The Large Intracellular Pool of Asialoglycoprotein Receptors Functions during the Endocytosis of Asialoglycoproteins by Isolated Rat Hepatocytes

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The function of intracellular asialoglycoprotein receptors during the endocytosis of asialo-orosomucoid in isolated hepatocytes was assessed by following changes in the occupancy of intracellular receptors. Unoccupied total cellular (inside and surface) or surface receptors were quantitated at 0 °C by the binding of $^{125}$I-asialo-orosomucoid in the presence or absence, respectively, of digitonin. Freshly isolated cells had about 17% of their total receptors on the surface. After incubation at 37 °C, the receptor distribution changed to 25 to 50% on the cell surface and 50 to 75% inside the cell. At 37 °C, the average total number of receptors/cell was $4.5 \times 10^6$. Dissociation constants, determined from equilibrium binding studies in the presence or absence of digitonin to assess total or surface receptors, were identical ($5.4 \pm 1.4$ and $5.6 \pm 1.1 \times 10^{-8}$ M, respectively). In the presence of asialo-orosomucoid at 37 °C, there was both a time- and a concentration-dependent decrease in surface and intracellular receptor activity. This receptor activity decrease was reversed by removing asialo-orosomucoid from the medium or by washing the digitonin-permeabilized cells with ethylene glycol bis(β-aminoethyl ether)-$N,N,N',N'$-tetraacetic acid prior to quantitation of receptor activity. Within 1 to 2 h in the presence of excess asialo-orosomucoid, a steady state was attained in which approximately 70% of the intracellular receptors were occupied. The kinetics of receptor activity recovery on the cell surface after internalization of a pulse of ligand is different than the rate of recovery of internal receptor activity. The results suggest that all of the internal asialoglycoprotein receptors are functional and participate during endocytosis. Internal receptors may be functionally equivalent to those on the surface or they may serve a reservoir or routing function for internalized ligand.

The endocytosis of asialoglycoproteins by hepatocytes in vivo and in vitro (1) or by cultured hepatoma cells (2, 3) is presently being used by many investigators as a model system for the study of the mechanisms of receptor-mediated endocytosis and receptor recycling. Pricer and Ashwell (4), in experiments which involved subcellular fractionation of whole rabbit liver, demonstrated that about 90% of the cellular receptor activity was not on the cell surface but rather in Golgi, microsomal, and lysosomal membrane fractions. These results were subsequently confirmed by Tanabe et al. (5) and Steer and Ashwell (6) in studies which employed, respectively, subcellular fractionation of rat liver or detergent solubilization of isolated rat hepatocytes. Other investigators have also concluded that the majority of receptors are intracellular (7-9). In contrast, Geuze et al. (10) recently reported on indirect immunofluorescence and immunocytochemistry studies with frozen liver sections in which the receptor was detected only on the cell surface and was not intracellular. In addition, in the only hepatoma line, HepG2, shown to retain the asialoglycoprotein receptor (2), a similar receptor distribution was reported (3). In order to understand the mechanism by which the overall system functions, it is clearly important to resolve this question about the existence of intracellular receptors and, if they exist, to determine whether such receptors function during endocytosis. Stockert, Morell and co-workers (9, 11) have concluded that the intracellular receptors may not function since surface receptors were apparently not replaced during the uptake of galactosyl ligands by neuraminidase-treated hepatocytes (11) or after the perfusion of intact liver with antibody to the receptor (9). We have developed an independent method, which is simple and rapid, to quantitate the active receptors (i.e. capable of ligand binding) inside hepatocytes made permeable, but not solubilized, with the nonionic detergent digitonin. We also find that the majority of total cellular receptors are intracellular as assessed by this technique. In addition, the use of digitonin has enabled us to determine that after internalization of prebound asialoglycoprotein the dissociation of receptor-ligand complexes is biphasic (12). Half of the internalized complexes dissociate within 1 to 2 min after internalization ($k > 0.35 \; \text{min}^{-1}$), while the other half dissociate with an apparent half dissociate with an apparent $t_{1/2}$ of 50 min (12). Bridges et al. (13) have also concluded that internalized ligand remains associated with receptor inside the cell but then dissociates prior to degradation. In the present study, we have used the technique of permeabilizing cells with digitonin in order to monitor how the number of occupied and unoccupied cell surface and total cellular receptors changes with time during the endocytosis of asialoglycoprotein by isolated hepatocytes. We conclude that the majority of intracellular receptors are clearly functional since they become occupied with ligand in a time- and concentration-dependent manner during receptor-mediated endocytosis. Preliminary reports of some of these results have been presented (12, 14).

