Intracellular Segregation of Asialoglycoproteins and Their Receptor: A Prelysosomal Event Subsequent to Dissociation of the Ligand-Receptor Complex

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ABSTRACT Rat hepatocytes in monolayer culture rapidly internalized asialoglycoproteins and the receptors to which they are bound. Subsequent to endocytosis, the receptor-ligand complex is dissociated within an acidic endosome (Harford, J., K. Bridges, G. Ashwell, and R. D. Klausner, 1983, *J. Biol. Chem.* 258:3191–3197; Harford, J., A. W. Wolkoff, G. Ashwell, and R. D. Klausner, 1983, *J. Cell Biol.* 96:1824–1828). Here we show that addition of the proton ionophore monensin to the cells after dissociation has occurred results in intracellular rebinding of ligand molecules. With increasing time inside the cell, the ability of ligand to reassociate with receptor progressively decreases consistent with a segregation of receptor and ligand. The combination of colchicine and cytochalasin B appears to retard the process of segregation. In contrast, removal of sodium from the medium, while inhibiting degradation of ligand, does not affect the decrease in monensin-mediated rebinding. Nonetheless, both sodium deprivation and treatment with colchicine plus cytochalasin B result in the ligand remaining in a low density, nonlysosomal subcellular fraction. Thus, segregation, like dissociation, appears to occur in a pre-lysosomal endocytic compartment. Perturbation of the endocytic pathway by reduced temperature (18°C) was also explored. Our data are consistent with two temperature-sensitive steps: receptor-ligand dissociation is inhibited and there is an independent temperature-sensitive step involved in delivery of ligand to lysosomes. This second effect was localized as being beyond the point in the pathway sensitive to sodium deprivation.

Receptor-mediated endocytosis is a process common to many species and cell types (1, 2). In one of the best characterized receptor-ligand systems, the hepatocyte receptor for desialylated glycoproteins (3, 4), interaction of an asialoglycoprotein with its cell-surface receptor is followed by internalization of the ligand-receptor complex (5, 6). Subsequently, ligand and receptor separate; the receptor returns to the cell surface while the ligand enters lysosomes in which it is degraded. Recent studies have demonstrated that dissociation of the receptor-ligand complex is mediated by acidification within a prelysosomal endocytic vesicle (5, 7). Binding of asialoglycoproteins to the purified receptor has a similar pH sensitivity (8). Intracellular dissociation of the receptor-asialoglycoprotein complex appears to be obligatory for entry of ligand into lysosomes and for receptor reutilization (7, 9).

Although it is clear that reduced pH mediates the dissociation of ligand from receptor, segregation of the two into separate cellular compartments is a distinct phenomenon. The mechanism for directing ligand to the lysosomes and receptor back to the plasma membrane remains obscure. In the present study, we examined the effects of several perturbants on the process of endocytosis of asialoglycoproteins. These perturbations include incubation of cells at reduced temperature, removal of sodium from the incubation medium, and exposure of cells to a combination of colchicine and cytochalasin B. Based upon localization of the effects of these treatments within the pathway, we propose that these manipulations provide tools for the analysis and resolution of the process of endocytosis and, most significantly, a biochemical assay for the segregation of ligand and receptor.

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MATERIALS AND METHODS

Short-term Hepatocyte Culture: Rat hepatocytes were isolated and cultured in monolayer at densities of 1–3 × 10^6/60 mm dish (7), and were used for studies between 16 and 24 h after plating. Before incubation with radiolabeled ligand, cultures were washed twice with 1.5 ml of modified serum-free medium and incubated at 37°C for 1 h in 3 ml of this medium. The modified medium contained either sodium or potassium and was composed of the following: NaCl or KCl, 0.132 M; 0.59 mM of NaH_2PO_4 or KH_2PO_4; 2.12 mM Na_2HPO_4 or K_2HPO_4; 1.2 mM $MgCl_2$; 0.81 mM $MgSO_4$; 27.8 mM glucose; 2.5 mM $CaCl_2$; 25 mM of HEPES. Where indicated, colchicine and cytochalasin B (both of Sigma Chemical Co., St. Louis, MO) were added as 1,000 × stock solution in ethanol to final concentrations of 40 μg/ml and 10 μM respectively.

