Galactose-specific Recognition System of Mammalian Liver: Receptor Distribution on the Hepatocyte Cell Surface

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ABSTRACT An isolated perfused liver system was used to study the distribution of asialoglycoprotein (ASGP) binding sites on rat hepatocyte cell surfaces. The number of surface receptors was quantitated by monitoring clearance of ^{125}I-labeled ligands from the perfusate medium under two conditions that blocked their internalization: low temperature (<5°C) or brief formaldehyde fixation. The cell surface distribution of binding sites was visualized in the electron microscope with either asialoorosomucoid covalently coupled to horseradish peroxidase (ASOR-HRP) or lactosaminated ferritin (Lac-Fer), both of which were bound with similar kinetics and to similar extents as ASOR itself. At low temperature or after prefixation, ASGP binding sites were present over much of the sinusoidal cell surface, but were concentrated most heavily over coated pits. Quantitation of ligand distribution at 4°C with Lac-Fer gave an ~70-fold greater density of ferritin particles over coated membrane than over uncoated regions. We obtained no evidence for gradual movement of ASGP receptors into or out of coated pits within the time-course of our experiments. Finally, the number and distribution of cell surface binding sites was unaffected by previous exposure to ASOR or by inhibition of endocytic vesicle-lysosome fusion and ASOR degradation at 16°C.

Desialylated glycoproteins (ASGPs) are removed from the circulation of mammals by the parenchymal cells of the liver (1). These cells possess a cell surface receptor that recognizes and binds molecules having exposed galactose, *N*-acetylgalactosamine, or glucose residues (1). After binding receptors, ASGPs are internalized via coated pits and coated vesicles and subsequently appear in a complex network of tubules and uncoated vesicles before reaching the lysosomes where they are degraded (2–4).

In our previous in vivo studies of ASGP internalization, little evidence was obtained for binding of the ligand to the hepatocyte cell surface outside of coated pits. However, the rapidity with which bound ASGPs entered the cell (5) precluded any conclusions regarding the localization of initial binding sites. Studies of receptor-mediated endocytosis in a variety of other systems have shown that ligand internalization can be inhibited by the use of low temperature, light fixation before binding, or a variety of inhibitors. Localization of epidermal growth factor (EGF) and α_2-macroglobulin (α_2M) in fibroblasts and human carcinoma cells under these conditions indicates that their initial binding to the cell surface occurs both in coated and uncoated regions of the plasma membrane (6–10). However, the binding of low-density lipoprotein(LDL)-ferritin at low temperature or after formaldehyde treatment shows a stronger preference for coated pits, a region of the cell surface comprising <2% of the total fibroblast surface area (11). Because internalization of all three ligands appears to occur primarily via coated pits and coated vesicles, movement of ligand-receptor complexes into coated pits has been suggested.

In this paper, we report the results of studies on ASGP receptor distribution under conditions that prevent ligand internalization. We have used the isolated, perfused liver system described by Dunn et al. (4), so that the temperature of hepatocytes and the perfusate composition can be controlled without disrupting normal cellular associations. Our finding is that ASGP binding sites are concentrated predominantly in

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coated pits under all conditions so far examined. A preliminary report of this work has been published elsewhere (12).

MATERIALS AND METHODS

Reagents

Lactoperoxidase (purified grade) and horse spleen ferritin (A grade) were obtained from CalBiochem-Behring Corp., American Hoechst Corp. (LaJolla, CA); horseradish peroxidase (type VI), 3-amino-1,2,4-triazole, 3,3'-diaminobenzidine (grade II), polyvinylpyrrolidone (PVP-40, pharmaceutical grade), and glucose oxidase (type V) were from Sigma Chemical Co. (St. Louis, Mo.); glutaraldehyde was obtained from EM Sciences (Fort Washington, Pa); Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Wilmington, Mass.

Isolated Liver Perfusion

Recirculating perfusion of rat livers was performed according to the procedure of Dunn et al. (4), using 125 ml of Krebs-Henseleit balanced salt solution containing 2% PVP-40 as the perfusate medium. The livers were perfused via a cannula inserted in the hepatic portal vein. Temperature was controlled by pumping the medium through a condenser maintained at the appropriate temperature by a recirculating water bath. The liver effluent was collected in a beaker through which 95% O_2/5% CO_2 was passed via gas-permeable fibers.

Single-pass perfusions were set up in a manner similar to that for recirculating perfusion except that the effluent solution was diverted before reentry into the liver and a fresh solution substituted. Liver temperature was determined by measuring the effluent medium as it flowed out of the organ.

Rats were fasted 12–24 h before sacrifice.

Binding and Dissociation of Ligand

The preparation of asialoorosomucoid and iodination of proteins have been described previously (2).

Ligands were added to the liver effluent, allowing maximal mixing with the perfusate before it entered the liver. The amounts and specific activities added are specified in the Results for each experiment. In some experiments, calcium present in the Krebs-Henseleit perfusion medium (2 mM) was removed by the addition of 2.5 ml of 0.5 M EDTA (final concentration of 10 mM EDTA) plus sufficient 5 N NaOH to maintain a constant pH. Because ASGP binding is strictly dependent on the presence of calcium (13), this treatment resulted in a prompt dissociation of accessible (i.e., noninternalized) ligand.

In protocols where the dissociation step was followed by subsequent binding of fresh ligand, the dissociated ligand and chelating agent were first rinsed out with at least 100 ml of fresh perfusate.

Prefixation

Isolated livers were fixed before binding by a single-pass perfusion with 150 ml of 2% formaldehyde (freshly prepared from paraformaldehyde) in Krebs-Henseleit/PVP-40 medium at 4°C and 25 ml/min. Excess fixative was then rinsed out by a single pass with 100 ml of cold perfusate medium before the organ was placed in the recirculating mode for subsequent binding studies.

Temperature-shift Experiments

Isolated livers were warmed rapidly from 2°–4°C to 30°–31°C by single-pass perfusion with 37°C medium that had not circulated through the condenser. The temperature of the liver effluent could be raised or lowered between 2°–4°C and 30°–31°C within 1.5–2 min after a temperature shift was begun. Switches between 2°–4°C and 16°C were accomplished by using two temperature-controlled water recirculators set at −7°C (for 2°–4°C perfusion) and 12°C (for 16°C perfusion).

Preparation and Visualization of EM Tracers

Procedures for the preparation of the asialoorosomucoid–horseradish peroxidase (ASOR-HRP) conjugate and of lactosaminated ferritin (Lac-Fer) have been described elsewhere (2). ^{125}I-Lac-Fer and ASOR-HRP, radioiodinated either in the ASOR moiety only or in both ASOR and HRP, were cleared from the perfusion medium by the isolated liver with kinetics similar to those found for ^{125}I-ASOR. Binding of iodinated ASOR, ASOR-HRP, or Lac-Fer gave similar numbers of surface ASGP receptors per cell (see Results for ^{125}I-ASOR data).

HRP cytochemistry was performed according to the protocol described earlier (2) or a slight modification of the procedure of Rodewald (14), in which incubations were for 2 h at 24°C and 3-amino-1,2,4-triazole (0.02 M) was added. The fixation protocol I of Franke et al. (15) was used in most Lac-Fer experiments to facilitate identification of coated membrane regions.