**EXPERIMENTAL PROCEDURES**

**Materials**—Human orosomucoid (α-acid glycoprotein) was a gift from Dr. M. Wickerhauser of the Plasma Derivatives Laboratory of

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the American Red Cross. Orosomucoid was desialylated by digestion with Clostridium perfringens neuraminidase (15). Sephadex G-25 and G-75 were from Pharmacia Chemical Co. Triton X-100, collagenase (type 1), BSA2 (fraction V), phosphotungstic acid, EGTA, and neuraminidase (type X) were from Sigma. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid was from Research Organics, Inc., Cleveland, OH. 1,10-Phenanthroline-5-sulfonic acid was from Pierce Chemical Co. Digitonin was from Matheson, Coleman and Bell. Male Sprague-Dawley rats (200-350 g when used) were obtained from Timco Breeding Laboratories, Houston, TX. Na125I (10-20 mCi/μg of iodine) was from Amerham Corp. 125I-Asialo-orosomucoid was prepared as described previously (16) according to the procedure of Fraker and Speck (17). Freshly isolated hepatocytes were suspended in Medium 1 (which contained a modified Eagle's medium (1) with a total Na+ content of 143 mM NaCl, 6.8 mM KCl, 2 mM CaCl2, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4. All other chemicals were reagent grade.

**Cell Preparation—**Hepatocytes were prepared by the collagenase perfusion procedure of Seglen (18) with several minor modifications (19). Briefly, the liver lobes were removed and placed in a modified KRB buffer (37°C). The lobes were minced with a surgical scalpel, then added to a suspension of freshly isolated cells (19, 20).

**Assessment of Intracellular and Surface Receptor Content in Hepatocytes—**The binding of 125I-asialo-orosomucoid to suspensions of isolated hepatocytes was performed at 0-4 °C using the centrifugation procedure described previously (19). Briefly, samples of cell suspension were diluted into ice-cold Medium 1/BSA, centrifuged, resuspended to 3-4 x 106 cells/ml in Medium 1/BSA with 1.5 μg/ml 125I-asialo-orosomucoid and incubated on ice for 60 min prior to washing. It is assumed that the binding of one molecule of asialo-orosomucoid monitors the activity and presence of one receptor molecule. Specific binding was assessed in the presence of at least a 50-fold excess of nonradioactive glycoprotein and was typically on the order of 90%. In the absence of digitonin, only the number of active receptors available on the cell surface is measured at 0 °C.

**Determination of Endocytotic Activity of Asialo-orosomucoid by hepatocytes—**Endocytotic activity was assessed by measuring the appearance of acid-soluble radioactivity (16). A sample of cell suspension (0.6 ml) was added to ice-cold Medium 1/BSA containing 20 mM EDTA or 7.5 mM EGTA to remove surface-bound but not internal glycoprotein (21). In some experiments, the cells were continuously exposed to the glycoprotein, whereas in other experiments the ligand was bound to the cell surface. In this latter case, cells were allowed to bind radioactive asialo-orosomucoid at 0 °C for 60 min, washed twice by centrifugation to remove nonbound glycoprotein, and then used. With ligand bound to the cells, there is only one wave of synchronous endocytosis as the cell surface is cleared (16, 21).

**Determination of the Rate of Degradation of Asialo-orosomucoid—**The degradation of 125I-asialo-orosomucoid was determined by measuring the appearance of acid-soluble radioactivity (16). A sample of cell suspension (0.6 ml) was added to ice-cold Medium 1/BSA containing 20 mM EDTA or 7.5 mM EGTA to remove surface-bound but not internal glycoprotein (21). After at least 15 min on ice, the precipitates were centrifuged for 10 min at 3000 rpm in a Beckman model TJ6 table-top centrifuge. Radioactivity in the supernatant fluid was then determined.

**General—**Protein was determined by the method of Bradford (22) or by using BSA as the standard. Centrifugations of cell suspensions were done at 800 rpm for 2 min in a GCL-1 table-top centrifuge (Sorval Instrument Co.). 125I-radioactivity was determined using a Beckman gamma 4000 spectrometer.