Studies of the Uptake and Degradation of ^{125}I-Asialo-orosomucoid: Asialo-orosomucoid (ASOR)[1] was radiolabeled with ^{125}I by a chloramine-T method (10). In all studies, 1 μg of ^{125}I-ASOR was added to hepatocyte monolayers in 1 ml of ice-cold serum-free medium. Nonspecific binding was assessed in cultures to which 100 μg of unlabeled ASOR was added to the incubation. The cells were incubated at 4°C for 60 min. Unbound ligand was removed by four washes with 1.5 ml of cold medium. The third of these washes contained 0.5 mM *N*-acetylgalactosamine (GalNAc) as previously described (5). The washed, surface-labeled hepatocytes were incubated at 37°C or, in studies on the effect of temperature, at 18°C or 23°C. Degradation of ligand was quantitated as radioactivity remaining soluble upon addition of aliquots of the incubation media to equal volumes of 20% trichloroacetic acid, 4% phosphotungstic acid. After two washes of the cell monolayers with 1.5 ml of cold medium, surface-bound ligand was quantitated as radioactivity released by incubation of cells at 4°C with 20 mM EGTA in 0.15 M NaCl, 0.02 M Tris-Cl, pH 7.6. After this removal of the residual surface-bound ligand, cells were washed twice with 1.5 ml of cold 0.28 M sucrose, 2 mM $CaCl_2$, 0.01 M Tris-Cl, pH 7.6. Cell monolayers were then subjected to the solubilization-precipitation assay shown previously to distinguish between ligand bound to the receptor and that which had dissociated from the receptor (5). Briefly, cells were solubilized by addition of 1 ml/dish of an ice-cold solution containing 1% Triton X-100, 1 μg/ml of unlabeled ASOR, 50 mM $CaCl_2$, 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.6. The dishes were scraped with a rubber policeman and the contents added to a 1 ml of saturated ammonium sulfate (adjusted with Tris to pH 7.6) at 25°C. The precipitated material was filtered onto GF/C filters (Whatman Inc., Clifton, NJ) and washed twice with 1.5 ml of 45% saturated ammonium sulfate containing 20 mM $CaCl_2$. The amounts of radioactivity associated with the filters and with the filtrate plus washers were determined.

To eliminate variability due to differences in number of cells plated, plating efficiency, or recovery the data for cell surface ligand and degraded ligand, were expressed as percentages of the total radioactivity in the dish. Similarly, internal receptor-bound ligand was expressed as a percentage of the total internalized ligand in each dish. In all cases, data represented averages of duplicate culture dishes and nonspecific radioactivity obtained in analogous determinations using culture dishes to which a 100-fold excess of unlabeled ASOR had been added have been subtracted in the calculations. By normalization for content of radioactivity, duplicate dishes generally differed from one another by <10%. Nonspecific cell-associated radioactivity was found to be generally <10% of the total cell-associated ligand.

Percoll Gradient Fractionation of Hepatocytes: Hepatocytes were fractionated on Percoll density gradients as described (7, 9). Briefly, cells were homogenized in 0.28 M sucrose, 2 mM $CaCl_2$, 0.01 M Tris-Cl, pH 7.6 by 20 up and down strokes in a tight Dounce homogenizer. The homogenates were centrifuged at 280 *g* for 10 min and the supernatants made 20% in Percoll (Pharmacia Fine Chemicals, Piscataway, NJ). These mixtures were centrifuged at 10,000 *g* in a Beckman 65 rotor to a final $\omega^2 t$ value of 4.55 × 10^9. The resultant gradients were fractionated and radioactivity in each fraction was determined. The lysosomal enzyme acid β-hexosaminidase was measured as previously described (7). Under all conditions used in this paper, all of the detectable hexosaminidase activity was found to reside in the bottom ten fractions of the Percoll gradients.

RESULTS

Internalization and Degradation of ^{125}I-ASOR under Control Conditions

Warming monolayers of cultured hepatocytes whose surface asialoglycoprotein receptors had been occupied at 4°C by ^{125}I-ASOR resulted in rapid internalization of ligand-receptor complex. During 15 min at 37°C, ~80% of the pre-bound ligand moved into the cell, as judged by resistance to removal by 20 mM EGTA (7). After internalization, ^{125}I-ASOR rapidly dissociated from its receptor as a consequence of endocytic vesicle acidification (7, 9). After 15 min of warming, ~60% of the internalized ligand was no longer receptor-bound. Degradation of ^{125}I-ASOR to acid-soluble products became evident only after 30 min at 37°C, with the majority of cell-associated ligand being degraded in 2 h. The kinetics of ligand movement from the cell surface to lysosomes including intracellular dissociation of ligand-receptor complex have been described in detail elsewhere (7). The proton ionophore, monensin, inhibits the process of ligand dissociation within the cell (9). For receptor reutilization to occur concomitant with ligand degradation, this dissociation step must necessarily be followed by separation of ligand and receptor into different cellular compartments. If unoccupied receptor and dissociation ligand coexist in an acidic endocytic vesicle, neutralization of this compartment by addition of monensin might be expected to result in reassociation of receptor and ligand. Indeed, we found that addition of monensin did result in apparent rebinding of intracellular dissociated ligand. Moreover, the extent to which the monensin-induced reassociation occurred was dependent upon the timing of the ionophore addition after warming of the cultured cells to 37°C (Fig. 1*A*). Shortly after intracellular dissociation, the addition of monensin led to a rapid and nearly complete rebinding of ligand to receptor. However, the percentage of released, undegraded ligand that is capable of rebinding to receptor decreased with time. We interpret this decrease as indicative of a physical segregation of ligand and receptor soon after the pH-mediated intracellular dissociation.