Quantitation of Lac-Fer Binding

Lac-Fer particles were counted at the electron microscope at a screen magnification of × 31,500 or from prints at a final enlargement of × 80,000. The sinusoidal region of hepatocytes was scanned by following the profile of the surface, including microvillar projections, and counting all Lac-Fer particles seen close to a membrane bilayer. Particles not clearly outside of the cell (i.e., those associated with grazing sections of the cell surface that may have been located just inside the membrane) were not counted, to avoid confusion with endogenous ferritin particles found free in the cytoplasm of all hepatocytes. Particles were scored as either (*a*) associated with uncoated membrane, including microvillar surfaces and smooth vesicle profiles near the cell surface, or as (*b*) associated with coated pits or coated vesicle profiles near the surface. The latter category was undoubtedly underestimated because any membrane region that was not obviously coated was scored as uncoated. Uncoated or coated vesicle profiles that were close to but not clearly contiguous with the surface membrane were included in the scoring for two reasons: (*a*) the EDTA dissociation experiments indicated that at low temperature virtually all bound ligand (>95%) was accessible to the chelator and hence at the surface; and (*b*) our previous work with livers fixed *in situ* indicated that ~50% of the coated vesicle profiles were not vesicles, but were areas of surface membrane whose continuity with the cell surface was not apparent because of the plane of section (see reference 2 for ruthenium red experiments demonstrating this).

Quantitation of Coated Pits and Vesicles

Estimation of the number of coated pits and vesicles at the sinusoidal surface of hepatocytes was done at the electron microscope at a screen magnification of × 12,500. Only hepatocytes whose entire periphery was visible and whose nuclei could be seen were scored in an attempt to eliminate those in which the plane of section only grazed the cell surface. Coated vesicle profiles that were not close to the cell surface (e.g., those deep within the cytoplasm in the Golgi-lysosome region) were not counted.

The fractional surface area contributed by coated pits at the sinusoidal surface was estimated as follows. Micrographs of the sinusoidal region of hepatocytes were taken at random at a standard magnification of × 10,000. Quantitative analysis was performed at a final magnification of × 27,000. The relative surface area of coated pits was determined by using a calibrated set of lines and recording the type of plasma membrane (uncoated vs. coated) that crossed the lines. 200 total crossings were recorded, two of which were over coated pits. A more extensive analysis was not performed, because of the very small area contribution of these structures. Rather, an upper limit of 2% of the total sinusoidal surface was assigned to coated pits.

RESULTS

Clearance and Metabolism of ^{125}I-Ligands by the Isolated Perfused Liver at 37°C

We first examined ^{125}I-ASOR clearance at 37°C to establish that the isolated liver was capable of normal binding and metabolism of ASGPs. Clearance of 0.02 nmol of ^{125}I-ASOR at 37°C occurred with a $t_{1/2}$ of 3–5 min and resulted in the removal of 70% of the total ligand from the medium by 15 min (Fig. 1). This rate was somewhat slower than the 1–1.5 min found in in vivo studies (2, 15–17), but was comparable to the results of Dunn et al. (4) with the isolated perfused liver. After the rapid initial decline in circulating radioactivity, a gradual increase was seen beginning at 15 min after ligand administration. This rise continued over the next hour and was accompanied by a decrease in the percent of acid-insoluble radioactivity in the perfusate, from 87 to 25%. These clearance kinetics and ligand solubility characteristics indicated that ^{125}I-ASOR was being internalized by cells in the liver, degraded to low molecular weight material (^{125}I and ^{125}I-monoiodotyrosine; see reference 3) and released back into the medium.

Low Temperature Perfusion

CONDITIONS THAT BLOCKED ^{125}I-ASOR CLEARANCE: To localize the initial ASGP binding sites on hepatocyte cell surfaces, it was necessary to first establish conditions

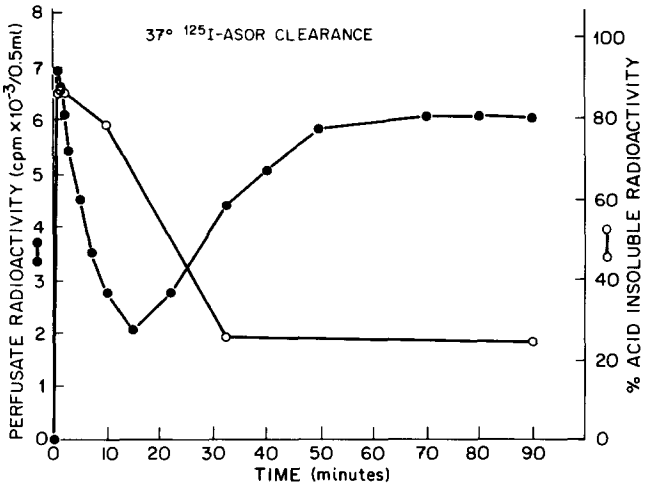

FIGURE 1 Clearance of ^{125}I-ASOR by an isolated perfused liver maintained at 37°C. The liver was allowed to equilibrate for 15 min at 37°C before the addition of 0.02 nmol (2.1×10^6 cpm) of ^{125}I-ASOR to the perfusion medium. 0.5-ml aliquots were taken at the indicated times, and radioactivity in each sample was counted. Each aliquot was cooled on ice before an equal volume of cold 10% TCA was added. After at least 1 h, the precipitates were centrifuged (15 min at 3,000 rpm), and the supernates and pellets were separated and counted. The increase in perfusate radioactivity starting at 15 min was attributable predominantly to acid-soluble material as evidenced by a decrease in the percentage of acid-insoluble radioactivity from 87% at 1 min to 25% at 30–90 min.

that allowed ligand binding but blocked ligand internalization. When the isolated liver was cooled to a temperature of 2°–5°C, the clearance kinetics of ^{125}I-ASOR (0.01 nmol) slowed to rates one third to one fourth of those seen at 37°C (see Fig. 2). In addition, no decrease in acid-insoluble radioactivity after the initial decline was seen at this temperature, even after 2 h of incubation. Furthermore, the addition of EDTA (final concentration 10 mM) to remove all free calcium from the perfusion medium resulted in a prompt rise of radioactivity in the perfusate back to the peak level seen after the initial addition of ligand (Fig. 2). Finally, all of the released ^{125}I-ligand was acid-insoluble. These results indicated that the ^{125}I-ASOR that was cleared from the perfusate at 2°–5°C had remained on the cell surface without internalization or subsequent degradation by hepatocytes.

The binding of ligand at low temperature was quite stable. Livers to which either 0.22 or 2.2 nmol of ^{125}I-ASOR was first bound for 60 min at 2–5°C were rinsed free of excess unbound ligand and then incubated for an additional 60 min each, first in the absence of circulating ligand, then in the presence of 2.2 nmol of unlabelled ASOR. Finally, sufficient EDTA was added to remove free calcium and dissociate accessible ^{125}I-ASOR. Perfusate radioactivity was monitored throughout and no detectable loss of bound ASOR from the cell surface occurred until the final EDTA step, at which time virtually all of the radioactivity that had originally been cleared from the circulation was released back into the perfusate (data not shown).

