counted in a gamma counter (PWE 4800; Philips Electronic Instruments, Inc., Mahwah, NJ). The density distribution in the gradient formed was measured using density marker beads (Pharmacia Fine Chemicals).

DAB Cytochemistry

A modification of the method, originally developed by Courtoy et al. (1984) was used. A 4.5-mM solution of DAB (Fluka AG, Buchs, Switzerland) in homogenization buffer was prepared, adjusted to pH 7.2 with 1 N NaOH, and filtered through a 0.22-μm filter (Millipore Corp., Bedford, MA). 250 μl postnuclear supernatant was mixed with 300 μl DAB solution and 3 μl of 6% H_2O_2 and incubated for 15 min at room temperature in the dark. A 250-μl control sample from the same homogenate was incubated with 300 μl homogenization buffer alone. Subsequently, 500 μl of the mixtures were fractionated on Percoll density gradients as described above.

To quantitate the degree of the density shift of each ligand, the distribution patterns of ligand after incubation with and without DAB were laid on top of each other. The surplus of ligand left in the bottom fractions as a consequence of DAB cytochemistry was divided by the total amount of endosomal ligand (fraction 1–17). To calculate the percentage of intracellular ^{131}I-transferrin not sorted from ASOR/HRP, the degree of ^{131}I-transferrin shift at 2, 5, or 10 min was divided by the degree of ^{125}I-ASOR/HRP shift at 2 min of incubation of the cells at 37°C. The ^{125}I-ASOR/HRP shift at 2 min was taken as the reference point, because at each time point the normal endosomal density distribution of ^{131}I-transferrin resembles that of ^{125}I-ASOR/HRP at 2 min.

Results

Binding of ^{125}I-ASOR/HRP to the Asialoglycoprotein Receptor

^{125}I-ASOR/HRP was used as a ligand for the ASGP receptor. The conjugation method used to prepare ASOR/HRP produces only low molecular weight conjugates, probably because of the limited accessibility of reactive amino groups of the heavy glycosylated ASOR molecule. Also, the relative high affinity of ASOR for ASGP receptors (Fig. 1) compared with other galactose terminal ligands such as asialofetuin and galactosylated bovine serum albumin makes the ASOR/HRP conjugate an ideal bifunctional molecule with respect to its receptor binding and peroxidase activity. To determine the specificity of binding and the number of binding sites for this ligand at the plasma membrane, HepG2 cells were incubated

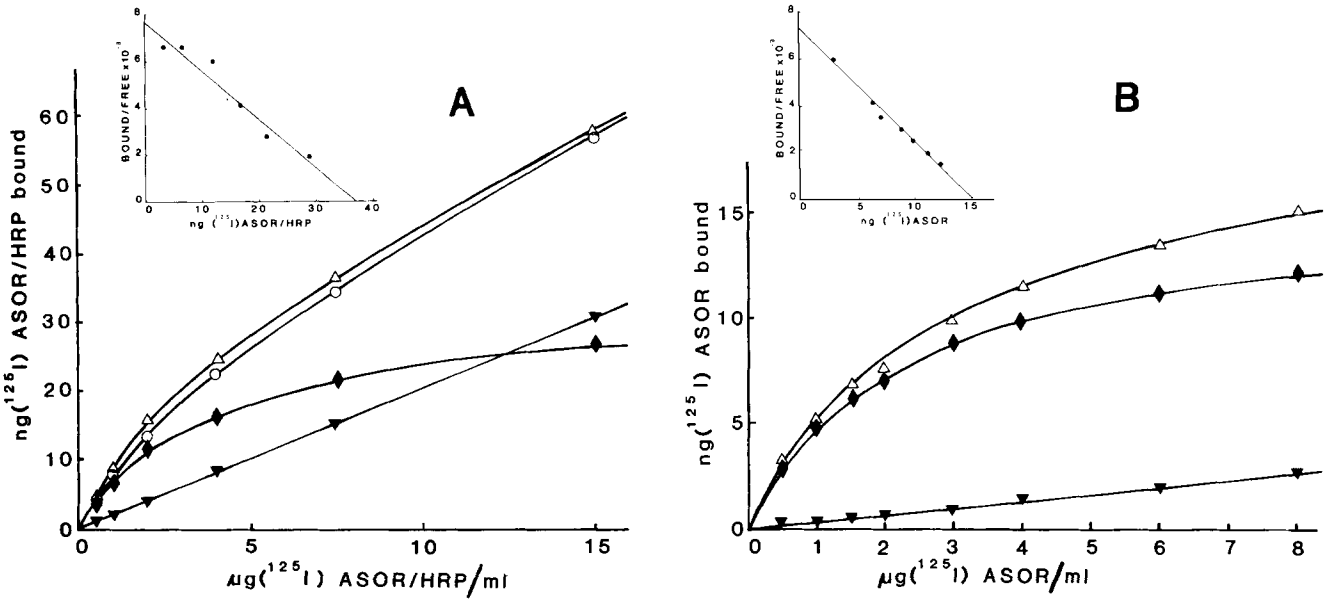

Figure 1. Saturation binding of ^{125}I-ASOR, and ^{125}I-ASOR/HRP to HepG2 cells. Tissue culture dishes (35-mm) were incubated for 30 min at 37°C in binding medium to deplete surface receptors from ligand. Then the cells were incubated for 60 min at 0°C with various concentrations of ^{125}I-ASOR/HRP (*A*), or ^{125}I-ASOR (*B*). Excess ligand was washed away, and the cells were dissolved in 1 N NaOH. Total binding (*open triangle*), binding with excess of nonlabeled HRP (*open circle*), and binding with excess of nonlabeled ASOR (*solid triangle*) were measured as described in the text. The specific binding (*solid diamond*) was calculated by subtracting binding in the presence of excess ASOR from the total binding. (*Insets*) Scatchard plots of the specific receptor binding of both ligands.