Effects of Colchicine/Cytochalasin B on Internalization and Degradation of ^{125}I-ASOR

When either colchicine (40 μg/ml) or cytochalasin B (10 μM) was added to the incubation media, there was little if any effect on binding of ^{125}I-ASOR or on internalization of pre-bound ligand and only a minimal reduction in the dissociation of internalized ligand from receptor. After 60 min at 37°C, ~60% of internalized ^{125}I-ASOR had been released in the presence of either drug as compared to ~75% in control cultures. However, ligand degradation was reduced ~50% by each of the drugs. When a mixture of colchicine and cytochalasin B was added to hepatocytes, the extents of internalization and release were unchanged from that seen with either drug alone, but there was virtually no degradation of internalized ligand (Fig. 2). Kolset et al. (11) reported that both colchicine and cytochalasin B inhibit asialofetuin degradation in freshly isolated hepatocytes by impeding access of the protein to lysosomes. However, these investigators observed only partial inhibition even with 1 mM colchicine or 200 μM cytochalasin B. Moreover, no additive effect on degradation was found. We believe that these differences are due primarily to experimental design. Kolset et al. (11) exposed cells to ligand for 15 min at 37°C before addition of the drugs and further incubation at 37°C. Under their conditions a portion of the ligand may have progressed sufficiently along the pathway of endocytosis to be insensitive to these agents. In the present study, the drugs were added before warming the cells and consequently appear to be considerably more effec-

[1] *Abbreviations used in this paper:* ASOR, asialo-orosomucoid; GalNAc, *N*-acetylgalactosamine.

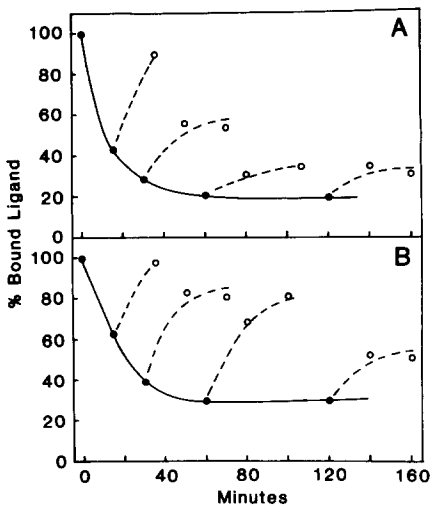

FIGURE 1 Rebinding of intracellular dissociated ligand upon addition of monensin: effect of colchicine and cytochalasin B. (*A*) The surface receptors of hepatocytes in monolayer culture were occupied by incubation with ^{125}I-ASOR (1 μg/ml) at 4°C, in sodium medium as described in Materials and Methods. After washing to remove unbound ligand the cells were warmed to 37°C for the indicated times. At 15, 30, 60, or 120 min, some of the cultures were made 50 μm in monensin (○) and the incubation was continued. Cells were washed in ice-cold medium including a wash with 20 mM EGTA to remove cell-surface ligand. Intracellular ^{125}I-ASOR was then subjected to the solubilization-precipitation assay described previously (5). The time course of intracellular dissociation without monensin addition is also shown (●). Data are expressed as the percentage of the intracellular ligand that is receptor-bound and represent the average of duplicate culture dishes. Nonspecific values determined in cultures to which a 100-fold excess of unlabeled asialo-orosomucoid was added have been subtracted in the calculation. (*B*) Experimental procedures were as in *A* except that colchicine (40 μg/ml) and cytochalasin B (10 μM) were included in the medium. At time zero under both conditions, ~25,000 cpm/dish of ^{125}I-ASOR were specifically cell associated. After 15 min in control medium or medium containing colchicine plus cytochalasin B, 73% and 67% respectively of these ligand molecules had been internalized.

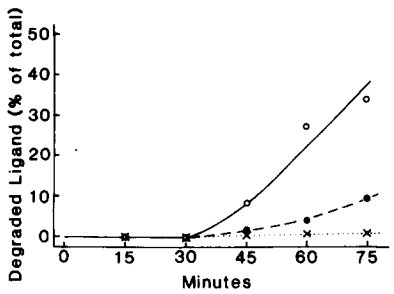

FIGURE 2 Effect of sodium replacement or the combination of colchicine and cytochalasin B on ^{125}I-ASOR degradation by cultured hepatocytes. Experimental design was similar to that described in Fig. 1. Cells were incubated at 37°C in sodium medium (○), sodium medium with colchicine (40 μg/ml) and cytochalasin B (10 μM) (x), or in potassium medium (●). Degradation of ligand was assessed as acid-soluble radioactivity in the medium. Values for total cell-associated ligand at time zero averaged 37,130 ± 4,730 cpm/dish. Data are expressed as percentage of the total ligand degraded and represent the average of duplicate determinations. Values obtained upon including a 100-fold excess of unlabeled asialo-orosomucoid have been subtracted.

