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Fate of receptor and ligand during endocytosis of asialoglycoproteins by isolated hepatocytes

(receptor reutilization/compartmental analysis)

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ABSTRACT **The endocytosis leading to degradation of ^{125}I-labeled asialo-orosomucoid specifically bound to the surface of freshly isolated hepatocytes was monitored as a function of time at 37°C. Experimental values were determined for the rates of internalization, dissociation of the receptor–ligand complex, and degradation of the labeled ligand. Compartmental analysis and computer modeling revealed that the data were compatible with dissociation of ligand from receptor preceding ligand degradation. The rate coefficient for internalization was calculated to be an order of magnitude greater than that for receptor–ligand dissociation. Ligand internalization did not result in concomitant depletion in the total number of cell surface receptors. Our data are taken to indicate that ligand remains associated with the receptor after internalization, that the complex is dissociated prior to degradation, and that new, unoccupied receptors are promptly returned to the cell surface from an internal pool.**

In receptor-mediated endocytosis, binding of ligands to cell surface receptors is followed by rapid internalization (reviewed in ref. 1). The mechanism involved in this process has eluded definition despite extensive efforts directed at its understanding. Questions as fundamental as when and where receptor and ligand dissociate lack definitive answers. In addition, current information is inadequate to determine whether observed reutilization of receptors involves only recycling of cell surface molecules or whether an internal pool of receptors also participates.

Earlier studies have identified a receptor in the plasma membrane of mammalian hepatocytes that binds and mediates internalization and degradation of desialylated serum glycoproteins (reviewed in ref. 2). In the present study we have utilized isolated hepatocytes and followed a synchronous wave of ligand internalization to address the above questions.

Our results indicate that ligand remains associated with receptor as it moves into the hepatocyte but dissociates prior to degradation. The data have been analyzed in terms of a compartmental model from which kinetic parameters related to intracellular traffic in ligand have been computed. Evidence is also presented supporting the replacement of internalized receptors with unoccupied receptors from an internal pool.

METHODS

Occupation of Cell Surface Receptors with Ligand. Hepatocytes were isolated from male, 200-g Sprague–Dawley rats (Taconic Farms, Germantown, New York) by perfusion with collagenase (Worthington, type III) as described (3), except that cells were washed with ice-cold medium [Waymouth's 752/1 (GIBCO)/25 mM Hepes, pH 7.2] containing 2.5% heat-inactivated horse serum (GIBCO) and 1.7 mM additional $CaCl_2$. Greater than 88% of the isolated cells excluded 0.05% trypan blue. Preparation of asialo-orosomucoid and labeling with ^{125}I to specific activities of $1.0–1.4 \times 10^7$ cpm/μg have been described (4). The same ligand was labeled with ^{3}H to a specific activity of 8×10^5 cpm/μg by using the method of Tack *et al.* (5). Hepatocytes ($6–10 \times 10^6$/ml) were incubated with shaking in ice-cold medium containing radiolabeled asialo-orosomucoid (1 μg/ml). After 60 min of incubation, cells were centrifuged at 50 × *g* for 30 sec and the resultant cell pellet was washed twice with 50 ml of ice-cold medium followed by a wash with 10 ml of ice-cold medium containing 0.5 mM *N*-acetylgalactosamine (GalNAc). Although this concentration of GalNAc displaced <10% of the bound ligand, omission of this wash led to greater variability and generally lower values for bound ligand in the solubilization–precipitation assay described below. The final cell pellet after this "low GalNAc wash" was suspended in ice-cold medium to approximately 5×10^6 cells per ml.

Assessment of Internalization and Degradation of Ligand. Washed hepatocytes with radiolabeled asialo-orosomucoid occupying their cell surface receptors were warmed as indicated by transfer to a 50-ml plastic tube in a 37°C water bath. The amount of radioactivity that could not be displaced from intact cells was taken as a measure of ligand internalization. To displace ligand, samples (0.3 ml) of the cell suspension were added to 0.3 ml of 100 mM EGTA/20 mM Tris·HCl, pH 7.6/150 mM NaCl at 4°C in a 1-ml Microfuge tube. After 10 min on ice, cells were centrifuged at 8000 × *g* for 1 min in a Beckman B Microfuge and the amount of radioactivity in the resultant supernatant and cell pellet was determined. Comparable results were obtained when 100 mM GalNAc (final concentration) was used in place of the EGTA. The same concentration of *N*-acetylglucosamine or buffer alone was ineffective. When ^{125}I-labeled asialo-orosomucoid (^{125}I-asialo-orosomucoid) was used, a Packard Auto-Gamma spectrometer was employed. Samples containing [^{3}H]asialo-orosomucoid were added to 10 ml of Aquasure (New England Nuclear) and the ^{3}H content was determined in a Beckman LS8100 liquid scintillation spectrometer.