in the presence of various concentrations of ^{125}I-ASOR/HRP for 1 h at 0°C (Fig. 1 *A*). At this temperature uptake is completely blocked, so that only surface receptors are labeled. Nonspecific ^{125}I-ASOR/HRP binding was measured in the presence of an excess of nonlabeled ASOR (400 μg/ml) and amounted to 30% of the total binding at 4 μg/ml ASOR/HRP. Excess of nonlabeled HRP (400 μg/ml) did not reduce ASOR/HRP binding. Thus, saturable binding of ^{125}I-ASOR/HRP was exclusively dependent on binding to the ASGP receptor and reached a plateau at a concentration of ~10 μg/ml ^{125}I-ASOR/HRP. The exact number of binding sites can only be roughly determined, since the ligand was not fully homogeneous (see Materials and Methods). Scatchard analysis (Scatchard, 1949) indicates that there are 240,000 ASGP receptors at the cell surface, postulating a homogeneous $ASOR_1/HRP_1$ population. Since the ASOR/HRP preparation also contained ~10% $ASOR_2/HRP_1$ complexes, the real amount of surface ASGP receptors could probably be somewhat less than 240,000 per cell. As a control, a similar titration was performed using ^{125}I-ASOR (Fig. 1 *B*). Saturation binding was achieved at a concentration of ~5 μg/ml, which is about equimolar to the saturation concentration of ASOR/HRP. Analysis of these data show that there are 200,000 plasma membrane ASGP receptors per cell saturated for 50% at 5.2×10^{-8} M ASOR after 1 h at 0°C. This number is well in agreement with that found for ASOR/HRP and close to the 150,000 ± 20,000 binding sites per cell surface reported elsewhere (Schwartz et al., 1981).

Fractionation of Endocytosed ^{125}I-ASOR/HRP on Percoll Gradients

To standardize the density distribution in the Percoll gradient of endocytosed ASGP, prebound ^{125}I-ASOR/HRP was allowed to enter the cell for various periods of time at 37°C. Subsequently, the cells were fractionated on Percoll density gradients (Fig. 2). If the cells were kept on ice, a single peak of ^{125}I-ASOR/HRP-containing vesicles with a mean density of ~1.045 g/ml was observed. This peak represented exclusively plasma membrane-bound ligand, as removal of Ca^{2+} or lowering of the pH to 5 before homogenization completely removed this peak (not shown). In the absence of Ca^{2+} ions or at pH 5, ASGP-receptor complexes are unstable (Schwartz et al., 1981). After 2 min of incubation at 37°C most of the prebound ligand was detected in vesicles focusing at a mean density of 1.050 g/ml (Fig. 2). Washing the cells in the presence of EGTA or at pH 5 removed the radioactivity present at a density of 1.045 g/ml, but did not affect the peak at 1.050 g/ml (not shown), indicating an intracellular localization. Prolonged incubation at 37°C gave rise to labeling of denser vesicles up to 1.09 g/ml after 30 min, while at the same time plasma membrane-bound label disappeared. After 60 min of incubation at 37°C, some label had left the cells, probably because of lysosomal degradation and release of the degradation products in the medium (Simmons and Schwartz, 1984). Label in the top fractions of the gradients represents non-membrane-bound ^{125}I-ASOR/HRP. If excess of unlabeled ASOR was present during ^{125}I-ASOR/HRP binding, the nonspecific bound radioactivity was recovered at the top gradient fractions (Fig. 5 *A*). It was also possible that some endocytosed ligand originating from leaky vesicles contributed to the radioactivity present in these fractions.

DAB-induced Density Shift

If HRP-containing microsomes are incubated with DAB and H_2O_2 before sucrose density fractionation, a density shift can be obtained due to HRP-catalyzed polymerization of

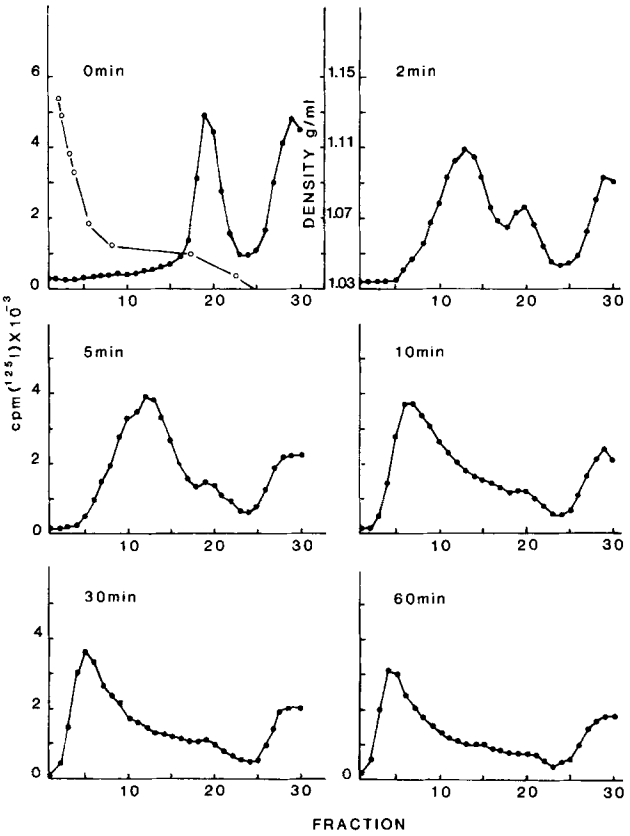

Figure 2. Percoll gradient density distribution of ^{125}I-ASOR/HRP. Culture dishes (6 cm) were pretreated as described in Fig. 1. Then ligand binding was performed during an incubation period of 60 min at 0°C in medium supplemented with 4 μg/ml ^{125}I-ASOR/HRP. Excess ligand was washed away at 0°C, and the cells were incubated for 0, 2, 5, 10, or 60 min at 37°C before cell fractionation. The density distribution of ^{125}I-ASOR/HRP (*solid circle*) was measured as described in Materials and Methods. The density of the fractions (*open circle*) was measured with density marker beads.