tive. Furthermore, when the experiment was performed in this fashion, an additive effect of colchicine and cytochalasin B on degradation was observed; the mixture of cholchicine and cytocyclasin B inhibited degradation of ligand but had little effect on the preceding steps in the pathway of endocytosis. We therefore sought to determine whether these agents inhibited the physical segregation of ligand from receptor. The results, shown in Fig. 1 *B*, suggest a marked inhibition in the rate of ligand-receptor segregation. At 60 min, >70% of the released ligand rebinds to the receptor after addition of monensin as compared to <20% in control cells. It is clear that, whereas colchicine and cytochalasin B retard the process of segregation, they do not absolutely prevent it (Fig. 3). After 2 h at 37°C, only ~35% of the released ligand rebinds after the addition of monensin in colchicine/cytochalasin B–treated cultures. Identical results were obtained using 1 μg/ml of colchicine. The higher concentration was used because the loss of ability to reassociate with receptor was only slowed and not completely inhibited at 1 μg/ml. However, the nature of the inhibition was not altered by increasing the colchicine concentration to 40 μg/ml.

Effects of Substitution of Potassium for Sodium on Internalization and Degradation of ^{125}I-ASOR

Recently, Baenziger and Fiete (12) reported that endocytosis of asialoglycoproteins was altered by replacement of sodium with potassium in the incubation medium. Evidence was presented indicating that ligand did not enter lysosomes under these conditions. Having assays for several of the steps in the overall pathway of endocytosis, we sought to determine which step was sensitive to the ionic composition of the medium. Substitution of an equivalent molar amount of potassium for sodium in the incubation media resulted in a negligible reduction in internalization. The amount of internalized ligand after 15 min of warming in potassium medium was between 83% and 96% of control values. Moreover, only ~10% of specifically bound ^{125}I-ASOR remained on the cell surface at 60 min as compared to 5% in control medium. Release of internalized ligand from receptor was similar in the two media. However, degradation of ^{125}I-ASOR was reduced by >80% (Fig. 2). By these criteria, the "high K^+" (12) block appeared to be quite similar to inhibition by the combination of colchicine and cytochalasin B. However, the assay for monensin-induced rebinding revealed a clear distinction in their respective sites of action. After 60 min at 37°C in

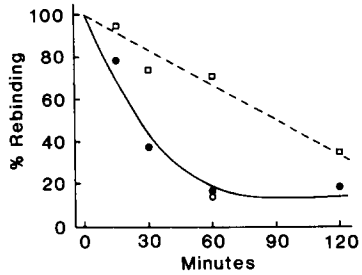

FIGURE 3 Time-dependent loss of the ability of ^{125}I-ASOR to reassociate with receptor following monensin addition. Data in this figure are derived from the experiment in Fig. 1 and are expressed as the percentage of the dissociated ligand that rebinds upon addition of 50 μM monensin at the indicated time. Incubation was in sodium medium (●), sodium medium containing colchicine and cytochalasin B (□), or potassium medium (○).

potassium medium, the addition of monensin produced A level of rebinding indistinguishable from that seen in sodium medium (~20%) and in sharp contrast to the high degree (~70%) of reassociation observed in cultures treated with colchicine and cytochalasin B (Fig. 3).

The Nature of the Potassium Inhibition

To determine whether the potassium effect was the result of inhibitory action of potassium or a requirement for sodium, we undertook the following experiments. Using ligand degradation as an end point, we titrated the concentration of potassium in a series of buffers containing reciprocally various amounts of sodium and potassium. The results, shown in Fig. 4*A*, demonstrate that at potassium concentrations >80 mM or sodium concentrations <55 mM there was a sharp decline in ligand degradation. Note that 30% of the control level of degradation was maintained, even in the medium containing only potassium. The inhibition of degradation was rapidly reversed by washing cells in sodium-containing medium, thus ruling out inhibition by cell death (see Table I). Furthermore, preincubation of hepatocytes for up to 2 h in sodium-free medium did not alter the subsequent ability of the cells to bind and internalize ligand or to acidify endosomes as judged by receptor-ligand dissociation. A sodium-free buffer in which sucrose iso-osmotically replaced all monovalent cations affected endocytosis in a manner identical to that of the sodium-free buffer containing potassium salts (Fig. 4*B*). The concentration of sodium required for 50% of maximal ligand degradation was ~20 mM. These results are most consistent with a requirement for sodium for this step in endocytosis rather than a direct inhibition by potassium. Replacement of sodium with either lithium or choline revealed that lithium salts were able to support nearly normal endocytosis and degradation