ESTABLISHMENT OF BINDING CONDITIONS REQUIRED TO SATURATE CELL SURFACE RECEPTORS: We next determined the conditions at 4°C necessary to saturate the available ASGP binding sites to ensure that we were visualizing the majority of ASGP receptors in our EM localization experiments.

The time-course of ^{125}I-ASOR-HRP clearance from the perfusate is shown in Fig. 3. Similar results were obtained when ^{125}I-ASOR was used as the ligand. An initial rapid phase of binding was followed by a gradual decrease to a plateau at around 2 h. In several experiments, the decrease in circulating ASOR-HRP between 1 and 2 h averaged ~15–20% of the total amount of ASOR-HRP bound over the 2-h period. A 1-h incubation was chosen as a standard and more convenient time

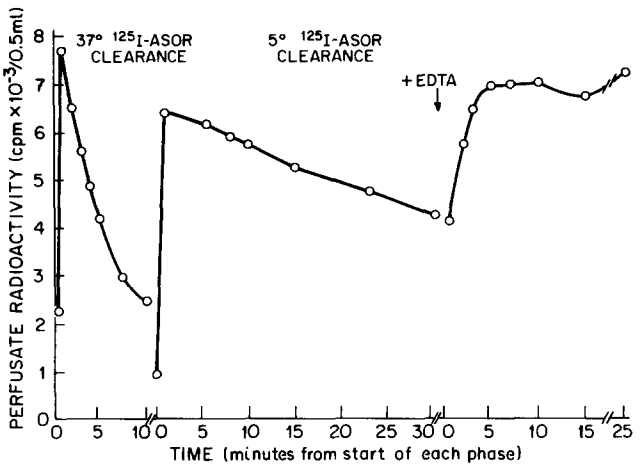

FIGURE 2 Effect of low temperature on clearance of ^{125}I-ASOR by the isolated perfused liver. After the liver was equilibrated for 15 min at 37°C, 0.01 nmol (3.2×10^6 cpm) of ^{125}I-ASOR was added to the perfusate, and 0.5-ml aliquots were taken at the indicated times. After 10 min, the liver was cooled to 5°C and a similar aliquot of ^{125}I-ASOR was added. Clearance was monitored for 30 min, then EDTA was added to dissociate accessible ligand, and aliquots were taken over the next 25 min. Removal of calcium by EDTA resulted in a prompt dissociation of the bound ASOR, indicating that internalization of the ligand had not taken place.

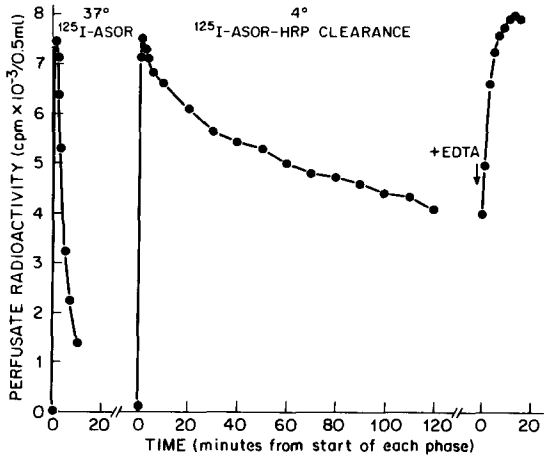

FIGURE 3 Time-course of ^{125}I-ASOR-HRP binding to the isolated perfused liver at 2°–5°C. After equilibration for 15 min at 37°C, 0.02 nmol (2.1×10^6 cpm) of ^{125}I-ASOR was added to the perfusate, and the incubation continued for 10 min, at which time the liver was cooled to 4°C and fresh perfusion medium was added. 2.2 nmol (4.8×10^5 cpm) of ^{125}I-ASOR -HRP (labeled in the ASOR moiety only) plus 0.02 nmol of ^{125}I-ASOR was added, and clearance was monitored for 120 min. Clearance at low temperature shows a rapid initial decline, then levels off to approach a plateau between 60 and 120 min. The addition of EDTA at 4°C resulted in a prompt release of ligand back into the perfusate. The extent of decrease in circulating radioactivity between 60 and 120 min in this experiment was somewhat greater than the usual 15–20%. The liver weighed 9.21 gm.

for subsequent binding and localization studies at the low temperature.

We then examined the effect of added ligand concentration on the amount of ASOR bound by the liver in 60 min at 2°–4°C. The results are presented in Fig. 4. Because the actual weight of the liver used in these experiments was not known until the end of the experiment, the amount of ASOR bound is expressed as the estimated number of surface receptors per cell, and the amount of ASOR added as a ratio of ASOR input to liver weight. Although the amount of ligand bound increased rapidly at low levels of added ASOR, binding began to approach a plateau of ~300,000 surface receptors per cell at ~4.4 nmol of added ligand (ratio: 20–30). Many assumptions are inherent in the calculation of this figure, including the following: (*a*) that 1 g of liver, wet weight, contains 1.38×10^8 parenchymal cells (18); (*b*) that the molecular weight of ASOR is 45,000, of ASOR-HRP is 90,000, and of Lac-Fer is 460,000; (*c*) that all ligand cleared was specifically bound; (*d*) that only hepatocytes, and not endothelial or Kupffer cells, bound the ligands; (*e*) that one receptor bound one ligand molecule, despite the multivalency of the ligand; and (*f*) that all regions of the liver were uniformly perfused. Because of these uncertainties, the actual numbers of receptors per cell can only be regarded as an estimate. Nonetheless, the values obtained were quite consistent in many different experiments using several different preparations of ligand.

The majority of experiments to be described below were performed with 2.2 nmol of ASGP added for a 60-min incubation time. Because 2.2 nmol of added ligand resulted in 70–80% of maximal binding at 60 min (Fig. 4: the dark bar on the abcissa indicates those experiments in which 2.2 nmol of ligand were added), and because a 60-min incubation gave 80–85% of the maximal binding at 120 min (Fig. 3), we estimated that we were visualizing 55–70% of the total cell surface receptors in our EM localization experiments.

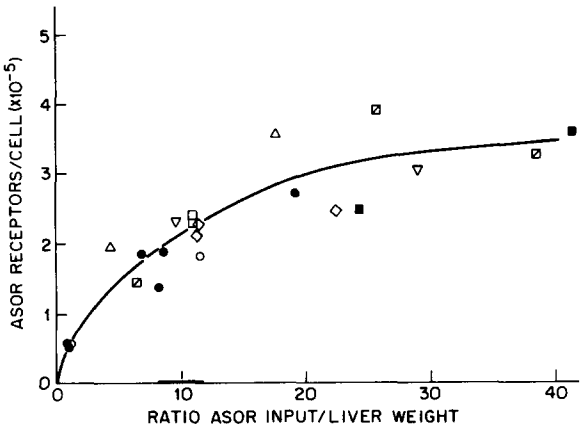

FIGURE 4 Effect of ligand concentration on the binding of ^{125}I-ASOR to the isolated perfused liver. The amount of ASOR bound is expressed on the ordinate as the estimated number of receptors per cell and the amount of ASOR added as the ratio of μg of ASOR input to the wet weight of the liver in grams. The dark bar indicates those ratios obtained with the addition of 2.2 nmol of ASOR, the standard ligand level used for most experiments. ^{125}I-ASOR (0.01 nmol) plus varying amounts of unlabeled ASOR (0.22–6.6 nmol) was added to the perfusate of isolated livers and allowed to circulate for 60 min at 4°C. EDTA was then added to a final concentration of 10 mM to ensure that all ligand was at the cell surface. In some experiments, dissociated ligand and EDTA was rinsed out and further rounds of binding were performed on the same liver (these points indicated by different open symbols for each liver). Filled symbols represent points derived from single rounds of ASOR (circles) or Lac-Fer (squares) binding, each to a different liver. Saturation of surface binding was not complete until the amount of ligand added was at least 4–5 nmol (ratios of 20 or more), giving a saturation level of ≅300,000 receptors/cell.