DAB inside the vesicle (Courtoy et al., 1984). We used a similar cytochemical procedure in combination with cell fractionation on Percoll gradients. The postnuclear supernatant derived from cells labeled at 4°C with ^{125}I-ASOR/HRP and chased for 2 min at 37°C was split in two equal portions, one of which was incubated with DAB and H_2O_2 containing homogenization buffer and the other with homogenization buffer alone. Subsequently, both parts were fractionated on Percoll gradients (Fig. 3 *A*). Plasma membrane-bound ^{125}I-ASOR/HRP (fraction 18–22) did not shift upon DAB incubation, presumably because plasma membranes did not vesiculize, or vesiculized in an outside-out orientation. However, endocytosed material (mean density, 1.045 g/ml) was shifted towards a mean density of ~1.09 g/ml. The DAB cytochemistry procedure increased both the density and the heterogeneity in density of ^{125}I-ASOR/HRP-containing microsomes. The steep increase in density at the bottom of the Percoll gradient, however, caused the formation of a relatively narrow peak of this material. If H_2O_2 was omitted during the DAB incubation, a minor density shift towards a mean density of 1.053 mg/ml was obtained, possibly because of endogenously formed H_2O_2. If ^{125}I-ASOR instead of ^{125}I-ASOR/HRP was used as a ligand, the density of ligand-containing vesicles did not change upon DAB incubation (Fig. 3 *B*). Our conclusion is that the density shift of ^{125}I-ASOR/HRP-containing vesicles upon DAB cytochemistry is entirely dependent on peroxidase activity.

The Rate of Sorting of Endocytosed Transferrin and ASOR/HRP

The DAB density shift principle was used to distinguish between vesicles containing both transferrin and ASOR/HRP, and vesicles containing only one of the ligands. Cells were incubated with ^{131}I-transferrin (4 μg/ml) and ^{125}I-ASOR/HRP (4 μg/ml) at 0°C. Under these conditions the plasma membrane transferrin binding sites were nearly saturated with transferrin (Ciechanover et al., 1983*a*) and less than half of the 200,000 ASGP binding sites were occupied by ASOR/HRP (Fig. 1). After ligand binding at 0°C, excess ligand was removed, and the cells were incubated at 37°C for 2, 5, or 10 min. After 2 min at 37°C, the density distribution of endocytosed ^{131}I-transferrin is nearly identical to that of ^{125}I-ASOR/HRP (Fig. 4, upper left panel). In contrast to ^{125}I-ASOR/HRP containing vesicles, ^{131}I-transferrin containing vesicles did not increase in density upon prolonged incubation periods at 37°C. After 10 min at 37°C a significant portion had already completed the endocytotic cycle and was released from the cells, presumably as apotransferrin. This can be concluded from the decreased total amount of label present at a density of 1.045 g/ml compared with earlier time points. At each time point the ^{125}I-ASOR/HRP-containing vesicles could be shifted towards densities of ~1.09 g/ml after DAB incubation (Fig. 4, right panels). To quantitate the density shift of ^{125}I-ASOR/HRP-containing vesicles, the ratio of shifted and total endosomal ^{125}I-ASOR/HRP radioactivity was calculated and used as a measure for calculating

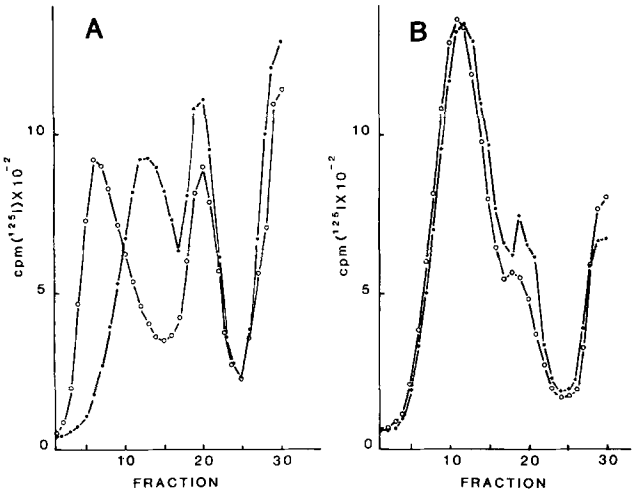

Figure 3. The effect of DAB cytochemistry on the density distribution of ^{125}I-ASOR/HRP- and ^{125}I-ASOR-containing vesicles. Tissue culture dishes (9 cm) were treated as described in Fig. 1. Subsequently the cells were incubated for 60 min at 0°C in medium containing 4 μg/ml ^{125}I-ASOR/HRP (*A*), or 2 μg/ml ^{125}I-ASOR (*B*). After the nonbound ligand was washed away, the cells were incubated for 2 min (*A*) or 5 min (*B*) at 37°C before homogenization. Half of the postnuclear supernatant was incubated with (*open circle*), and half without (*solid circle*) DAB before fractionation. The densities in the gradient were as in Fig. 2.