To assess the amount of ligand degradation, samples (0.3 ml) of the cell suspension were added to 0.3 ml of ice-cold 20% trichloroacetic acid/4% phosphotungstic acid in a 1-ml Microfuge tube. After 10 min on ice, the precipitate was centrifuged and the amount of radioactivity in the supernatant and pellet was determined.

Solubilization–Precipitation Assay for Receptor-Bound Versus Unbound Ligand. To distinguish receptor-bound ^{125}I-asialo-orosomucoid from unbound ligand, an adaptation of "Assay A" of Hudgin *et al.* (6) was utilized. Samples (0.3 ml) of the cell suspension were added to tubes containing 0.9 ml of ice-cold

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Abbreviation: GalNAc, *N*-acetylgalactosamine.

1.33% Triton X-100 (New England Nuclear)/20 mM Tris·HCl, pH 7.6/150 mM NaCl/50 mM $CaCl_2$ and either 1.33 μg of unlabeled asialo-orosomucoid per ml (to prevent binding of unbound ^{125}I-ligand) or 133 mM GalNAc (to displace receptor-bound ^{125}I-ligand). After 10 min on ice, 1.2 ml of saturated ammonium sulfate adjusted to pH 7.6 with Tris was added. Following an additional 30 min on ice, each sample was filtered through a double thickness of GF/C filter (Whatman) that had been soaked previously in 45% saturated ammonium sulfate/20 mM $CaCl_2$/0.5% bovine serum albumin (fraction V, Sigma) adjusted to pH 7.6 with Tris. Precipitated material on the filters was washed three times with 0.5 ml of this same solution. The amounts of radioactivity on the filters and in the combined filtrate plus washes were determined. Use of a single thickness of filter resulted in filtrates that were variably cloudy in contrast to the clear filtrates obtained with the double GF/C.

Measurement of ^{125}I-Asialo-Orosomucoid Binding to Hepatocytes. To hepatocytes (5×10^6/ml) suspended in ice-cold medium was added 0.1 vol of ^{125}I-asialo-orosomucoid (10 μg/ml) in 150 mM NaCl/20 mM Tris·HCl, pH 7.6. The amount of ^{125}I-ligand bound to the cell surface receptors at a given time was determined by diluting 0.2 ml-samples of the cell suspension into 1 ml of ice-cold medium and quickly filtering through a GF/C filter previously soaked in medium. The filtered cells were washed with an additional 1 ml of ice-cold medium and the cell-associated ^{125}I was measured. Nonspecifically bound ^{125}I-ligand was defined as that which could not be displaced by dilution of the sample into ice-cold medium containing 100 mM GalNAc.

RESULTS

When incubated with ^{125}I-asialo-orosomucoid at 4°C in the presence of calcium, isolated hepatocytes bind but do not internalize the ligand. Under these conditions, the surface-bound protein can be readily displaced by EGTA or with the hapten sugars galactose or GalNAc (3). However, the rate of unassisted dissociation is slow (7, 8), thereby allowing cell suspensions to be washed free of unbound ligand without loss of receptor occupancy. When these washed hepatocytes were warmed to 37°C, ligand rapidly became resistant to displacement with EGTA and, during prolonged times of warming, labeled metabolic breakdown products accumulated in the medium.