LOCALIZATION OF ASOR-HRP BINDING: Having established the conditions at 4°C resulting in maximum binding of ^{125}I-ASOR and ASOR-HRP without internalization, we next used the cytochemical tracer ASOR-HRP to visualize the ASGP binding sites on hepatocyte cell surfaces. Incubation of livers at 2°–5°C with 2.2 nmol of ASOR-HRP for 60 min, followed by processing of the tissue for the detection of HRP activity, revealed a nonuniform distribution of HRP staining on hepatocyte cell surfaces (Fig. 5 *A* and *B*). ASOR-HRP was concentrated in pits of 90–350 nm length at the surface of the cells, often at the base of microvilli. The rest of the cell surface was lightly stained in many, but not all, regions of the tissue. Stained pits were occasionally seen on the lateral surface of hepatocytes. Not all pits contained ligand, and stained pits could occasionally be found in close proximity to unstained ones (Fig. 5 *A*). Both the dark staining of pits and the lighter staining outside were eliminated when the liver was exposed to 10 mM EDTA for 10 min before fixation (Fig. 5 *D*), indicating that the staining observed was a result of ASOR-HRP binding to the cell surface. Kupffer and endothelial cells also showed calcium-dependent staining of pits in addition to a light staining of the cell surface.

Because we were concerned that some of the observed binding might be nonspecific, especially the low level seen outside of coated pits, a number of controls were performed. (*a*) Addition of a 60-fold excess of ASOR, together with ^{125}I-ASOR or ^{125}I-ASOR-HRP, drastically reduced clearance of the radiolabeled ligands from the perfusate medium, and eliminated both the heavy HRP staining of pits and the light staining of the rest of the hepatocyte cell surface. (*b*) In contrast, a similar excess of fully sialylated orosomucoid (OR) or mannan (a mannose-terminating glycoprotein that inhibits binding to endothelial and Kupffer cells) had little effect on clearance from the perfusate or distribution of ASOR-HRP on hepatocyte cell surfaces, although mannan reduced the staining observed on endothelial and Kupffer cells.

Exposure of livers to low levels of ASOR-HRP (0.22 nmol) resulted in <20% of maximum binding. Nonetheless, the same distribution of binding sites as that seen at higher levels (2.2 nmol of ASOR-HRP) was found, suggesting that binding sites both inside and outside of pits have similar affinities for the ligand.

EXAMINATION OF ASOR-HRP DISTRIBUTION AFTER DIFFERENT INCUBATION TIMES: To determine whether a slow movement of bound ligand was occurring during the 60-min incubation at 2°–5°C, we examined ASOR-HRP distribution after shorter incubation times. Binding of 2.2 nmol of ASOR-HRP for 20 (Fig. 5 *C*) or 40 min resulted in generally lighter staining both inside and outside of pits, with no obvious changes in the relative staining intensity of the two areas. Two attempts at binding for shorter times did not give sufficient staining for clear visualization of the ligand.

Next, ASOR-HRP was bound for 60 min at the low temperature, excess unbound rinsed out, and the livers incubated for an additional 60 min at a low temperature before fixation. The same result was obtained as when livers were fixed immediately after the initial 60-min binding. That is, regions were seen in which the entire cell surface was lightly stained, while surface