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2 The abbreviations used are: BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; PBS, phosphate-buffered saline.

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**RESULTS**

**The Effect of Digitonin on 125I-Asialo-orosomucoid Binding by Isolated Rat Hepatocytes—**Freshly isolated cells in the presence of increasing concentrations of digitonin were incubated with 125I-asialo-orosomucoid to measure the amount of specific binding by asialoglycoprotein receptors. At very low concentrations of digitonin (<0.01%), there was no change in the observed specific binding. At or above about 0.008% (65 μM) detergent, all cells stain with trypan blue. However, a large increase in binding was observed from approximately 0.01-0.05% digitonin (Fig. 1). Above about 0.05%, the measured receptor content reached a plateau (see Fig. 1). The amount of released cell protein was constant at about 50% from 0.02-0.09% digitonin (not shown). In the studies presented here, a detergent concentration of 0.055% has been employed.

The increase in the specific binding of 125I-asialo-orosomucoid in the presence of digitonin represented approximately a 6-fold increase in the total number of receptors detected per cell. However, the partition of asialoglycoprotein receptors between the cell surface and the cell interior is different in freshly isolated cells compared to cells which are first incubated at 37 °C. We find that the number of cell surface receptors increases severalfold when freshly isolated cells are incubated at 37 °C (19, 20). Freshly isolated cells have an average of 16.0 ± 5.3% (± s.e.m) of their total receptor pool, measured in the presence of 0.055% digitonin, on the cell surface. In contrast, hepatocytes which are first incubated at 37 °C for 45 min have approximately 45% of their total receptor pool on the cell surface (see below).

**Antiserum to the receptor but not nonimmunized serum was able to block the ability of digitonin-treated cells to bind 125I-asialo-orosomucoid (Table I). Since 92% of the total binding activity was abolished by the antiserum and this was also approximately the level of specific binding with 125I-asialo-orosomucoid, we conclude that virtually all of the specific binding activity in digitonin-treated hepatocytes is mediated by the asialoglycoprotein receptor.

Control experiments were performed to assess the possibil-
Our data show that some receptors or receptor-ligand complexes may be solubilized during exposure to the detergent. Cells were first allowed to bind \(^{125}\)I-asialo-orosomucoid at 0 °C, washed free of excess glycoprotein, and then treated with 0.055% digitonin on ice. In a series of experiments, the average recovery of total bound ligand was 90.5 ± 6.2% (n = 6), while the average specific binding was 92.4 ± 2.2%. A similar result was obtained in the presence of 0.10% digitonin. Therefore, at the most, only about 10% of pre-existing receptor-ligand complexes are lost during the treatment with digitonin. By inference, we assume that the same is true for unoccupied receptors although we cannot test this directly.

Affinity of Digitonin-exposed Binding Sites for \(^{125}\)I-Asialo-orosomucoid—The saturation curves for the binding of \(^{125}\)I-asialo-orosomucoid by hepatocytes in the presence and absence of digitonin are very similar (Fig. 2A), with half-maximal values at about 0.2-0.3 \(\mu\)g of protein/ml (approximately 5.0-7.5 nM). Equilibrium binding experiments using \(^{125}\)I-asialo-orosomucoid at 0 °C were performed to determine whether the additional intracellular receptors detected in the presence of digitonin have the same binding characteristics as the receptors initially present on the cell surface. Analyses of Scatchard plots (24) indicate that the affinity of the surface and total cellular receptors are virtually identical (Fig. 2B).

Table I summarizes information from several experiments. In any one experiment, the \(K_d\) values for total or surface receptors were the same within about ±4%. Dissociation constants with and without digitonin were, respectively, 5.4 ± 1.4 \(\times 10^{-9}\) M (n = 5) and 5.6 ± 1.1 \(\times 10^{-5}\) M (n = 3). The total cellular receptor content was on the order of 4.5 \(\times 10^{10}\) receptors/cell and approximately 55% of the total cellular receptors were intracellular in these experiments (note that these cell suspensions were first treated at 37 °C to increase and stabilize the number of surface receptors; 19, 20). In a large number of experiments, the percentage of total cellular receptors inside the cell ranged from about 50-75% for cells equilibrated at 37 °C.