To examine the intermediate stages between surface binding and degradation, we probed the relationship of ligand and receptor using an assay originally designed (6) for measuring binding by soluble receptor preparations. Briefly, washed hepatocytes, their surface receptors occupied with ^{125}I-asialo-orosomucoid, were solubilized in an ice-cold buffer containing Triton X-100. The receptor–ligand complex was precipitated by addition of an equivalent volume of saturated ammonium sulfate and separated from any free ^{125}I-asialo-orosomucoid by filtration. The solubilization–precipitation assay was tested in preliminary experiments utilizing washed rat liver membranes to which labeled ligand had been bound. When these membranes were solubilized in Triton X-100 and applied to a Sephadex G-100 column, greater than 90% of the radioactivity was recovered in the excluded volume, well separated from ^{125}I-asialo-orosomucoid. Upon precipitation with 50% saturated ammonium sulfate, essentially the same amount of radioactivity was scored as receptor–ligand complex. In experiments with loaded hepatocytes, between 80% and 90% of the associated ligand appeared as receptor-bound in the solubilization–precipitation assay. The ability to account for membrane- and cell-associated ligand in a near-quantitative fashion assured us that little, if any, receptor bound material was scored erroneously as unbound ^{125}I-asialo-orosomucoid.

To insure that no false positives were recorded for receptor-bound ligand in the assay, increasing amounts of free ^{125}I-asialo-orosomucoid were included in the solubilization buffer to which ligand bearing hepatocytes were added. In addition, excess unlabeled ligand was necessary to prevent binding by intracellular receptors previously cryptic and exposed by the detergent treatment. The experimentally determined amounts of receptor-bound ligand remained constant in the face of large amounts of added free ^{125}I-asialo-orosomucoid (Fig. 1). Moreover, unbound ligand was readily detected even when present at levels corresponding to only 10% of the bound material.

We then proceeded to examine endocytosis. Hepatocytes with their surface receptors occupied by ^{125}I-ligand were warmed to 37°C and, at various times, samples for three determinations were removed. One sample was used to assess internalization of ligand as judged by resistance to displacement with EGTA; the second was precipitated with 10% trichloroacetic acid/2% phosphotungstic acid as a measure of degradation; the third was subjected to the solubilization–precipitation assay. Changes in the three measurements were calculated from the initial values at time zero and are presented in Fig. 2. By the criterion of EGTA resistance, at least 50% of the cell surface radioactivity became internalized by 5 min at 37°C. Essentially no acid soluble material appeared during this period. The acid-insoluble radioactivity at each time point was examined by $NaDodSO_4$/polyacrylamide gel electrophoresis and was found to contain no proteolytic fragments of the ^{125}I-asialo-orosomucoid. No loss of surface bound radioactivity into the incubation medium occurred as a result of warming the cells.

Prior to any evidence of ligand degradation, a time-dependent increase was observed in radioactivity resistant to precipitation by 50% ammonium sulfate. This was interpreted to be ligand that had been internalized and subsequently released from the receptor. The percentage of internalized ligand that appeared to have been released continued to increase and peaked at about 20% between 15 and 30 min at 37°C. It should be noted that this value represents the sum of released intact ligand and acid-soluble radioactivities. After 60 min at 37°C, the degraded material constituted most of the total released radioactivity. It is apparent from Fig. 2 that the rate at which ligand becomes EGTA resistant exceeds the rate of release by ap-

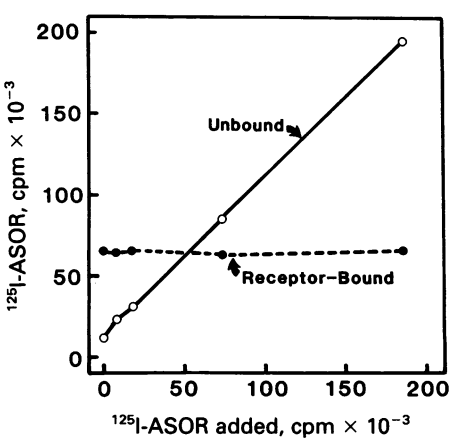

FIG. 1. Solubilization–precipitation assay for receptor-bound versus unbound ligand. The surface receptors of isolated hepatocytes were occupied with ^{125}I-asialo-orosomucoid (ASOR) and samples (0.3 ml) were subjected to the solubilization–precipitation assay with the indicated amounts of ^{125}I-ASOR (1.4×10^7 cpm/μg) added to the assay mixtures. Radioactivity in the filtrate was scored as unbound (○——○). Radioactivity on the filters that was specifically dissociated by 100 mM GalNAc was considered receptor-bound (●----●). Data shown represent the average of duplicate determinations.