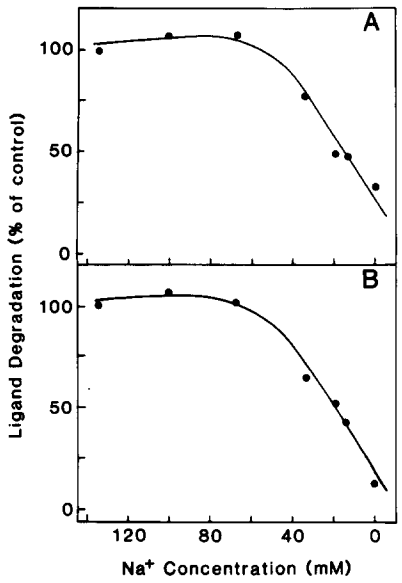

FIGURE 4 Dependence of ^{125}I-ASOR degradation upon sodium. Ligand degradation after 60 min at 37°C was assessed by determination of acid-soluble radioactivity in the media as described under Materials and Methods. Data are expressed as the percentage of degradation seen in sodium medium (135 mM Na^+) and represent the average of duplicate culture dishes. Isotonicity was maintained by replacement of sodium with potassium (*A*) or sucrose (*B*). In this experiment, degradation in sodium medium was 36% of the approximately 66,000 cpm/dish of ^{125}I-ASOR specifically cell-associated.

TABLE I

Effect of Reduced Temperature on Steps in the Endocytosis Pathway that Follow Segregation of Ligand and Receptor

Incubation 1 45 min		Incubation 2 60 min		
Temp	*Ion*	*Temp*	*Ion*	*% Degraded*
37°C	K^+	37°C	K^+	5.9
37°C	K^+	37°C	Na^+	21.6
37°C	K^+	18°C	K^+	1.5
37°C	K^+	18°C	Na^+	0.2

Cells were incubated for 45 min at 37°C in potassium medium (incubation 1) as described in the text. Under these conditions, ligand and receptor dissociate and segregate, but ligand does not enter lysosomes. Cells were then washed with either potassium or sodium medium and incubated for 60 min at either 37°C or 18°C (incubation 2). Degraded ligand was quantitated as described under Materials and Methods and expressed as a percentage of total ligand. In this experiment total cell-associated ligand was 88,750 ± 7,135 cpm/dish. Analogous determinations were performed using cultures to which a 100-fold excess of unlabeled ASOR had been added. These values were subtracted in the calculation of the percentage of ligand degraded. *Temp*, temperature.

whereas, in choline medium, degradation was intermediate between that with sodium and that with potassium (data not shown).

Delivery of ASOR to Lysosomes

Reduced degradation of ^{125}I-ASOR in sodium-free medium or in the presence of colchicine plus cytochalasin B could result from reduced delivery of ligand to lysosomes, as has been described with NH_4Cl (7) or monensin (9). Alternatively, inhibition of lysosomal protease activity, as seen with leupeptin (7), might be responsible. To distinguish between these possibilities, we determined subcellular distribution of radioactivity by Percoll density gradient centrifugation of the cell homogenates. We have previously demonstrated that this method clearly separates lysosomes from plasma membranes and endocytic vesicles (7). As demonstrated in Fig. 5, after a 60-min incubation in sodium medium, the majority of radioactivity resided near the bottom of the gradient, corresponding to the location of the lysosomal beta-hexosaminidase activity (enzyme activity data not shown). There was little radioactivity in this region when ^{125}I-ASOR was incubated with cells in the presence of a mixture of colchicine and cytochalasin B or with media in which potassium replaced sodium; radioactivity remained near the top of the gradients in the region corresponding to endocytic vesicles. The results presented in Fig. 3 together with the density gradients indicate that the intracellular segregation of ligand and receptor is a pre-lysosomal event. In potassium medium at the time examined, segregation has occurred (Fig. 3) but the ligand resides in a low density nonlysosomal subcellular fraction well separated in the gradient from lysosomes (Fig. 5, upper panel).

Effect of Reduced Temperature on the Endocytosis of ASOR

The assay for receptor-ligand dissociation described here as well as those reported earlier (5) provide for an increasingly precise definition of the steps involved in receptor-mediated endocytosis. Weigel and Oka (14) reported that there is little degradation of internalized ASOR at 20°C or below. Dunn et al. (15) have suggested that reduced temperature specifically reduced fusion between endocytic vesicles and lysosomes following the uptake of asialofetuin in perfused rat liver. We