Surface and Total Receptor Occupancy in Hepatocytes Exposed to Nonradioactive Asialo-orosomucoid—Hepatocytes exposed to different concentrations of asialo-orosomucoid showed both a time- and a concentration-dependent decrease in the number of available (unoccupied) receptors on the cell surface (Fig. 3A). At lower concentrations of asialo-orosomucoid, there was a transient decrease in the available receptor content on the surface and then an increase with time. For example, at 0.10 \(\mu\)g/ml glycoprotein, there was a drop of approximately 60% in the number of unoccupied surface receptors within about 5 min. Over the next 50-60 min, however, the number of unoccupied receptors on the hepatocyte surface increased and returned essentially to the value present at the start of the experiment. This concentration of asialo-orosomucoid (0.10 \(\mu\)g/ml) represented only about 3-4 times the amount of surface receptors on the total population of cells in suspension. The lowest concentration of asialo-orosomucoid used in Fig. 3 (0.01 \(\mu\)g/ml) corresponded to the equivalent of about 30-40% of the total surface receptors.

### Table I

<table>
<thead>
<tr>
<th>Serum Concentration (v/v)</th>
<th>(^{125})I-Asialo-orosomucoid bound (fmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonimmune serum</td>
</tr>
<tr>
<td>0%</td>
<td>555 (100)</td>
</tr>
<tr>
<td>0.5%</td>
<td>554 (99)</td>
</tr>
<tr>
<td>10%</td>
<td>498 (90)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, per cent of control.
TABLE II
Equilibrium binding of $^{125}$I-asialo-orosomucoid to hepatocytes at 0°C with and without digitonin

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dissociation constant</th>
<th>Receptor number</th>
<th>Receptor ratio (surface/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Minus</td>
<td>Plus</td>
</tr>
<tr>
<td>1</td>
<td>$\times 10^{-8}$ M</td>
<td>3.6</td>
<td>5.1</td>
</tr>
<tr>
<td>2</td>
<td>$\times 10^{-8}$ M</td>
<td>5.1</td>
<td>5.0</td>
</tr>
<tr>
<td>3</td>
<td>$\times 10^{-8}$ M</td>
<td>5.6</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>$\times 10^{-8}$ M</td>
<td>7.4</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>$\times 10^{-8}$ M</td>
<td>5.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Average</td>
<td>$\pm 1.1$</td>
<td>5.4</td>
<td>4.5</td>
</tr>
<tr>
<td>S.D.</td>
<td>$\pm 1.4$</td>
<td>$\pm 0.8$</td>
<td>$\pm 1.2$</td>
</tr>
</tbody>
</table>

Available in the suspension and this concentration caused only a very small transient decrease in the number of available surface receptors. The highest concentration of asialo-orosomucoid employed (1.5 μg/ml) was the equivalent of over a 50-fold excess of glycoprotein relative to the total number of initial surface receptors and resulted in a very rapid and large decrease of available surface receptors (Fig. 3A). In this case, at the attainment of the apparent steady state, the per cent occupancy of surface receptors was about 85%.

In the same experiment, a similar trend was observed when the total number of unoccupied surface and internal receptors was assessed by measuring their availability to $^{125}$I-asialo-orosomucoid at 0 °C in the presence of digitonin (Fig. 3B). At the lowest concentration of asialoglycoprotein, there was virtually no effect detected on the size of the total cellular receptor pool, whereas at the intermediate concentration, there was a transient 40% decrease and then a recovery which occurred with significantly different kinetics than that observed for the recovery of surface receptors (compare to Fig. 3A). With the highest concentration of asialo-orosomucoid, there was a much more extensive decrease in the total number of available receptors which continued for a longer time and which approached a steady state by approximately 60 min (Fig. 3D). The free surface and total cellular asialoglycoprotein receptor content at the start of the experiment was 476 and 2050 fmol/mg of cell protein, respectively, after normalizing for the percentage of total cellular protein lost during the digitonin treatment. That is, approximately 23% of the total cellular receptors were on the cell surface. At the end of 60 min in the presence of 1.5 μg/ml nonradioactive asialo-orosomucoid, the per cent occupancy of the surface and internal receptor pools was calculated to be 82 and 70%, respectively, and the overall occupancy of total cellular receptors was 73%. At the apparent steady state attained by 60 min in the presence of excess exogenous asialoglycoprotein, only 30% of the intracellular receptors were unoccupied. In other similar experiments, a true steady state level of occupancy of internal receptors of about 70% was achieved after 1-2 h. Therefore, a majority of the intracellular asialoglycoprotein receptors participate in some way in the internalization and/or intracellular routing of asialo-orosomucoid.