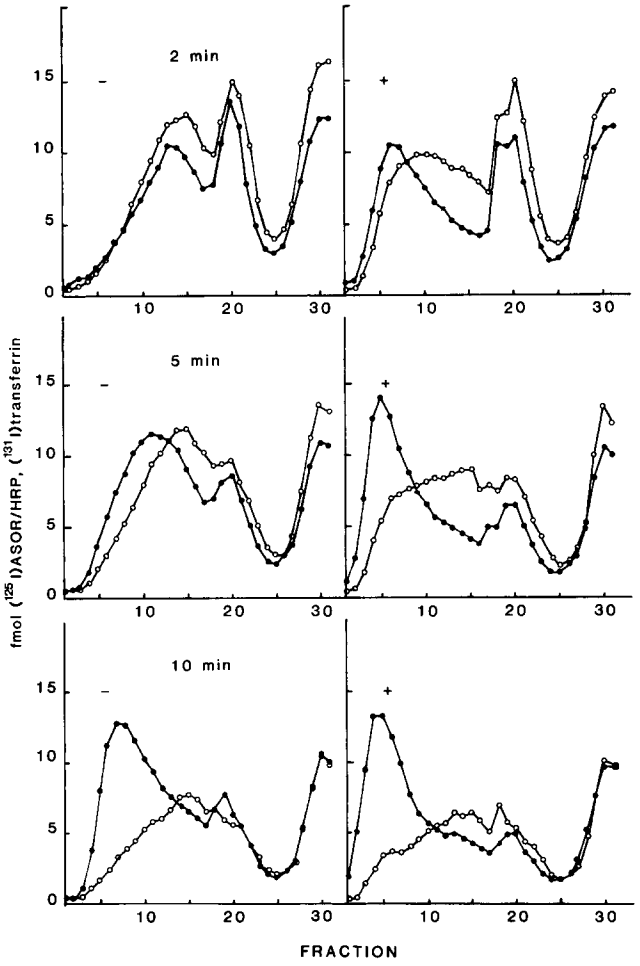

Figure 4. Sorting of ^{125}I-ASOR/HRP and ^{131}I-transferrin during single cycle endocytosis. Tissue culture dishes (9 cm) were treated as described in Fig. 1 to deplete cell surface receptors from their ligands. Subsequently, the cells were incubated for 60 min at 0°C in medium containing 4 μg/ml of both ^{125}I-ASOR/HRP (*solid circle*) and ^{125}I-transferrin (*open circle*). Excess of ligands was washed away and the cells were incubated for 2, 5, or 10 min at 37°C. Equal aliquots of the postnuclear supernatant were incubated with (+), or without (−) DAB before fractionation. The densities in the gradient were as in Fig. 2.

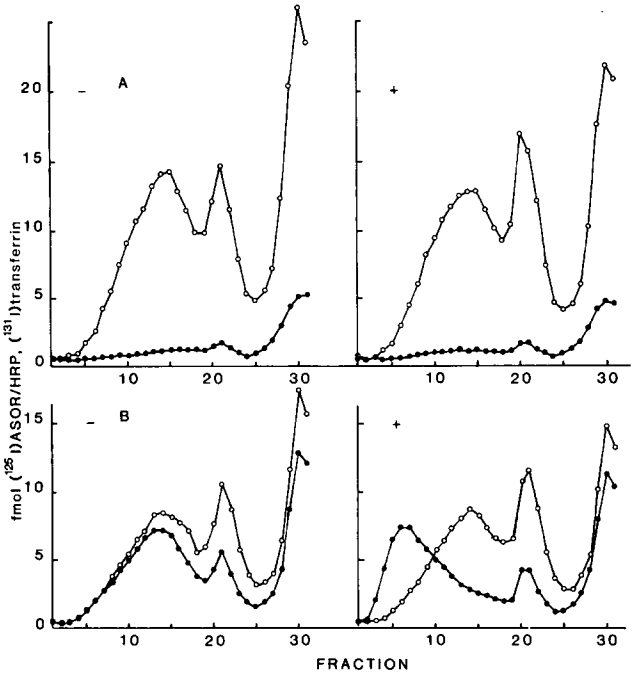

Figure 5. Specificity of the DAB-induced density shift procedure. (*A*) A 9-cm culture dish was treated as described in Fig. 4, except that besides ^{125}I-ASOR/HRP (*solid circle*) and ^{131}I-transferrin (*open circle*), 400 μg/ml ASOR was also present during ligand binding at 0°C to prevent specific ^{125}I-ASOR/HRP binding. The cells were incubated for 2 min at 37°C before fractionation. (*B*) Two culture dishes (6 cm) were treated as described in Fig. 4. One culture dish was incubated in medium supplemented with 4 μg/ml ^{125}I-ASOR/HRP, and the other one in medium with 4 μg/ml ^{131}I-transferrin for 1 h at 0°C. Excess of ligands were removed and the cells were incubated for 2 min at 37°C. The two cell cultures were scraped, pooled, and homogenized together. The distribution of ^{125}I-ASOR/HRP (*solid circle*), and ^{131}I-transferrin (*open circle*) was measured after incubation with (+) or without (−) DAB and Percoll gradient fractionation. The densities in the gradients were as in Fig. 2.

the ^{131}I-transferrin shift. The degree of ^{131}I-transferrin shift after 2, 5, and 10 min incubation at 37°C was similarly determined, and is given as a percentage of the degree of shift of ^{125}I-ASOR/HRP at 2 min. After 2 min at 37°C, 60% of the intracellular ^{131}I-transferrin coshifted with ^{125}I-ASOR/HRP to denser fractions, implicating a localization of most endocytosed ^{131}I-transferrin in vesicles in which ASOR/HRP was also present. After 5 min, 40% of internalized ^{131}I-transferrin shifted together with ^{125}I-ASOR/HRP, and after 10 min only 20% ^{131}I-transferrin shifted towards denser fractions. These data show that after internalization, most, if not all, ^{131}I-transferrin initially resides in ASOR/HRP-containing vesicles, and that the two ligands are sorted with a half-time of ~2 min, after warming to 37°C.