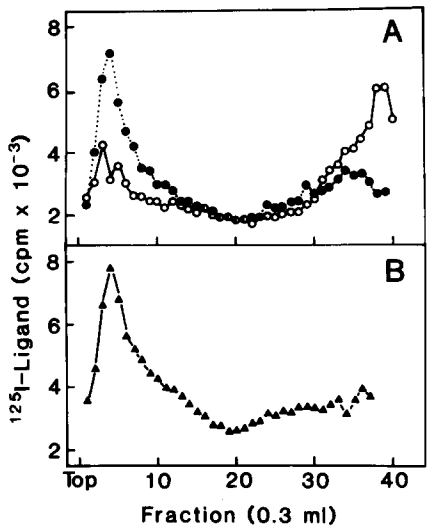

FIGURE 5 Subcellular fractionation of cultured hepatocytes: effect of sodium replacement or the combination of colchicine and cytochalasin B. Monolayer cultures were incubated at 4°C with ^{125}I-ASOR and washed free of unbound ligand. After 60 min at 37°C, cultures were subjected to homogenization and Percoll density gradient centrifugation. Cells were washed prior to homogenization with ice-cold buffer containing 50 mM *N*-acetylgalactosamine and hence the gradients shown depict the distribution of intracellular ligand. (*A*) Incubation in sodium medium (○) or potassium medium (●). (*B*) Incubation in sodium medium containing colchicine (40 μg/ml) and cytochalasin B (10 μM). Under all conditions the lysosomal marker enzyme β-hexosaminidase resided in the bottom 10 fractions of the gradient.

used the above assays to determine more precisely where in the pathway the effect of reduced temperature is manifested. To this end, we warmed cultures to 18°C after washing out unbound ligand. Internalization of ^{125}I-ASOR remained rapid at 18°C although it was slightly reduced relative to 37°C (Fig. 6*A*). However, release of internalized ligand from the receptor was markedly reduced (Fig. 6*B*) and by 1 h >70% of internalized ligand remained receptor-bound. This inhibition of ligand dissociation was accompanied by virtual absence of degradation (Fig. 6*C*), similar to that seen with amines or monensin. Results at 23°C were intermediate between those at 18°C and those at 37°C although the inhibition of degradation at 23°C appeared more pronounced than that of dissociation. These results with reduced temperature are consistent with our earlier suggestion that dissociation of the ligand-receptor complex is required for ligand degradation (7, 9).

The effect of reduced temperature on delivery of ligand to lysosomes was addressed in the following way. Hepatocytes were allowed to internalize ^{125}I-ASOR for 45 min at 37°C in potassium medium wherein both dissociation and segregation of the receptor-ligand complex were shown to occur (see above). After potassium medium was replaced by sodium medium either at 18°C or at 37°C, incubation was continued for 60 min. Degradation of ligand was observed at 37°C but none was seen at 18°C (Table I). Given that lysosomal enzymes of the liver are still quite active at 18°C (15), we believe that these results support the contention of Dunn et al. (15) that fusion with lysosomes is inhibited at reduced temperature. In our experiments, in which a wave of ligand is followed through sequential steps in the pathway of endocytosis, the influence of a perturbant is seen at its most proximal site of action. The sequential use of two inhibitors can be employed to discern more distal blocks or to demonstrate the equivalence of two perturbing conditions. Such experiments, for example, have indicated that monensin and chloroquine act at the same point in the process (9). From the results described above, it would appear that reduced temperature has at least two effects: one proximal to the sodium-requiring step and involving inhibition of ligand dissociation and a more distal effect on delivery of ligand to lysosomes.