Fig. 3 also illustrates the significant kinetic difference between the rates at which the surface and total cellular receptors become occupied. For example, with 1.5 μg/ml asialo-orosomucoid in the medium, the surface receptor population reaches a steady state occupancy in less than 5 min (Fig. 3A), whereas there is still a significant rate of decrease in the number of available total cellular receptors occurring at 30 min (Fig. 3B). Likewise, the kinetics of the recovery of active receptors on the cell surface (Fig. 3A) is much more rapid than the recovery of total cellular receptors (Fig. 3B). At 0.10 μg/ml asialo-orosomucoid, the surface receptor content has recovered by 50% after approximately 18 min, whereas the total cellular receptor pool takes on the order of 40 min to attain a 50% recovery. This difference probably reflects the time necessary for a substantial fraction of the occupied internal receptors to dissociate from the internalized asialo-orosomucoid, consistent with recent reports on the rate of dissociation of intracellular receptor-ligand complexes (12, 13). Reversibility of the Apparent Receptor Activity Loss in the Presence of Nonradioactive Asialo-orosomucoid—The decrease in the number of active receptors detected with $^{125}$I-

![Fig. 3](http://www.jbc.org/)
asialo-orosomucoid in cells which have been incubated first with nonradioactive asialoglycoprotein reflects the occupancy of asialoglycoprotein receptors with the nonradioactive glycoprotein. In support of this, the apparent loss of receptor activity can be reversed by either washing the cells free of nonradioactive asialo-orosomucoid or by treating the digitonin-permeabilized cells with EGTA prior to the measurement of available receptor number. In the former experiment (Fig. 4), the onset of the recovery of active asialoglycoprotein activity can be reversed by either washing the cells free of available receptor number. In the former experiment (Fig. 4), the onset of the recovery of active asialoglycoprotein

![Graph](image1)

**Fig. 4.** Reversibility of the decrease in total cellular receptor occupancy after removing exogenous asialo-orosomucoid. Suspensions of freshly isolated hepatocytes (2 x 10^6/ml in Medium 1/BSA) were incubated at 37 °C for 45 min, after which PBS (○) or nonradioactive asialo-orosomucoid in PBS (■) was added to a final concentration of 3.0 μg/ml. After 30 min (arrow), a portion of the latter cell suspension (■) was centrifuged, washed once at 37 °C, and resuspended to the original cell concentration in Medium 1/BSA at 37 °C, and the incubation was continued. At the indicated times, samples were taken for the determination of the total cellular content of unoccupied receptors by measuring specific 125I-asialo-orosomucoid binding in the presence of digitonin as described in Fig. 1 and under "Experimental Procedures."  

![Graph](image2)

**Fig. 5.** Effect of an EGTA wash on the occupancy of total cellular and surface receptors of hepatocytes exposed to asialo-orosomucoid. Freshly isolated cell suspensions were incubated at 37 °C for 45 min and exposed to 3.0 μg/ml nonradioactive asialo-orosomucoid, and the unoccupied total cellular (○) or surface (■) receptor content was then determined using 125I-asialo-orosomucoid as described in Fig. 1 except for the following. The washed cell samples were incubated on ice for 20 min with (○) or without (■) 0.055% digitonin in Buffer 1/Ca^2+, and then washed and resuspended in ice-cold Medium 1/BSA with (○) or without (■) 7.5 mM EGTA (protein recovery is unaffected by this treatment). After 10 min, the cells were pelleted by centrifugation, washed again, and resuspended on ice with 125I-asialo-orosomucoid for the determination of unoccupied receptors.