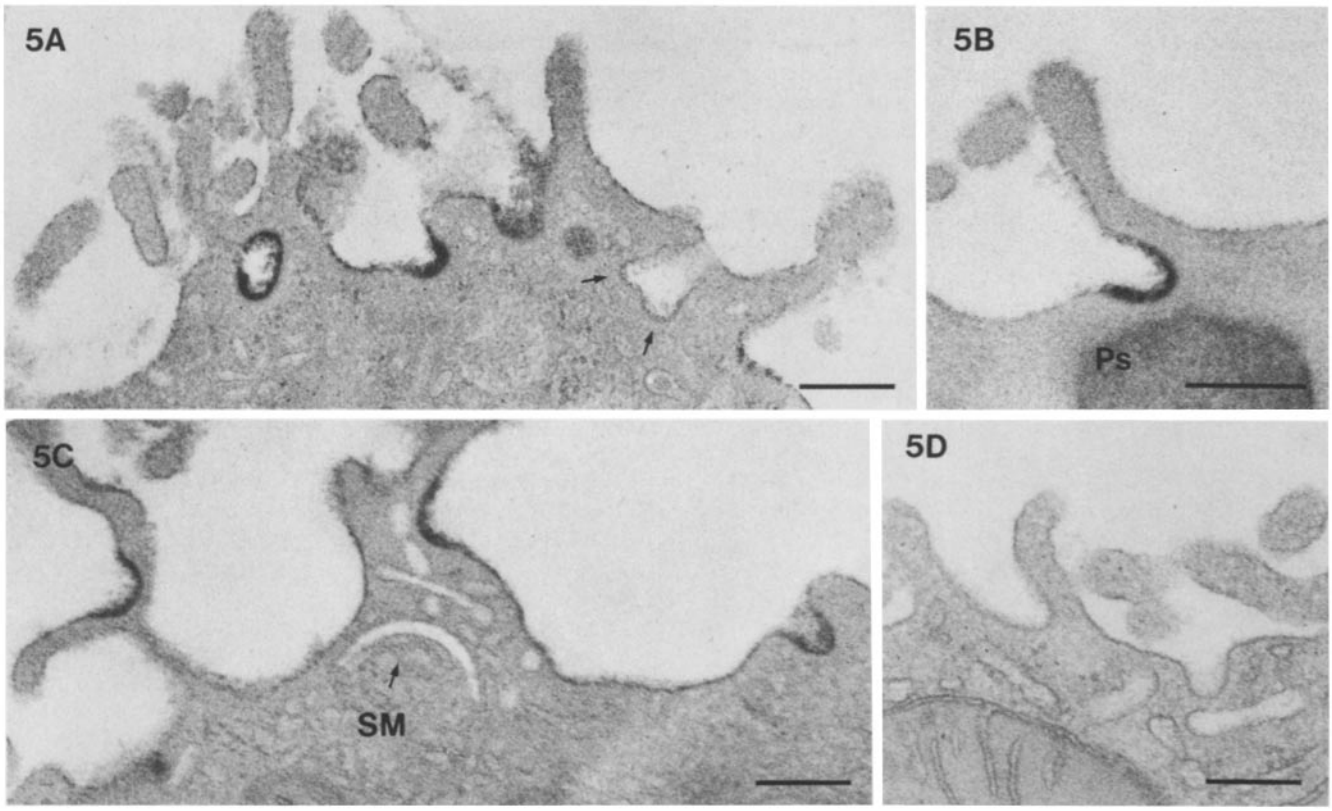

FIGURE 5 Electron micrographs of the peripheral cytoplasm of hepatocytes in livers fixed after incubation with 2.2 nmol ASOR-HRP at 2°–5°C. In all experiments, excess unbound ligand was rinsed out before the liver was fixed and processed for HRP cytochemistry. In *A*, *B*, and *D*, ASOR-HRP was bound for 60 min; in *C*, the liver was rinsed and fixed 20 min after ligand administration. Although HRP activity is concentrated in pits, a light staining is seen over most of the cell surface (most readily seen in *A* and *C*, and questionable in *B*). The staining outside of pits is particularly evident in areas where grazing sections pass across the periphery of microvillar projections, as in the central portion of *A*. The peroxisome seen in *B* (*Ps*) stains heavily because the catalase activity of these organelles was not inhibited by aminotriazole in this experiment. The liver shown in *D* was treated with 10 mM EDTA after the ASOR-HRP incubation. This treatment removed all surface activity, both inside and outside of pits. In *A*, heavily stained pits are seen in close proximity to unstained ones (unstained pits marked with arrows in *A*). In *C*, smooth-surfaced membrane vesicles and tubules (*SM*) are prominent in the hepatocyte periphery. *A*, *C*, and *D*: × 50,000. Bar, 0.25 μm. *B*, × 80,000. Bar, 0.2 μm.

pits showed heavy deposits of HRP reaction product. This result argues against a gradual shift of ligand molecules into pits from the lightly stained areas.

LOCALIZATION OF LAC-FER BINDING: QUANTITATION OF LIGAND DISTRIBUTION: Although the ASOR-HRP conjugate was extremely useful as an indicator of overall distribution of ASGP binding sites, this ligand did not allow the binding to be readily quantitated. In addition, we were not able to visualize coated membrane regions after HRP cytochemistry was performed. Therefore, we turned to the use of the particulate tracer Lac-Fer to estimate the relative numbers of ASGP receptors associated with coated vs. uncoated membrane regions.

Binding of Lac-Fer (8 nmol for 60 min or 14 nmol for 125 min) at 2°–5°C revealed a similar distribution of ASGP binding sites as the ASOR-HRP experiments. 58% of the total Lac-Fer particles counted (362 out of 628 total: counts from two experiments were similar and were therefore pooled) were associated with coated membrane regions which comprise <2% of the total surface membrane area (see Fig. 6). The remaining 42% of Lac-Fer was associated with uncoated membrane, making the concentration of Lac-Fer over coated regions at least 67-fold higher than the concentration over uncoated membrane. Because coated pits were often difficult to identify with certainty in the Lac-Fer experiments, we consider this value (67-fold higher) to be a lower limit of ASGP concentration in coated pits of hepatocytes.

Using the data from these morphological experiments, the number of Lac-Fer particles bound per hepatocyte was calculated to be 5,400 to 12,000.[1] This value is approximately equal to 5% of that obtained from biochemical studies with ^{125}I-Lac-Fer (see Fig. 4). Two factors are known to contribute to this discrepancy: (*a*) binding of Lac-Fer to sinusoidal lining cells, which could account for no more than 25% of the total Lac-Fer bound, according to ASOR competition experiments (not shown); (*b*) loss of 40–50% of the bound Lac-Fer during processing of the tissue for EM. With these corrections, we still obtain a lower estimate of Lac-Fer particles bound using morphologic techniques (16% of that expected from biochemical data). However, other factors that are more difficult to

[1] This number was obtained using 17.7 μm for the diameter of hepatocytes, a section thickness of 75 nm (250–320 sections/cell), an average of 5–11 coated pit profiles for hepatocyte per section, an average of two Lac-Fer particles/pit profile and 42% of total Lac-Fer bound outside of pits.

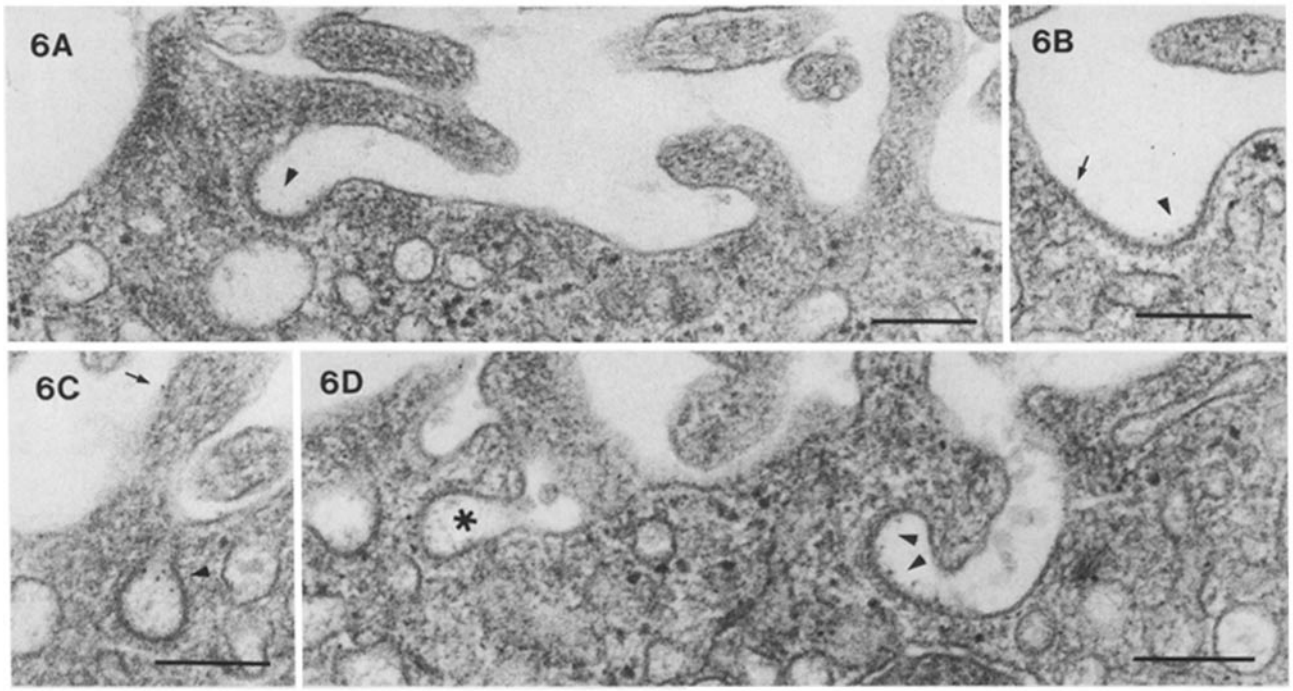

FIGURE 6 Electron micrographs of the peripheral cytoplasm of hepatocytes in livers fixed after incubation with Lac-Fer (8 nmol) for 60 min at 4°C. Excess Lac-Fer was rinsed out with 4°C perfusion medium before the tissue was fixed by procedure I of Franke et al. (15). Binding of Lac-Fer occurred preferentially in coated pits (indicated by arrowheads: 58% of total particles counted). Ferritin particles associated with uncoated membrane are indicated by small arrows. An asterisk indicates a coated pit in which no clearly discernible ferritin particles are found. The number of Lac-Fer per pit is higher in these selected micrographs than the average values seen. However, it should be noted that empty pits contributed to the low average value of two Lac-Fer per pit used in the quantitation of Lac-Fer binding. *A*, × 70,000; *B–D* × 80,000. Bars, 0.2 μm.

quantitate also contribute to an underestimation by morphology: (*a*) uncertainty in the identification of some Lac-Fer particles because of their poor contrast against the background material; (*b*) elimination from quantitation of any Lac-Fer particles not clearly seen to be on the outer aspect of a membrane cross-section; (*c*) underestimation of the number of coated pit profiles per section because of the difficulty in recognizing coats in some areas. Taking all of these factors into consideration, the quantitation of Lac-Fer binding site concentration in coated pits must be interpreted with caution, because the possibility of selective loss exists. Nevertheless, the similarity in binding pattern of ASOR-HRP in pits of similar size and distribution as coated pits reinforces the Lac-Fer data.