To ascertain that nonspecifically bound ASOR/HRP did not interfere with the density shift of endocytosed ^{131}I-transferrin, a control experiment was performed in which an excess of nonlabeled ASOR was added to the binding medium to prevent receptor binding of ^{125}I-ASOR/HRP. After 2 min of incubation of prelabeled cells at 37°C, almost no ^{125}I-ASOR/HRP was endocytosed. Only very little ^{125}I-label was recovered in membrane fractions after Percoll gradient fractionation (Fig. 5 *A*). Under these conditions no endocytosed ^{131}I-transferrin shifted after incubation of the homogenate with DAB (Fig. 5 *A*, right panel).

To show that the density of ^{131}I-transferrin-containing vesicles was not influenced by the presence of ASOR/HRP in different vesicles during DAB cytochemistry, a mixing experiment was performed. Two cell culture plates were labeled with ^{125}I-ASOR/HRP or ^{131}I-transferrin, respectively, and incubated for 2 min at 37°C. The cells were scraped, combined, homogenized, and DAB cytochemistry was performed. No ^{131}I-transferrin could be shifted to a higher density upon DAB incubation, indicating that intravesicular colocalization of the two ligands was an absolute requirement for co-shifting. We conclude that sorting between receptor-mediated endocytosed transferrin and ASOR/HRP is an intracellular event, occuring within minutes after internalization.

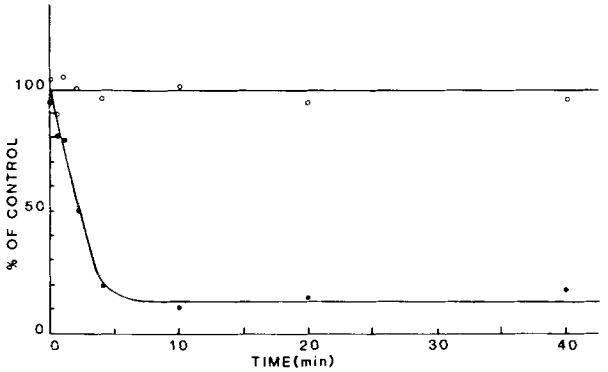

Figure 6. The effect of primaquine on transferrin receptor recycling. Culture dishes (35 mm) were incubated at 37°C for various periods of time in medium supplemented with 40 μg/ml unlabeled transferrin with (*solid circle*), or without 0.3 mM primaquine (*open circle*). The cell surface-bound transferrin was removed at 0°C by incubating the cells for 10 min at pH 4.5 (25 mM Na acetate, pH 4.5, 150 mM NaCl, 50 μM desferrioxamine, 5 mM $CaCl_2$), and 10 min at pH 7.2 in binding medium, respectively. Then the amount of surface transferrin receptors was measured by incubating the cells in the presence of 4 μg/ml ^{125}I-transferrin at 0°C. Nonspecific binding, determined in the presence of 100-fold excess unlabeled transferrin, was subtracted from all values. The specific binding is plotted in a percentage of the binding sites present at the beginning of the incubation period.

Primaquine Blocks Transferrin Receptor Recycling

Primaquine, like other weak bases, raises the pH of acidic subcellular compartments such as endosomes and lysosomes. The acidic environment is likely to be a prerequisite for receptor–ligand dissociation and lysosomal degradation. In HepG2 cells both ASGP receptor recycling and ASGP lysosomal degradation are blocked in the presence of 0.3 mM primaquine (Schwartz et al., 1984*a*; Strous et al., 1985).

To study the effect of primaquine on the recycling of the transferrin receptor, cells were incubated in the presence or absence of 0.3 mM primaquine and in the presence of a saturating concentration of nonlabeled transferrin for various periods of time at 37°C. Transferrin was added to the incubation medium to eliminate possible differences in transferrin saturation of surface receptors as a consequence of the effect of primaquine. After this preconditioning, cell surface receptors were cleared from transferrin at 0°C by alternate incubations at pH 4.5 and pH 7.2. This procedure removes nearly all plasma membrane–bound transferrin (Ciechanover et al., 1983*a*). Subsequently, the number of specific transferrin binding sites was determined at 0°C. The total plasma membrane–located receptor population decreased to 50% within 2 min to reach a plateau of 15% after 6 min (Fig. 6). This shows that in the presence of 0.3 mM primaquine, transferrin receptors rapidly enter the cell, but do not return to the plasma membrane.

The Effect of Primaquine on the Sorting of Transferrin and ASOR/HRP

Since primaquine blocks the recycling of transferrin and ASGP receptors, as well as the dissociation of ASGP from its receptor, we have also tested the effect of primaquine on the sorting of the two ligands. If 0.3 mM primaquine was present during a 10-min incubation of cells at 37°C, prelabeled with ^{131}I-transferrin and ^{125}I-ASOR/HRP at 0°C, a broad endosomal peak with a mean density of 1.05 g/ml was observed for both labeled ligands after Percoll density gradient fractionation (Fig. 7 *A*). The primaquine-induced endosomal swelling, as determined by morphological studies (Geuze et al., 1984), is a possible explanation for the altered density distribution of ligand-containing vesicles in the Percoll gradient. DAB treatment of the homogenate resulted in a density shift of ^{125}I-ASOR/HRP to a density comparable to experiments in which no primaquine was used (Fig. 7 *B*). In addition, all ^{131}I-transferrin exhibited a similar density distribution upon DAB cytochemistry. Our conclusion is that the ^{131}I-transferrin is localized in ASOR/HRP–containing vesicles, at least up to 10 min at 37°C in the presence of primaquine.