![Graph](image3)

**Fig. 6.** Relative changes in surface and total cell receptor occupancy and in glycoprotein uptake and degradation during the continuous exposure of hepatocytes to asialo-orosomucoid. Cell suspensions were incubated at 37 °C for 45 min in Medium 1/BSA at which time either PBS alone (○, ■) or nonradioactive (△, ○) or iodinated (△, ■) asialo-orosomucoid in PBS was added to a final concentration of 0.15 μg/ml. A, availability of specific surface and total cellular receptors. At the indicated times, samples were taken for the determination of available (unoccupied) total or surface receptors using 125I-asialo-orosomucoid with (○, △) or without (●, ■) 0.055% digitonin, respectively. The dashed lines are the PBS controls (○, ■). B, endocytosis and degradation of 125I-asialo-orosomucoid. Internalization (△) and degradation (■) were determined as described under "Experimental Procedures."  

receptors began immediately after the nonradioactive glycoprotein was removed at 37 °C and within 90 min about 70% of the lost receptor activity was restored. During this time, active degradation of internalized protein occurred and the cellular content of asialoglycoprotein decreased (see below). In the latter experiment (Fig. 5), the disappearance of both surface and total cellular receptor activity was completely reversed by first treating the permeabilized cells with EGTA in the cold. This treatment strips asialo-orosomucoid from the receptor (19, 21). The receptor number detected after the EGTA wash was greater than at the start of the experiment (prior to the addition of asialoglycoprotein as we have reported before (19). This was true even on cells which were incubated first at 37 °C. The decrease in the number of active total cellular or surface receptors in the presence of asialo-orosomucoid is therefore due to the formation of receptor-ligand complexes.

*The Kinetics of Endocytosis, Degradation, and Surface and Total Cellular Receptor Occupancy Changes during...*
Continuous Endocytosis—Hepatocytes were exposed at 37 °C to a pulse of radioactive or nonradioactive asialo- orosomucoid (0.15 μg/ml) equivalent to approximately 7.8 times the total number of initial surface receptors. Within 5 min, the number of unoccupied surface receptors reached its nadir and then began to increase (Fig. 6A). By 40 min, the number of free surface receptors was equal to that in the control and then actually increased above this value before again stabilizing at the control level by 2 h. The number of unoccupied total cellular receptors decreased for about 10–15 min, and then leveled off and increased to the control level over another 100 min (Fig. 6A). In an identical parallel incubation, the accumulation of intracellular 125I-asialo- orosomucoid by hepatocytes proceeded linearly for about 30 min (Fig. 6B) and leveled off at the time of full recovery of unoccupied surface receptors (Fig. 6A). This also corresponded to the time (30 min) at which degradation began and the cellular asialo- orosomucoid content began to decrease (Fig. 6B).

In this experiment, therefore, by 30–40 min, the following changes have occurred. (i) An amount of asialo- orosomucoid equivalent to about 2.5 times the number of total cellular receptors has been internalized. (ii) Endocytosis has ceased. (iii) Surface receptors are again unoccupied. (iv) The lag period for delivery of ligand to lysosomes is over and degradation is underway. (v) About 40% of the intracellular receptors (23% of the total) are still occupied, although about 10 times more asialo- orosomucoid molecules (9.0 x 10^5 versus 9.6 x 10^5 molecules/cell) are not bound to receptors but rather are free inside the cell and not yet delivered to lysosomes.

Changes in Surface and Total Cell Receptor Occupancy during Endocytosis of Prebound Glycoprotein—In similar experiments (not shown), cells were first allowed to bind nonradioactive asialo- orosomucoid on ice, washed, and put at 37 °C, and the available receptors (detected with 125I-asialo- orosomucoid) were then quantitated at 0 °C in the presence and absence of digitonin. As expected, the recovery of active surface receptors was rapid and correlated well with the rapid rate of endocytosis previously reported under these conditions (16, 21). On the other hand, the recovery of internal receptor activity, reflected by the total available receptor content measured in the presence of digitonin, occurred more slowly, consistent with the results shown in Fig. 3.

**DISCUSSION**

The steroid glycoside digitonin has been used to increase cell permeability to small ions and metabolites (25, 26), to release intracellular enzymes (27), and to afford better electron microscopic visualization of cytoskeletal components (28). At low concentrations, digitonin is believed to make membranes permeable by binding to cholesterol (29). At high concentrations, it may behave more like a typical weak nonionic detergent, capable of solubilizing membrane phospholipids and proteins. Intracellular membranes that presumably lack cholesterol are not permeabilized by digitonin. For example, neither lysosomes nor mitochondria release their soluble enzymatic contents even at digitonin concentrations as high as 0.3% (27).