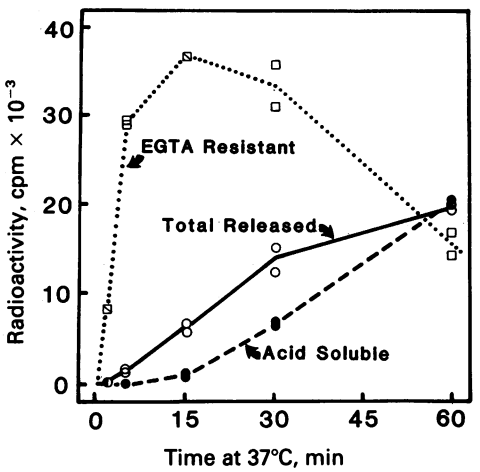

FIG. 2. The fate of surface-bound ligand upon warming of cells. The surface receptors of isolated hepatocytes were occupied with ^{125}I-asialo-orosomucoid (1.4×10^7 cpm/μg). After washing, the cell suspension (5×10^6/ml) was warmed to 37°C. At the indicated times, samples (0.3 ml) were removed for assessment of ligand internalization (□·····□) and degradation (●----●). Identical samples were subjected to the solubilization–precipitation assay and the increase in unbound radioactivity occurring as a result of warming (○——○) was determined. Duplicates of each determination are shown.

proximately 1 order of magnitude, thereby implying that the ligand enters the cell as a complex with receptor.

The appearance of a precursor–product relationship in the curves of Fig. 2 suggested that ligand, initially bound at the cell surface, was internalized in association with the receptor from which it was subsequently released and then degraded. In combination, our assays of EGTA resistance, acid solubility, and association of ligand with receptor allowed us to assign values at every time point for each of these presumed states of the ligand. These data were analyzed in terms of this compartmental model by using the Consaam computer program (9) to test whether the apparently dissociated ligand was of necessity an intermediate in the intracellular traffic in ligand (Fig. 3). Such modeling allowed us to ask whether the data were numerically consistent with all of the internalized ligand being released from the receptor prior to ligand degradation. The basic elements of ligand transfer mentioned above are present but the acquisition of EGTA resistance did not truly follow a single exponential. Therefore, an additional surface compartment (designated **21**) was included. However, since the rate of exit from this added compartment was only 2% of that from the major surface compartment, it represented a minor component that could be ignored in practice. The lag before the appearance of acid-soluble radioactivity required that an obligate delay (designated **4**) be inserted between release from the receptor and ligand degradation. The physiological meaning of this 8-min "aging" of ligand is unclear but it may reflect intracellular translocation of ligand before entry into the compartment responsible for proteolysis.

In Fig. 4, the discrete points plotted are those from the experiment shown in Fig. 2; the curves are the best fit of these data predicted by the model. The calculated rate coefficients for ligand transfer into and out of each compartment have been computed and are shown in Fig. 3. The rate coefficient for internalization (0.2 min^{-1}) is in close agreement with the first-order rate constant determined by Tolleshaug (8). It is clear from visual inspection as well as from the sum of the squares of deviations that an excellent fit was achieved. Several alternate modeling schemes proved to be inferior to the simple model of Fig. 3. Attempts to model a pathway in which ligand was released from the receptor and then bound again to another receptor molecule intracellularly (at compartment **4**) yielded a considerably worse fit—i.e., the sum of the squares was doubled. Likewise, if as little as 20% of the internalized ligand were assumed to bypass the released state and go directly from being receptor-bound to being degraded, an approximately 3-fold in-