Discussion

Our results show that transferrin is sorted from ASGPs within minutes after internalization. The DAB density shift procedure used to monitor this process is a modification of the method originally developed by Courtoy et al. (1984). Plasma membranes, endosomes, and density-shifted material labeled with ^{125}I-ASOR/HRP were separated with a very high reproducibility by single-step Percoll gradient centrifugation. A shift in density of ^{131}I-transferrin–containing vesicles upon DAB cytochemistry indicated a colocalization with ASOR/HRP. If prelabeled cells were incubated for 2 min at 37°C, the density shift after DAB treatment of ^{131}I-transferrin–containing vesicles did not entirely equal that of ^{125}I-ASOR/HRP. An explanation for this phenomenon is that the observed peak of ^{131}I-transferrin after DAB cytochemistry is composed of ^{131}I-transferrin–containing vesicles with and without ASOR/HRP. The percentages of sorted cell-associated ^{131}I-transferrin are 60, 40, and 20% of total radioactive transferrin present intracellularly after 2, 5, and 10 min. Taking into account that the half-time of secretion of endocytosed transferrin is ~5 min (Ciechanover et al.,

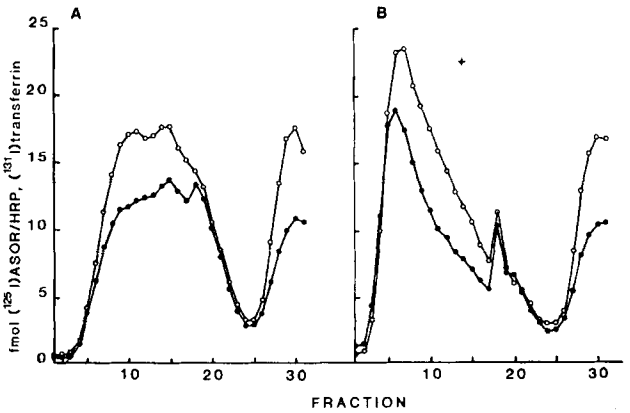

Figure 7. The effect of primaquine on the sorting of ^{131}I-transferrin and ^{125}I-ASOR/HRP. A 9-cm culture dish was treated exactly as described in Fig. 4, except that after binding of ^{125}I-ASOR/HRP (*solid circle*) and ^{131}I-transferrin (*open circle*) the cells were incubated at 37°C for 10 min in the presence of primaquine. Equal aliquots of the postnuclear supernatant were incubated with (+) or without (−) DAB before fractionation. The densities in the gradients were as in Fig. 2.

1983*a*), it can be calculated that prebound transferrin and ASOR are sorted with a half-time of 2 min.

The mean time of internalization at 37°C in HepG2 of prebound ^{125}I-ASOR is 2.2 min (Schwartz et al., 1982). The half-time of internalization of surface-bound transferrin is reported to be 3.5 min (Ciechanover et al., 1983*a*). Both receptors have nearly similar internalization kinetics (Ciechanover et al., 1983*b*). Taken together with our results, it can be concluded that both ligands are sorted immediately after internalization. Using Percoll gradient cell fractionation, we found distributions of both ligands between plasma membranes and intracellular locations that are fully in agreement with the data mentioned above. Our results clearly show that the divergence in routing of transferrin and ASGP occurs intracellularly and not at the plasma membrane. In isolated rat hepatocytes (Bridges et al., 1982) and HepG2 cells (Simmons and Schwartz, 1984) ASGPs remain associated with their receptor for several minutes during the process of internalization, as demonstrated by ammonium sulphate precipitation and saponin permeabilization, respectively. Thus, sorting between ASGPs and their receptor and between ASGPs and transferrin occurs within the same time span, and, therefore, possibly in the same compartment. Using double-label immunoelectron microscopy, Geuze et al. (1983*a*) demonstrated in rat liver that sorting of ASGP and its receptor occurs in a prelysosomal tubulovesicular network, termed CURL. Therefore, CURL is indeed the most likely candidate for transferrin-ASGP sorting. This, however, still needs morphological confirmation.

At 37°C, prebound transferrin is secreted, with a half-time of 5 min, as apotransferrin into the medium (Ciechanover et al., 1983*a*). Electron microscopic studies demonstrate the appearance of ASGP in lysosomes as early as 5 min after internalization at 37°C, whereas the majority of ligand is localized in secondary lysosomes after 15 min in both HepG2 (Geuze et al., 1983*b*) and rat hepatocytes (Wall et al., 1980; Geuze et al., 1983*a*). These data are in agreement with our results, which show that after 10 min at 37°C the total amount of cell-bound ^{131}I-transferrin decreased and the intracellular transferrin retained its localization in the gradient, whereas the endocytosed ^{125}I-ASOR/HRP migrated to heavier fractions, compared with earlier time points.

Both ASGP and transferrin routing are not unidirectional processes. In both rat hepatocytes (Weigel and Oka, 1984) and HepG2 cells (Simmons and Schwartz, 1984), a large slowly dissociating pool of endocytosed ligand–receptor complexes returns to the cell surface. In HepG2 50% of initially internalized ASGP returns receptor-bound to the cell surface in a half-time of 24 min. Up to 28% of internalized ^{125}I-ASOR returns undegraded non-receptor-bound to the incubation medium in a half-time of 84 min. The intracellular route of the transferrin receptor complex is also not unidirectional. Several data indicate that at least part of this complex recycles through Golgi compartments. Morphologically, surface-labeled transferrin receptor has been shown capable of entering the Golgi complex (Hopkins, 1983; Willingham et al., 1984; Woods et al., 1986). Resialylation of surface-desialylated transferrin receptor occurs in a halftime of 2 h (Snider and Rogers, 1985), implicating receptor passage through a sialyltransferase-containing compartment. A possible involvement of the Golgi in transferrin receptor recycling is likely to be only partial because of the low efficiency of resialylation of the transferrin receptor as observed by Snider and Rogers (1985). Our data on the rapid sorting between transferrin receptors and ASGPs are in agreement with the receptor recycling kinetics reported by Ciechanover et al. (1983*a*). Since this process is almost completed after 10 min at 37°C (Fig. 4) it is not likely that transferrin recycling occurs through the different ASGP routes referred to above. However, the possibility that identical routes, albeit at different rates, are followed cannot be excluded.