The ability of digitonin to increase the level of specific binding of asialo- orosomucoid in isolated hepatocytes clearly reflects the exposure of additional bona fide active receptor molecules since this increase is not observed with other types of cells 1 and is eliminated by antibody against the receptor. At least 90% of the receptors (assessed as receptor-ligand complexes) initially on the cell surface are unaffected by the treatment with digitonin. By inference, therefore, we assume that at least 90% of the intracellular receptors are detected in the presence of digitonin. However, the total cellular receptor number measured in the presence of digitonin is likely to be an underestimate. Potentially active receptors might still be in membrane compartments not made permeable by digitonin or in conformations or orientations not accessible to 125I-asialo- orosomucoid. In addition, not all receptor molecules on the surface of freshly isolated cells are able to bind ligand (19) and this may also be the case inside the cell.

The initial subcellular fractionation studies of Pricer and Ashwell (4) demonstrated that only about 10% of the total hepatic receptor activity could be recovered in a plasma membrane fraction; the remainder was in Golgi endoplasmic reticulum, and microsomal membrane fractions. Subsequent studies employing subcellular fractionation of whole liver (5), the use of receptor antibody in whole perfused liver (9), metabolic and surface labeling of receptor in cultured cells (8), solubilization of isolated cells with Triton X-100 (6, 7), sonic disruption of isolated cells (7), and, most recently, permeabilization of isolated cells with digitonin (14) have all also shown that the majority of asialo- orosomucoid receptor activity is inside the hepatocyte and not on the surface. 3 By the use of the digitonin technique, fully isolated rat liver receptors were found to have greater than 80% of their total receptors inside the cell, whereas in cells which were first equilibrated at 37 °C for 45 min, 50–75% of the total receptors were intracellular.

Given that the majority of receptors are intracellular, the question of whether these internal receptors function is extremely important in elucidating both the mechanism(s) by which they function and their route(s) of intracellular translocation. This question is particularly critical for studies in which the subcellular localization of receptor is monitored by ultrastructural techniques (e.g. Ref. 10), since the presence of nonfunctional receptors would preclude interpretation. In this report, we have shown that the total number of unoccupied cellular receptors, quantitated in permeabilized hepatocytes by the binding of 125I-asialo- orosomucoid at 0 °C, decreases when intact cells are first exposed to nonradioactive asialo- orosomucoid at 37 °C. This decrease in the total receptor number/cell is proportional to the time of exposure and to the concentration of external asialo- orosomucoid. The observed receptor number decrease reflects occupancy rather than the destruction or loss of receptors, since the decrease is transient at low concentrations, can be reversed in intact cells by removing the glycoprotein, and full receptor activity is restored simply by washing the digitonin-permeabilized cells with a Ca2+ chelator.

After several hours in the presence of excess asialo- orosomucoid in the medium, a steady state situation is obtained in which at least 70% of the intracellular receptors are present as receptor-ligand complexes and 30% are unoccupied. Initially, under either prebound or early continuous ligand uptake conditions, about half of the receptor-ligand complexes that enter the cell dissociate very rapidly and half very slowly (12). Current studies 3 indicate that, as a steady state receptor occupancy is reached, a larger fraction of internalized receptor-ligand complexes is processed by the fast dissociation pathway. If this did not occur, then the ability of the system to internalize ligand would continually decrease as a progressively larger fraction of the total receptor pool became occupied. In fact, the cell surface receptor number does not change during endocytosis (Fig. 5) and a rapid rate of endocytosis can be sustained for a long time. For this same reason, it is also necessary that, at a steady state, the rate of dissociation of

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3 We also find that cultured hepatocytes have greater than 50% of their total receptor number inside the cell, although under our culture conditions, the receptor number/cell (both total and surface) decreases continuously during time in culture (~50%/day). 4 J. A. Oka and P. H. Weigel, submitted for publication.
receptor-ligand complexes inside the cell must be greater than equal to the rate of internalization of these complexes. We have determined the first order kinetic rate constants for internalization and the fast dissociation process to be, respectively, \(0.27 \text{ min}^{-1}\) and \(>0.35 \text{ min}^{-1}\), which satisfies this condition. When the overall receptor system is operating at a steady state, there should be some percentage of its intracellular receptors unoccupied. We believe, therefore, that at least 70% and most likely all of the internal asialoglycoprotein receptors are functional during endocytosis. Bridges et al. (13) reported a slow dissociation \((k = 0.023 \text{ min}^{-1})\) of internalized complexes but not as a fast component. The reason for this is unclear, but there were substantial experimental differences between the two studies.