Ligand Distribution after Prefixation

Incubation of cells at low temperatures appears not to prevent movement of some membrane molecules within the bilayer (19). Therefore, the clustering of ASGP receptors in coated pits that we observed at low temperature may have resulted from movement induced by ligand binding. We were therefore prompted to examine other methods for blocking ligand uptake to reinforce our conclusions from the low temperature experiments. The use of formaldehyde fixation was suggested by the results of Willingham et al. (8) in which α_2M receptors showed a less clustered distribution after brief fixation than they did at 4°C.

CONDITIONS THAT BLOCKED LIGAND UPTAKE: A brief treatment by single-pass perfusion of 2% formaldehyde in Krebs-Henseleit perfusion medium at 4°C was found sufficient to completely block internalization of ^{125}I-ASOR at 37°C (see Fig. 7). This is, exposure of a prefixed liver to ASOR at 37°C, followed by subsequent exposure to EDTA, resulted in complete dissociation of bound ligand. In contrast, exposure of control livers to ASOR at 37°C resulted in clearance and degradation (Fig. 1). In addition, prefixation had no effect on the extent of binding (i.e., the numbers of cell surface receptors per cell determined at 2°–5°C before or after formaldehyde treatment) or kinetics of binding at either 37° or 2°–5°C. These results indicated that formaldehyde prefixation blocked internalization (even at 37°C) and could therefore be used to visualize a representative population of ASGP receptors at the hepatocyte cell surface. However, the EM localization experiments were performed at low temperatures because morphological damage occurred if formaldehyde-treated livers were subsequently warmed at 37°C.

LOCALIZATION OF ASOR-HRP BINDING AFTER PREFIXATION: Binding of 2.2 nmol of ASOR-HRP for 60 min at 2°–5°C to formaldehyde-treated livers revealed a distribution of ASGP binding sites that was essentially identical to that seen at low temperature without prefixation (compare Fig. 8 with Fig. 5*A–C*). The tracer was again concentrated in pits at the cell surface, with light staining of the surface outside of pits in many areas.

Other Approaches to Blockage of Ligand Internalization

We have attempted to inhibit endocytosis using, in addition to low temperature and prefixation, a variety of reagents reported by others to be effective. To date, of 10–15 mM methylamine, 1 mM phenylglyoxal, 0.4 mM *N*-ethylmaleimide, 600 μM dansylcadaverine, and 10 mM NaF plus 1 mM KCN, none has prevented ASOR internalization. We are continuing

to investigate the use of these and other inhibitors in our study of ASGP endocytosis.

Modulation of ASGP Receptor Activity and Effects on Cell Surface Receptor Number and Distribution

The localization experiments described above were performed on livers that had not been previously exposed to ASGPs under conditions allowing binding and internalization (i.e., elevated temperatures). Prior physiological exposure of each liver to circulating ASGPs was therefore an unknown variable. Because active internalization of ASGPs might be expected to influence the disposition of these receptors within the various subcellular compartments, including the cell surface, we investigated the effects of receptor activity on the number and distribution of ASGP cell surface binding sites.

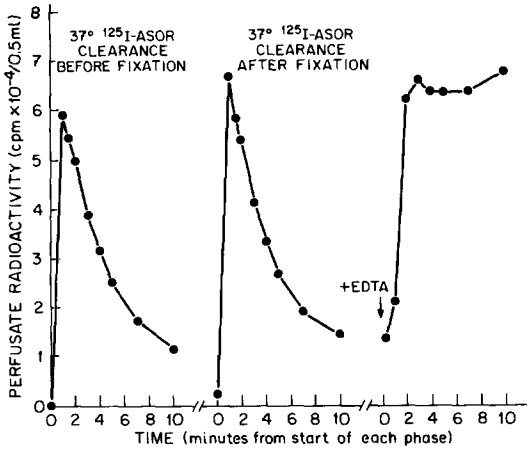

FIGURE 7 Effect of formaldehyde prefixation of clearance of ^{125}I-ASOR by the isolated perfused liver. The liver was allowed to stabilize at 37°C for 10 min before 0.01 nmol of ^{125}I-ASOR (1.2 × 10^6 cpm) were added to the perfusate. After monitoring clearance for 10 min, we prefixed the organ at 4°C by single-pass perfusion with 150 ml of 2% formaldehyde in Krebs-Henseleit medium. Fixative was rinsed out, and the liver warmed to 37°C before a second ^{125}I-ASOR clearance was performed exactly as before. After the end of the second clearance period, EDTA was added to 10 mM to dissociate surface-bound ligand. Prefixation had no effect on the kinetics of clearance, but the EDTA dissociation indicated that all of the bound ASOR was still at the cell surface.

EFFECT OF PREEXPOSURE TO ASOR ON CELL SURFACE ASGP RECEPTORS: First, when an excess (4.4 nmol) of unlabeled ASOR was perfused through a liver for 20 min at 37°C before glutaraldehyde fixation and processing for optimal visualization of coated pits, no increase was observed in the number of coated pits or coated vesicles found in exposed livers when compared to controls perfused 20 min at 37°C without added ASOR (see Materials and Methods for the method of scoring used). Therefore, it appears that the process of ASGP internalization does not induce a marked increase in the frequency of these structures.

Second, the distribution of ASOR receptors appeared not to be influenced by previous internalization of ASOR, because deliberate introduction of the ligand at 37°C before cooling and ASOR-HRP binding and visualization, resulted in a surface staining qualitatively identical to that of unexposed cells. This distribution was obtained whether the livers were preexposed to trace amounts of ASOR (0.01 nmol or 5,900 molecules/cell for 10 min at 37°C) or to amounts 8.7-fold in excess over the number of cell surface receptors, assuming 300,000 receptors/cell (4.4 nmol or 2.6 × 10^6 molecules/cell for 30 min at 37°C). In the latter experiment, 27% of the added ligand (702,000 molecules/cell or 2.3 times the number of receptors at the cell surface) was cleared from the medium. Neither form of ASOR preexposure altered the number or distribution of cell surface receptors.