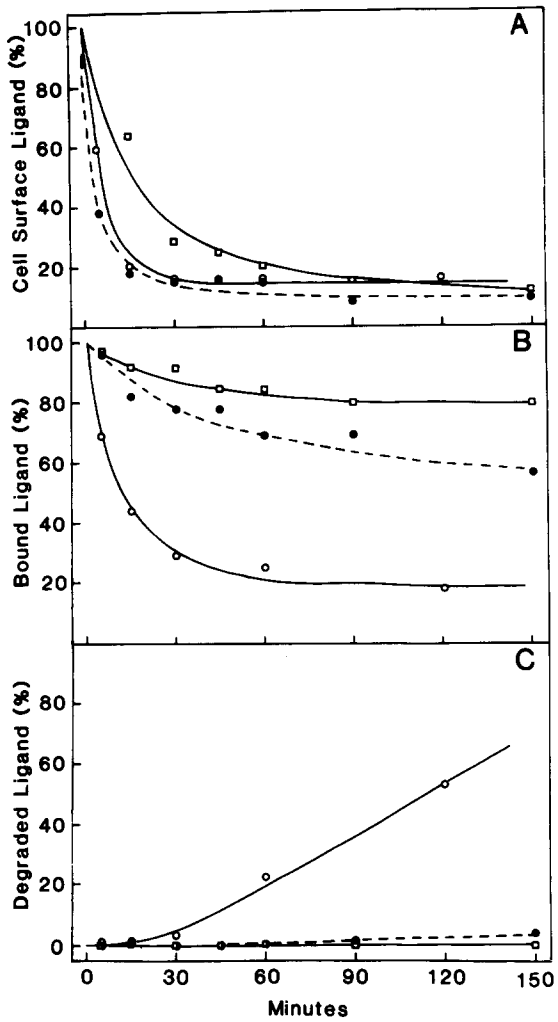

FIGURE 6 Effect of reduced temperature on the pathway of ^{125}I-ASOR catabolism. The surface receptors of hepatocytes in monolayer culture were occupied with ligand by incubation with 1 μg/ml ^{125}I-ASOR in sodium medium for 60 min at 4°C. Cell-associated ^{125}I-ASOR was 64,600 ± 7,900 cpm/dish. At 4°C, no internalization of ligand occurs. After washing in ice-cold medium to remove unbound ligand the cultures were warmed to 18°C (□), 23°C (●) or 37°C (○). At the indicated times after warming, ligand remaining on the cell surface was determined by displacement with ice-cold 20 mM EGTA, 150 mM NaCl, 20 mM Tris-Cl, pH 7.6 (*A*). Intracellular ligand (EGTA-resistant) was subjected to the solubilization-precipitation assay and the amount of receptor-bound radioactivity determined (*B*). Acid-soluble radioactivity in the incubation medium was determined as a measured of ligand degradation (C). All data represent the average of duplicate determination. Data in *A* and C are expressed as the percentage of the total ligand that is cell surface-associated and degraded respectively. Data in *B* are expressed as the percentage of internalized ligand remaining receptor-bound. Values obtained from cultures to which a 100-fold excess of unlabeled ASOR was added have been subtracted in the calculations.

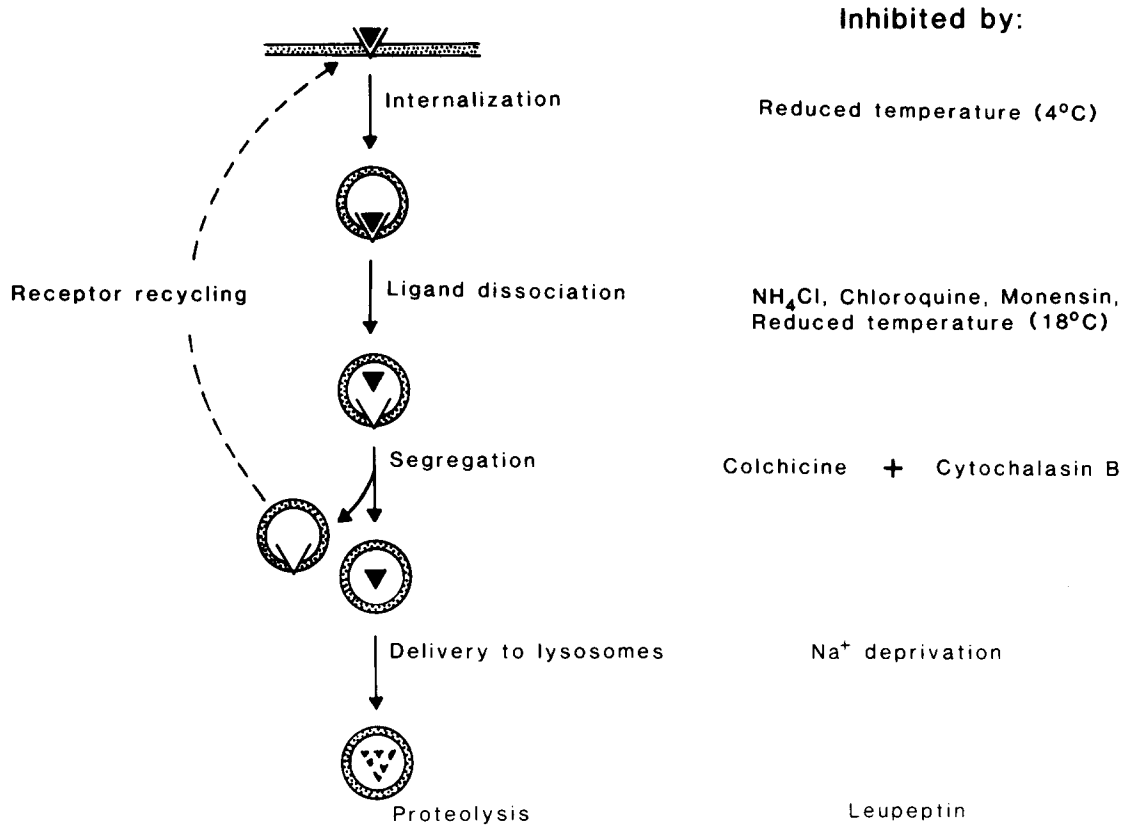

FIGURE 7 Schematic diagram of receptor-mediated endocytosis of asialoglycoproteins and its inhibitors. Based on these and other studies (7, 9), five discrete steps in uptake and catabolism can be quantitated. Inhibitors of each of these steps have been identified. These steps and their respective inhibitors are listed to the right of the schematic. Inhibitors are assigned on the basis of their most proximal site of action as a wave of pre-bound ligand moves through the pathway; inhibition of other more distal steps is not excluded. Also not excluded are as yet unidentified steps between those shown here.