Two major possibilities exist for the functional role(s) of the internal asialoglycoprotein receptors. 1) The simplest situation is that all receptors are ultimately equivalent in their functional potential and that the internal receptors are in various stages in the overall functional cycle and correspondingly intracellular locations on their way back to the cell surface. In this case, a particular receptor molecule would be found, at progressive times, on the cell surface, then in an unknown number of internal compartments (in series or in parallel), and finally on the surface again, ready to begin another cycle. In this model, there would presumably be more intracellular receptors than surface receptors because of the nature (especially the relative rates) of the processes within the cell required (i) to dissociate receptor and ligand, (ii) to direct their separate trafficking (the former to the cell surface and the latter to the lysosomes), and (iii) to prepare the system for the next functional cycle. A priori, this latter process must require the input of metabolic energy (30) since otherwise a true receptor functional cycle (which is the sense in which the term recycling is employed), as opposed to a reversible reaction or process, would violate the laws of thermodynamics. The intracellular steps needed for these processes could certainly be more extensive in number and complexity than internalization per se and could therefore require more time, thus holding a greater fraction of receptors inside the cell rather than on the surface when the functioning system has reached a steady state. An important result from this study is that the intracellular receptors have binding characteristics for \([\text{125I}-\text{asialo-orosomucoid}]\) indistinguishable from the surface receptors. The observed half-maximal values for saturation of binding and the association constants calculated from equilibrium binding data for the two receptor populations are virtually identical. The binding equivalence of surface and internal receptors is consistent with the premise that there is a functional equivalence in the intact hepatocyte.

2) An alternative possibility is that the intracellular receptors serve either as a holding reservoir for internalized ligand, or as a shuttle system which directs some or all of the incoming ligand to the intracellular processing machinery mentioned above. In this model, all asialoglycoprotein receptors are not functionally equivalent. The bulk of the intracellular receptors, for example, would not appear on the cell surface during a limited number of functional cycles. This model is less favored since receptors are apparently internalized with the bound asialoglycoprotein (13, 31) and the transfer of ligand from surface to intracellular receptors is not an appealing possibility. However, as discussed above, all internalized receptor-ligand complexes are processed in the same manner inside the cell\(^4\) (12). The significance of there being two intracellular pools of internalized complexes is not clear but is consistent with a reservoir or shuttle function for at least some of the internal receptors.

Other workers have provided evidence that the internal receptors do not function during endocytosis in isolated rat hepatocytes (9, 11). In one study, neuraminidase-treated hepatocytes were shown to be capable of internalizing desialylated mucin, whereas these cells could not internalize asialo-orosomucoid (11). After neuraminidase-treated cells were allowed to internalize desialylated mucin for 50 min, they were still not able to bind or internalize asialo-orosomucoid, from which it was concluded that no new active (nondesialylated) receptors had appeared on the cell surface. However, it is likely that, under the conditions of these experiments, the many other desialylated cell surface glycoproteins would not have been removed from the surface and would, therefore, have competed with subsequently added asialo-orosomucoid for either new or old (damaged) receptors. Such competition could be very effective, which is presumably why neuraminidase-treated cells lose receptor activity. Therefore, even if new receptors had been inserted into the plasma membrane, they probably could not have been detected. In another study, receptor activity was not recovered after perfusion of intact liver with antibody against the receptor (9). This result would not be inconsistent with a functional role for intracellular receptors if the normal receptor cycle was altered by the bound antibody.

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**Note Added in Proof**—Recent experiments have led us to propose that possibility 2 above is correct; that all cellular asialoglycoprotein receptors are not functionally equivalent. This reconciles our present conclusion with that of Stockert et al. (9, 11) since in this model, most of the functional intracellular receptor pool does not recycle and does not exchange with the cell surface receptor pool.

**REFERENCES**

The large intracellular pool of asialoglycoprotein receptors functions during the endocytosis of asialoglycoproteins by isolated rat hepatocytes.


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