The effect of lysomotropic agents on membrane flow has been reviewed (Dean et al., 1984). They inhibit lysosomal protein degradation (Carpentier and Cohen, 1976; Wibo and Poole, 1974) and neutralize the acidic environment of lysosomes and endosomes (Maxfield, 1982; Ohkuma and Poole, 1978; Poole and Ohkuma, 1981; Tycko et al., 1983). Recently the dose-response characteristics of the lysomotropic agent primaquine on ASGP receptor recycling in HepG2 cells has been published (Strous et al., 1985; Schwartz et al., 1984*a*). Receptor recycling is completely blocked in the presence of 0.3 mM primaquine. Similar effects have been reported for the ASGP receptor in rat hepatocytes (Tolleshaug and Berg, 1979; Berg and Tolleshaug, 1980). The effect of weak bases on the recycling of the transferrin receptor is not clear. In K562 cells up to 0.1 mM chloroquine did not affect transferrin recycling (Stein et al., 1984), whereas ammonium chloride has been demonstrated to slow down transferrin receptor recycling in HepG2 cells (Ciechanover et al., 1983*a*). Our results show that transferrin receptor recycling is inhibited in the presence of 0.3 mM primaquine (Fig. 6). The amount of surface binding sites decreased to 15% in 6 min at 37°C. In 2 min the plasma membrane receptor population was reduced to 50% of its original value. This is within the half-time of 3.5 min determined for transferrin uptake in non-primaquine-treated HepG2 cells (Ciechanover et al., 1983*a*), and implies that 0.3 mM primaquine does not impede transferrin receptor internalization. After 10 min of internalization of prebound ^{125}I-ASOR/HRP and ^{131}I-transferrin in the presence of 0.3 mM primaquine no sorting was found at all (Fig. 7), whereas under conditions without primaquine almost total sorting was observed (Fig. 3). This strongly supports the notion that an acidic environment is a prerequisite for sorting.

We would like to thank Dr. Arie van der Ende for many suggestions. We are grateful to Tom van Rijn for help with the drawings.

The investigations were supported in part by a grant from the Foundation for Medical Research, MEDIGON, The Netherlands (900-053-061).

Received for publication 1 October 1986, and in revised form 22 December 1986.

References

Berg, T., and H. Tolleshaug. 1980. The effects of ammonium ions and chloroquine on uptake and degradation of ^{125}-I labeled asialofetuin in isolated rat hepatocytes. *Biochem. Pharmacol.* 29:917–925.

Breitfeld, P. P., C. F. Simmons, Jr., G. J. Strous, H. J. Geuze, and A. L. Schwartz. 1985. Cell biology of the asialoglycoprotein system: a model of receptor-mediated endocytosis. *Int. Rev. Cytol.* 97:47–95.

Bridges, K., J. Harford, G. Ashwell, and R. D. Klausner. 1982. Fate of receptor and ligand during endocytosis of asialoglycoproteins by isolated hepatocytes. *Proc. Natl. Acad. Sci. USA.* 79:350–354.

Carpentier, G., and S. Cohen. 1976. ^{125}I-labeled human epidermal growth factor. Binding, internalization, and degradation in human fibroblasts. *J. Cell Biol.* 71:159–171.

Ciechanover, A., A. L. Schwartz, A. Dautry-Varsat, and H. F. Lodish. 1983*a*. Kinetics of internalization and recycling of transferrin and the transferrin

receptor in a human hepatoma cell line: effect of lysosomotropic agents. *J. Biol. Chem.* 258:9681–9689.

Ciechanover, A. J., A. L. Schwartz, and H. F. Lodish. 1983*b*. The asialoglycoprotein receptor internalizes and recycles independently of the receptors for transferrin and insulin receptors. *Cell* 32:267–275.

Courtoy, P. J., J. Quintart, and P. Baudhuin. 1984. Shift of equilibrium density induced by 3,3′-diaminobenzidine cytochemistry: a new procedure for the analysis and purification of peroxidase-containing organelles. *J. Cell. Biol.* 98:870–877.

Courtoy, P. J., J. Quintart, J. N. Limet, C. deRoe, and P. Baudhuin. 1985. Polymeric IgA and galactose-specific pathways in rat hepatocytes: evidence for intracellular ligand sorting. *In* Endocytosis. I. Pastan, and M. C. Willingham, editors. Plenum Publishing Corp., New York. 163–188.

Dautry-Varsat, A., A. Ciechanover, and H. F. Lodish. 1983. pH and the recycling of transferrin during receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA.* 80:2258–2262.

Dean, R. T., W. Jessup, and C. R. Roberts. 1984. Effect of exogenous amines on mammalian cells with particular reference to membrane flow. *Biochem. J.* 217:27–40.

Geuze, H. J., J. W. Slot, G. J. Strous, H. F. Lodish, and A. L. Schwartz. 1983*a*. Intracellular site of asialoglycoprotein receptor ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. *Cell.* 32:277–287.

Geuze, H. J., J. W. Slot, G. J. Strous, and A. L. Schwartz. 1983*b*. The pathway of the asialoglycoprotein-ligand during receptor-mediated endocytosis: a morphological study with colloidal gold/ligand in the human hepatoma cell line, HepG2. *Eur. J. Cell Biol.* 32:38–44.