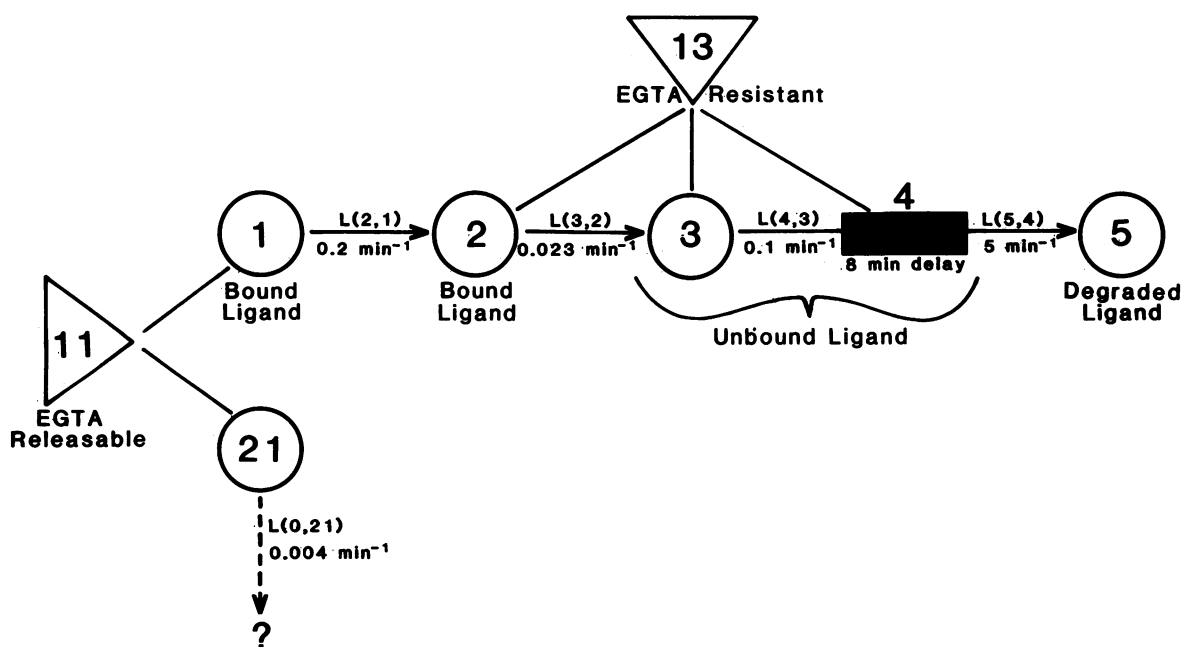

FIG. 3. Compartmental model for endocytosis and catabolism of asialoglycoprotein. Cell surface ligand, defined as EGTA releasable, is contained in two compartments (**1** and **21**) designated collectively as **11**. Internalized ligand is defined as EGTA resistant and is designated **13**. This compartment is composed of compartment **2** which is receptor-bound ligand and compartments **3** and **4** which are unbound ligand. Compartment **4** (rectangle) defines a delay function in that molecules mathematically "age" in this compartment. In compartments shown as circles, no such delay occurs and all molecules within these compartments have equal probabilities of exit per unit time. Compartment **5** consists of accumulated degraded ligand defined by acid solubility. The coefficient $L(X,Y)$ denotes the L for movement from Y to X.

crease in the sum of the squares resulted. In view of these calculations, we feel that the model in Fig. 3 is the most satisfactory of these relatively simple models. Clearly, more intricate models can be constructed in which these compartments are further subdivided, but this will require a means of probing these subdivisions experimentally.

Calculations of average residency times for the ligand in the compartments defined by our model yielded values of 36 min for the internal receptor-bound state (compartment 2) and 16 min for the internal unbound state (compartments 3 plus 4). It should be clear that these values are not mathematically equivalent to half-times or any function of them. Half-times are only a valid concept for those processes that are defined by a single exponential, whereas the process of receptor-mediated endocytosis and ligand catabolism is considerably more complex. As noted above, even the step of ligand internalization which approximates a first-order process is better described by two exponentials.

The above results lead to certain predictions relevant to receptor reutilization. Because the receptor appears to enter the cell along with ligand and resides within the cell for a finite time, return of the same receptor molecules to the surface would have to await the intracellular dissociation of ligand. Therefore, one might expect to see depletion of cell surface receptors immediately after the initial wave of ligand internalization. Alternatively, if internalized receptors were replaced with sufficiently high efficiency by different receptor molecules, no such depletion would be detectable.