TEMPERATURE-SHIFT EXPERIMENTS: We next examined the distribution and number of surface binding sites in livers that were saturated with ASOR at low temperature and then warmed to induce synchronous internalization of the ligand. These experiments also tested the capacity of ligand bound in and out of coated pits at low temperatures to be subsequently internalized. 2.2 nmol of ^{125}I-ASOR were bound for 60 min at 2°–5°C, excess unbound ligand was rinsed away, and the liver was warmed to 31°C for 8 min before being returned to low temperature for EDTA treatment. After the warm-up, 85–90% of ^{125}I-ASOR was no longer dissociable by EDTA, indicating that most of the ligand was initially bound to the cell surface via functional receptors capable of rapidly internalizing the molecules bound to them. In a separate experiment, we found that 63% of the bound ligand was no longer

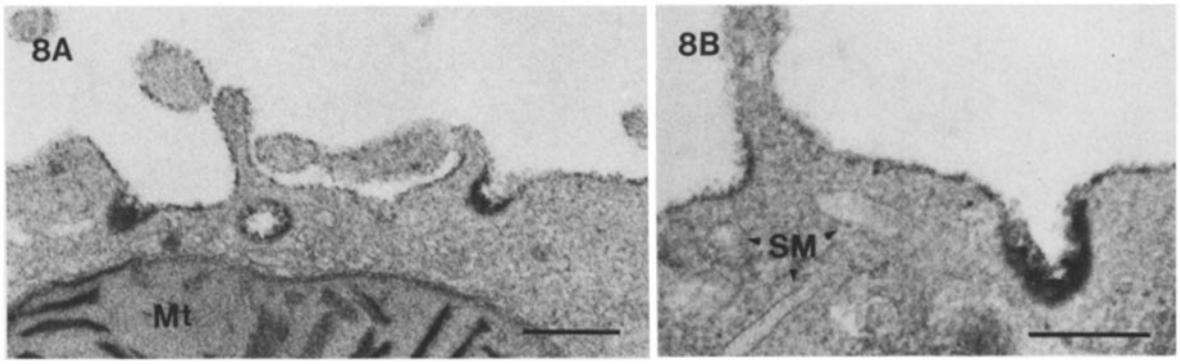

FIGURE 8 Electron micrographs of the peripheral cytoplasm of hepatocytes in livers fixed after formaldehyde prefixation and ASOR-HRP binding at 4°C. 2.7 nmol of ASOR-HRP were added to the perfusate of a liver that had been prefixed by single-pass perfusion with 2% formaldehyde. HRP activity can be detected over most of the cell surface, but is much more concentrated in pits than in other areas. The dark staining of mitochondrial (*Mt*) cristae results from the activity of cytochrome oxidase. (*A*) A series of darkly stained surface pits are seen along the sinusoidal surface of an hepatocyte. × 50,000. Bar, 0.25 μm. (*B*) Smooth-surfaced membrane vesicles and tubules (*SM*) are apparent in the peripheral cytoplasm of hepatocytes. × 80,000. Bar, 0.2 μm.

accessible to EDTA dissociation after a 3.5-min warm-up, giving a $t_{1/2}$ for internalization of <3.5 min (Table I). Furthermore, no significant change could be detected in the number of surface receptors during active internalization. This is, cells that had previously been exposed to 2.2 nmoles of ASOR at 4°C for 60 min, warmed to 31°C for 3.5 min, cooled, exposed to EDTA, and then rechallenged with ^{125}I-ASOR, bound 225,000 molecules of ligand per cell, as compared with 179,000 before warming. A similar result was obtained when ASOR binding was done at low ligand levels (Table I).

The constancy of cell surface receptor number after one round of ligand binding and internalization prompted us to compare the distribution of the new binding sites after such exposure to that found in control livers (i.e., unexposed cells). An experiment was performed in which 2.2 nmol of ASOR was added at 4°C for 60 min, the ligand was rinsed out, and the liver was warmed to 31°C for 4.5 min and then incubated with 2.2 nmol of ASOR-HRP for 60 min at 4°C. In this case only those cell surface receptors that had been vacated or that had appeared on the surface during the warm-up would be visualized, because the cytochemical tracer was added only during the second incubation. Nevertheless, the distribution of ASOR-HRP on the hepatocyte surface was the same as that seen at 4°C with no warm-up, i.e., a light staining of HRP reaction product over most of the surface and its concentration in pits. We therefore obtained no evidence for transient alterations in receptor number or disposition in the membrane during active internalization of asialoproteins. Nevertheless, it should be noted that our binding conditions were subsaturating, making a precise determination of actual receptor number difficult.

16°C PERFUSION: QUANTITATION OF ASGP RECEPTORS ON THE CELL SURFACE DURING INTERNALIZATION IN THE ABSENCE OF DEGRADATION: Temperature manipulation can be used to block certain steps in the endocytic process occurring after ligand internalization. Dunn et al. (5) have recently shown that livers maintained at 16°C will internalize ASGP but not degrade it, because of a failure of endocytic vesicles to fuse with lysosomes. We therefore used 16°C perfusion to investigate whether inhibition of the delivery of ligand to lysosomes would alter the number or distribution of cell surface ASGP receptors.

An isolated perfused liver maintained at 16°C was allowed to internalize ASOR at a very high ligand concentration (13.3 nmol of ASOR added) for 245 min, at which time 16% of the circulating ligand (780,000 molecules/cell) was cleared from the perfusate, and the decline in perfusate radioactivity approached a plateau. The liver was then rapidly cooled to 4°C, and any remaining surface-bound ASOR was removed with EDTA and rinsed out, together with unbound ligand. ASOR-HRP (2.2 nmol) plus a trace amount of ^{125}I-ASOR (0.013 nmol) was then bound for 60 min at 4°C. The number of cell surface receptors was measured at 202,000/cell, which was similar to the numbers obtained in untreated controls (150,000–250,000/cell). This result suggests that involvement of the lysosomal compartment is not essential to receptor replenishment on the cell surface.

TABLE I

Effect of Ligand Internalization on Subsequent Binding to the Cell Surface *

	ASOR added‡	ASOR bound or internalized		Percent of total ASOR bound or internalized
	nmol	*nmol*	*molecules/cell*	
First binding	2.2	0.63	179,000	28
	0.23	0.17	83,000	74
Internalized during warm-up	2.2	0.40	116,000	63§
	0.23	0.12	58,000	71§
Second binding	2.2	0.78	225,000	35
	0.23	0.17	83,000	74

* The experimental protocol was as follows: (*a*) bind 2.2 or 0.23 nmol ^{125}I-ASOR (3.2×10^6 cpm of ^{125}I-ASOR plus 100 μg or 10 μg unlabeled) at 4°C for 60 min; (*b*) rinse out excess ligand with fresh medium; (*c*) warm to 31°C for 3.5 min, then switch back to 4°C; (*d*) dissociate accessible ligand with EDTA; (*e*) rinse out released ligand and EDTA with fresh medium; (*f*) bind ASOR as in step *a*.

‡ 2.2 and 0.23 nmol represent 2.1 and 0.24 times the number of cell surface receptors (assuming 300,000 surface receptors/cell) because the liver weights were 15.2 and 13.7 gm, respectively.

§ Percent of total bound that was internalized during a 3.5-min warm-up to 31°C.