DISCUSSION

Recently Geuze et al. (16) have provided morphological evidence that receptor and ligand physically segregate from each other soon after internalization and move into separate vesicular structures. We believe that the rebinding experiments with monensin provide a biochemical assay for the process of receptor-ligand segregation. We have demonstrated that, for a limited time following pH-mediated ligand dissociation, both ligand and receptor reside in the same compartment as evidenced by their ability to reassociate upon neutralization by a proton ionophore. This ability to rebind to receptor is rapidly lost. We interpret these data to indicate either that conditions within the vesicle containing ligand and receptor become incompatible with their association or that the two are simply no longer in the same structure. Given that receptors are reutilized (17–19) and in light of the morphological evidence of Geuze et al. (16), we hold the latter to be a more plausible explanation. There is morphologic evidence that endosomes though heterogeneous may be several times larger than coated vesicles. Thus the question arises as to whether the time-dependent loss of monensin-mediated ligand rebinding (Fig. 3) might reflect a ligand dilution phenomenon. Simple calculations indicate that this is extremely unlikely. A single ligand molecule within a vesicle of 1,000 Å represents a concentration of ~5 μM. Even a volume increase by 1,000-fold would leave this single molecule of ASOR at a concentration well above its dissociation constant.

In cells incubated in sodium-containing medium, the rapid loss in the ability of the ligand to rebind was accompanied by movement of ligand into lysosomes. Under these conditions, it is difficult to determine whether segregation was completed in a pre-lysosomal structure. However, the data from cells incubated in potassium medium provide evidence that segregation is indeed accomplished in a low density pre-lysosomal compartment. Thus sodium did not appear to be required for internalization, acidification, or segregation. The major effect of sodium removal was on the movement of ligand from low density endocytic vesicles to the high density lysosomes. The inhibition by sodium deprivation revealed the existence of a late pre-lysosomal state in which ligand has already dissociated and segregated from receptor before delivery to lysosomes. These data are consistent with the report of Baenziger and Fiete (12) in which ligand did not enter lysosomes although receptors appeared to continue to recycle. Therefore, it seems that this sodium-requiring step occurs late in the endocytic pathway and is subsequent to the point where receptors are free to be recycled to the plasma membrane. The reason for the sodium requirement at this particular step in the pathway is still unclear.

The initial effect of reduced temperature (18°C) on receptor-mediated endocytosis of desialylated glycoproteins is similar to that seen upon inhibition of hepatocytes with monensin. This ionophore effectively neutralizes acidic endocytic vesicles, thereby inhibiting release of internalized ligand and subsequent entry into lysosomes. The studies reported here

indicate that release of internalized ligand is necessary but not sufficient for entry into lysosomes. Delivery to lysosomes was independently inhibited at 18°C. In the presence of colchicine and cytochalasin B, internalized ASOR was released from receptor at a nearly normal rate with but little transfer of ligand to lysosomes, and no detectable degradation. Significantly, segregation of receptor from ligand was markedly slowed in this situation, suggesting a role for microtubules and microfilaments in the segregation process. In the presence of these drugs the inhibition of degradation is most likely explained by the failure to deliver ligand to lysosomes. Whether this represents an additional and separate block to the inhibition of segregation cannot be determined from these studies. Thus, cytochalasin B and colchicine may also have a separate effect at or near the sodium-requiring step in the pathway in addition to retarding ligand-receptor segregation.

On the basis of these and earlier (7, 9) studies, five discrete steps in the endocytosis pathway can be identified and quantitated (Fig. 7). These are (*a*) internalization of the ligand-receptor complex into endocytic vesicles, (*b*) release of internalized ligand from receptor, (*c*) segregation of ligand and receptor, (*d*) entry of ligand into lysosomes, and (*e*) degradation of ligand within lysosomes. Inhibitors at each of these stages have now been identified. These include reduced temperature (4°C) as an inhibitor of internalization, monensin, amines, and reduced temperature (18°C) as inhibitors of release, a mixture of colchicine and cytochalasin B as an inhibitor of segregation, replacement of sodium by potassium or sucrose as an inhibitor of entry into lysosomes, and leupeptin as an inhibitor of degradation within lysosomes. These conditions or agents allow a functional dissection of the endocytic pathway. Further study of their effects should elucidate the relationship of these steps to each other and to the pathway of endocytosis as a whole.

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