To examine this, we measured surface receptors on cells shortly after internalization of surface bound ligand (Fig. 5). Hepatocytes were allowed to bind [^{3}H]asialo-orosomucoid at 4°C and washed free of unbound ligand. A portion of these washed cells was warmed to 37°C for 5 min and then rechilled to 4°C. Samples of the warmed and unwarmed cells were either washed with ice-cold medium (Fig. 5*A*) or stripped of bound ligand by washing with an ice-cold solution containing 10 mM EGTA (Fig. 5*B*). During the warming period, approximately 47% of the ^{3}H-ligand became EGTA resistant. Consistent with occupation of surface receptors by ^{3}H-ligand, the unwarmed, medium-washed cells bound little ^{125}I-ligand. However, medium-washed cells that had been warmed exhibited a markedly

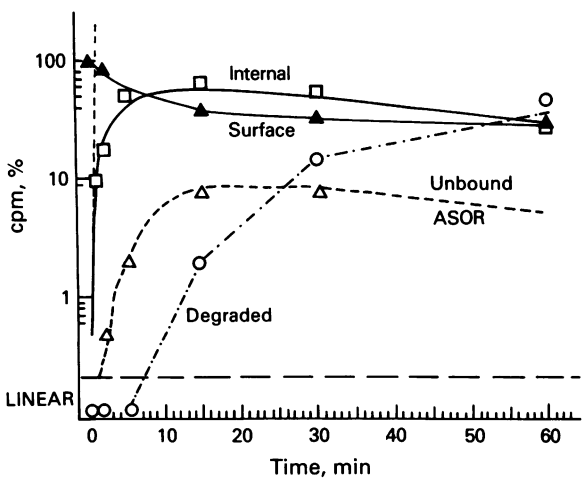

FIG. 4. Fit of experimental data to the compartmental model. Data calculated from the experiment shown in Fig. 2 are plotted as the discrete symbols. The curves represent the best fit of these data predicted by the model depicted in Fig. 3. Uncertainty as to the value for unbound ligand (ASOR) at 60 min (it was between 0% and 4%) led us to drop this point from the plot. Inclusion of any value in this range altered the calculated curves minimally.

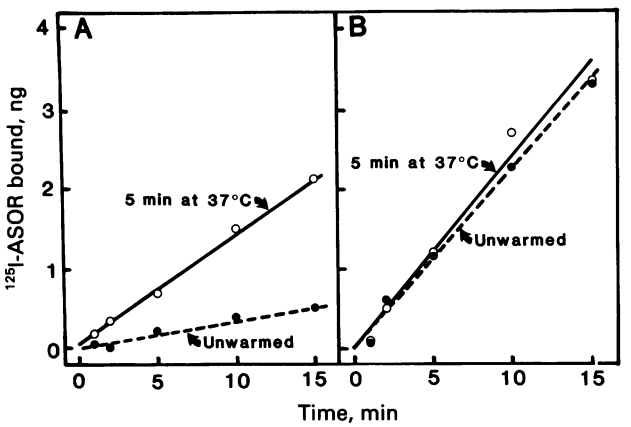

FIG. 5. Effect of ligand internalization on the number and state of occupation of cell surface receptors. The surface receptors of isolated hepatocytes were occupied with ^{3}H-asialo-orosomucoid (ASOR). After washing, one-half of the cell suspension (5×10^6/ml) was warmed for 5 min to 37°C. Portions of each half were then washed with either 5 vol of ice-cold medium (*A*) or 5 vol of ice-cold 10 mM EGTA/150 mM NaCl/20 mM Tris·HCl, pH 7.6 (*B*). After resuspension in ice-cold medium containing 10 mM additional $CaCl_2$, ^{125}I-ASOR (1 μg/ml) was added and the amounts bound at the indicated times were determined. The apparent rate constants, calculated by linear regression analysis of the data shown, were 0.04 ng/min (●----●) and 0.14 ng/min (○——○) in (*A*) and 0.23 ng/min (●----●) and 0.24 ng/min (○——○) in (*B*).

higher level of binding, indicating that unoccupied receptors had become available as a result of the warming. Interestingly, cells that were washed with EGTA displayed virtually identical initial rates of ligand binding independent of whether they had been warmed. These results indicated that no detectable depletion of surface receptors accompanied the internalization of half of the surface-associated ligand despite the earlier evidence suggesting that receptor also entered the cell. The initial rates of ^{125}I-ligand binding calculated from the slopes of the lines in Fig. 5 are proportional to the numbers of active surface receptors. It can be estimated from these that a nearly one-for-one replacement of internalized receptors with unoccupied receptors must have occurred. The source of these previously unavailable receptors is unknown although a relatively large pool of intracellular receptors has been reported (10).