Geuze, H. J., J. W. Slot, G. J. Strous, J. Peppard, K. Von Figura, A. Hasilik, and A. L. Schwartz. 1984. Intracellular receptor sorting during endocytosis: comparative immunoelectron microscopy of multiple receptors in rat liver. *Cell.* 37:195–204.

Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russell, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Ann. Rev. Cell Biol.* 1:1–39.

Hopkins, C. R. 1983. Intracellular routing of transferrin and transferrin receptors in epidermoid carcinoma A-431 cells. *Cell.* 35:321–330.

Klausner, R. D., G. Ashwell, J. Van Renswoude, J. B. Harford, and K. R. Bridges. 1983. Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. *Proc. Natl. Acad. Sci. USA.* 80:2263–2266.

Knowles, B. B., C. C. Howe, and D. P. Aden. 1980. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science (Wash. DC).* 209:497–499.

Limet, J. N., Y. J. Schneider, J. P. Vaerman, and A. Trouet. 1982. Binding, uptake and intracellular processing of polymeric rat immunoglobulin A by cultured rat hepatocytes. *Eur. J. Biochem.* 125:437–443.

Maxfield, F. R. 1982. Weak bases and ionophores rapidly and reversibly raise the pH of endocytic vesicles in cultured mouse fibroblasts. *J. Cell Biol.* 95:676–681.

Nakane, P. K., and A. Kawaoi. 1974. Peroxidase-labeled antibody: a new method of conjugation. *J. Histochem. Cytochem.* 22:1084–1091.

Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurements of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327–3331.

Poole, B., and S. Ohkuma. 1981. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. *J. Cell Biol.* 90:665–669.

Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* 51:660–672.

Schwartz, A. L., D. Rup, and H. F. Lodish. 1980. Difficulties in the quantification of asialoglycoprotein receptors on the rat hepatocyte. *J. Biol. Chem.* 255:9033–9036.

Schwartz, A. L., S. E. Fridovich, B. B. Knowles, and H. F. Lodish. 1981. Characterization of the asialoglycoprotein receptor in a continuous hepatoma line. *J. Biol. Chem.* 256:8878–8881.

Schwartz, A. L., S. E. Fridovich, and H. F. Lodish. 1982. Kinetics of internalization and recycling of the asialoglycoprotein receptor in a hepatoma cell line. *J. Biol. Chem.* 257:4230–4237.

Schwartz, A. L., A. Bolognesi, and S. E. Fridovich. 1984*a*. Recycling of the asialoglycoprotein receptor and the effect of lysosomotropic amines in hepatoma cells. *J. Cell Biol.* 98:732–738.

Schwartz, A. L. 1984*b*. The hepatic asialoglycoprotein receptor. *CRC Crit. Rev. Biochem.* 16:207–233.

Simmons, C. F., and A. L. Schwartz. 1984. Cellular pathways of galactose-terminal ligand movement in a cloned human hepatoma cell line. *Mol. Pharmacol.* 26:509–519.

Snider, M. D., and O. C. Rogers. 1985. Intracellular movement of cell surface receptors after endocytosis: resialylation of asialotransferrin receptor in human erythroleukemia cells. *J. Cell Biol.* 100:826–834.

Solari, R., and J. P. Kraehenbuhl. 1984. Biosynthesis of the IgA antibody receptor: a model for the transepithelial sorting of a membrane glycoprotein. *Cell.* 36:61–71.

Stein, B. S., K. G. Bensch, and H. H. Sussman. 1984. Complete inhibition of transferrin recycling by monensin in K562 cells. *J. Biol. Chem.* 259: 14762–14772.

Strous, G. J., A. DuMaine, J. E. Zijderhand-Bleekemolen, J. W. Slot, and A. L. Schwartz. 1985. Effect of lysosomotropic amines on the secretory pathway and on the recycling of the asialoglycoprotein receptor in human hepatoma cells. *J. Cell Biol.* 101:531–539.

Tolleshaug, H., and T. Berg. 1979. Chloroquine reduces the number of asialoglycoprotein receptors in the hepatocyte plasma membrane. *Biochem. Pharmacol.* 28:2919–2922.

Tycko, B., C. H. Keith, and F. R. Maxfield. 1983. Rapid acidification of endocytic vesicles containing asialoglycoprotein in cells of a human hepatoma line. *Cell Biol. Int. Rep.* 97:1762–1776.

Wall, D. A., G. Wilson, and A. L. Hubbard. 1980. The galactose-specific recognition system of mammalian liver: the route of ligand internalization in rat hepatocytes. *Cell.* 21:79–93.

Warren, T. L. 1959. The thiobarbituric acid assay of sialic acids. *J. Biol. Chem.* 234:1971–1975.

Weigel, P. H., and J. A. Oka. 1984. Recycling of the hepatic asialoglycoprotein receptor in isolated rat hepatocytes. Receptor-ligand complexes in an intracellular slowly dissociating pool return to cell surface prior to dissociation. *J. Biol. Chem.* 259:1150–1154.

Wibo, M., and B. Poole. 1974. Protein degradation in cultured cells. II. Uptake of chloroquine by rat fibroblasts and the inhibition of cellular protein degradation and cathepsin. *J. Cell Biol.* 63:430–440.

Willingham, M. C., J. A. Hanover, R. B. Dickson, and I. Pastan. 1984. Morphologic characterization of the pathway of transferrin endocytosis and recycling in human KB cells. *Proc. Natl. Acad. Sci. USA.* 81:175–179.

Woods, J. W., M. Doriaux, and M. G. Farquhar. 1986. Transferrin receptors recycle to cis and middle as well as trans Golgi cisternae in Ig-secreting myeloma cells. *J. Cell Biol.* 103:277–286.