DISCUSSION

The receptor for asialoglycoproteins *in vivo* (11) or in cultured hepatocytes (12) has a sufficiently long half-life to suggest that receptors are not destroyed during ligand catabolism. Receptor reutilization has been directly demonstrated by the finding that isolated hepatocytes internalized and degraded ligand in excess of their total receptor content even when *de novo* synthesis of receptors was blocked by inhibitors (3, 8). Several diverse hypotheses have been put forward for the mechanism by which receptor molecules are spared from destruction. For example, it has been postulated that receptor moves to the lysosomes with the ligand but avoids the proteolytic enzymes contained therein by undergoing a translocation of its binding site to the lysosome's exterior (11, 13). On the other hand, it has been suggested, based on electron micrographs of liver sections after uptake of lactosaminated ferritin, that ligand dissociates from the receptor soon after endocytosis in an intermediate vesicle of the peripheral cytoplasm (14). After dissociation, receptor would presumably be free to return to the plasma membrane rather than to accompany ligand to the lysosome. It has also been proposed that the receptor molecule responsible for ligand

binding functions without leaving the hepatocyte plasma membrane (15).

We have followed ligand–receptor interaction during the passage of ligand from surface binding to lysosomal degradation. Generally speaking, there are two major difficulties in a study of this type. First, when hepatocytes are incubated with ligand under physiological conditions, ligand internalization is highly asynchronous with ligand molecules at all stages of transit through the cell. Second, because ligand is labeled rather than receptor, the experimental handle on the receptor is lost upon dissociation from the ligand and labeled catabolic products rapidly diffuse from the cells. To circumvent these problems, we attempted to synchronize ligand internalization by first "loading" external receptors at 4°C and determining the relative amounts of bound and free ligand in various states on and inside the cells during the course of ligand metabolism. Data were then analyzed in terms of the kinetics of ligand flux through these states. This approach allowed us to evaluate the total flux through any compartment based on the experimentally determined data which gave only the quantity of ligand in the compartment at each time point. Thus, ligand was assigned by the combined use of three separate assays to be receptor-bound at the cell surface, receptor-bound within the cell, intact within the cell but dissociated from receptor, or degraded. This simple compartmental model (Fig. 3) fits well the available experimental data indicating that hepatocyte surface receptors were internalized together with asialo-orosomucoid but that, prior to degradation, ligand existed intracellularly in an unbound condition. The compartments described here refer to states of the ligand that can be distinguished experimentally from one another and can be treated as mathematical compartments in modeling. Any attempt to ascribe these states to cellular loci would be speculative.

Of particular interest is the finding that the cell surface suffers no net reduction in ability to bind asialoglycoproteins despite the internalization of receptor–ligand complex. This appears to be accomplished by replacement of endocytosed receptors from a pool of receptors previously unavailable to extracellular ligand and would appear to be at variance with the findings of Stockert *et al.* (15). These workers reported that neuraminidase treatment of hepatocytes altered the apparent specificity only of surface receptors and yet this alteration was maintained during protracted endocytosis. Based on this observation, it was concluded that receptors of the intracellular pool do not participate in endocytosis. Doyle *et al.* (16) reached a similar conclusion in studies with mouse L cells rendered active in asialoglycoprotein catabolism by insertion of the rat liver receptor into their plasma membrane. However, binding and degradation of ligand by these chimeric cells required a markedly longer time than that seen with rat hepatocytes. It is possible that intracellular receptors contribute to the rapidity with which hepatocytes handle asialoglycoproteins.

Confirmation of a portion of our results became known to us during the preparation of this manuscript. Using a somewhat different approach, Weigel (17) also concluded that receptors enter the hepatocyte with asialoglycoproteins. Also similar was his conclusion that internalized receptors were replaced by unoccupied receptors although the replacement he observed appeared to be considerably slower than the rate of endocytosis. This difference, as well as those mentioned above, are likely to reflect differences in experimental design. The system and approach described here should prove to be useful in this regard, as further clarification and expansion of the model become possible by the use of specific agents or conditions which perturb the overall catabolic process.

Note Added in Proof. While this paper was in press further evidence for the constancy of hepatocyte cell surface receptors during ligand internalization has appeared (18).

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