DISCUSSION

ASGP Receptors Are Concentrated in Coated Pits in the Absence of Internalization

The work reported in this paper was undertaken to investigate the distribution of ASGP receptors on the hepatocyte cell surface in the absence of ligand internalization. Both low temperature and brief formaldehyde fixation were used to block endocytosis, and both revealed the same pattern: ASGP binding sites were concentrated preferentially in coated pits but, in many areas, were also present over the rest of the cell surface.[2]

Nonrandom distribution of cell surface receptors has been described in other systems. In cultured human fibroblasts, LDL (11, 20, 21), α_2M (8, 9), and EGF (6) all showed some preference for binding to coated membrane regions at 0°–4°C. However, in none of these cases was the specificity for coated membrane absolute. That is, binding of ligands also occurred outside of these specialized areas of the plasma membrane. In the same studies, quantitation of surface binding sites revealed that the amount of ligand associated with coated pits was quite variable: from 10% of the total bound α_2M, yielding a five times greater concentration in coated pits than over the remaining surface (assuming the former to be 2% of the total cell surface area), to 34% of EGF (25 times greater) to 49–83% with LDL (47–244 times greater). In our experiments, 58% of the Lac-Fer particles were associated with coated pits, giving a 67 times greater concentration over these regions than over the remaining cell surface.

The results of prefixation studies on fibroblast surface receptor distribution varied with the ligand used. Formaldehyde treatment (2.5% in phosphate-buffered saline at 4°C for 45 min) did not change the LDL-ferritin distribution from that seen at 4°C without fixation (11), but reduced the clustering of α_2M receptors into coated pits (8; 0.2% HCHO in phosphate-buffered saline at 23°C for 5 min). Because fixation with formaldehyde (2% HCHO in Krebs-Henseleit/PVP 40 at 4°C for 6 min) had no discernible effect on ASOR-HRP distribution

[2] For purposes of discussion, we will assume that the ASGP receptor is responsible both for initial binding of the ligand to the cell surface as well as for its subsequent internalization, and use the terms "binding site" and "receptor" interchangeably.

at the hepatocyte cell surface, our results most closely parallel those obtained in the LDL receptor studies. However, Ziomek et al. (19) have shown, by varying the concentration of fixative, that the movement of integral membrane proteins in isolated mouse intestinal cells is slowed only at HCHO concentrations >4% (at 0°C for 15 min). In the absence of such titration studies, it is difficult to know whether the variability of receptor distributions in different systems is a result of the considerable variation in fixation conditions. An attempt at increasing the extent of fixation in our system by raising the temperature of the formaldehyde solution resulted in loss of ASGP binding activity.

The effect of incubation time on the observed ligand distribution is of interest because in all localization studies the binding times used have generally been quite long (2 h at 4°C for LDL-ferritin [11] and α_2M-ferritin [8], 40 min at 4°C for EGF-ferritin [7]). 20 min was the shortest binding time that allowed visualization of HRP activity in our experiments. However, staining intensity can vary from experiment to experiment, raising the possibility that shorter incubation times could be examined. Nevertheless, no difference in ASOR-HRP distribution was seen when incubation times with the ligand varied from 20 min to 2 h. From these data we can not say whether concentration of the ASGP receptor in coated pits in the absence of internalization occurs before or after the ligand attaches. In the former case, concentration into pits could occur either by lateral movement of membrane molecules after their random insertion into the bilayer, or by preferential insertion of receptor molecules into coated membrane regions. In the latter case, movement of the ligand-receptor complex would have to occur at low temperature and after formaldehyde fixation to be consistent with our observations. This possibility is suggested by the results of Ziomek et al. (19) on localization of alkaline phosphatase and leucine aminopeptidase in isolated mouse intestinal cells. These integral membrane glycoproteins undergo redistribution by lateral diffusion after cell dissociation, at either 4°C or after fixation with 0.5–3% formaldehyde. However, if ligand-induced redistribution did occur, it is not clear why a fraction of the occupied ASGP receptors would persist along uncoated regions of the sinusoidal membrane, even after long incubations at 4°C.

Coated Pits May Be Specialized for the Uptake of Different Ligands

The appearance of unstained pits in close proximity to stained ones reinforces similar observations made in our previous in vivo experiments, and suggests that coated membrane regions may be specialized for the uptake of different ligands. Nevertheless, it has been shown that α_2M and vesicular stomatitis virus can enter fibroblasts together within the same coated pits (10). If this finding can be generalized to the hepatocyte, it suggests that the concept of one receptor type per pit does not apply.

Consistency of Cell Surface Receptor Number: Receptor Reutilization

Several reports on the dynamics of the ASGP receptor have suggested that this membrane glycoprotein is spared degradation while the ligand it binds is catabolized within lysosomes. For instance, determination of the ASGP receptor $t_{1/2}$ in vivo by Tanabe et al. (22) yielded a value of ~88 h, either in the presence or absence of a large excess of circulating ASOR. Furthermore, in 180 min at 37°C, isolated hepatocytes treated with cycloheximide to block protein synthesis were capable of binding and metabolizing 34 times the cell surface binding capacity (or twice the total cellular binding capacity, if both internal and external receptors are included in the calculation). In the absence of cycloheximide, the cells metabolized 4 times the total cellular binding capacity. Such observations argue strongly for ASGP receptor reutilization. The subcellular compartments involved in this reutilization process are not known. At one extreme, the ASGP receptor may never leave the cell surface. At the other, it may enter the cell still complexed with its ligand and be processed through various subcellular compartments by an as yet unidentified salvage pathway before returning to the plasma membrane.

In our temperature-shift experiments, the number of ASGP receptors present at the cell surface appeared to be quite constant even when hepatocytes were actively engaged in ASGP internalization. Hence, the same number and distribution of receptors were seen after binding sites were saturated and the bound ligand was allowed to enter the cell during a brief warm-up. This result demonstrated that the cell maintained a numerically constant population of ASGP receptors at its surface. At least three alternatives could be responsible for this phenomenon: (*a*) the receptor could discharge its ligand to an intracellular pool without leaving the surface, (*b*) the receptor-ligand complex could be internalized and the same receptor molecule returned to the surface after ligand dissociation, or (*c*) the complex could be internalized and replaced by a different receptor molecule. The recent results of Stockert et al. (23) suggest that the same receptor molecule is reutilized, a result consistent with alternative *a* or *b*. These investigators reported that isolated hepatocytes whose accessible ASGP receptors were altered by neuraminidase treatment maintained a population of modified receptors at the cell surface. This occurred despite concurrent endocytosis of 20 times the surface binding capacity of desialylated ovine submaxillary mucin (an *N*-acetylgalactosaminyl-terminating glycoprotein that is still recognized by the neuraminidase-treated receptor). Thus, even though only 5–10% of the total binding capacity of the hepatocyte is present at the cell surface (24, 25) the large intracellular pool of ASGP receptors does not appear to supply the needed replacements. Our data indicate that the reutilization mechanism would have to occur very rapidly to avoid a transient decrease in the cell surface receptor population. Nevertheless, the possibility remains that such a decrease in surface receptors may have occurred, but was not detected in this experiment because reappearance of depleted receptors in the plasma membrane took place during the 60-min incubation of 2°–5°C necessary for quantitation of receptor number. If this were true, it would mean that such recycling was less sensitive to low temperature inhibition than the process of ligand internalization.

Lysosomes have been implicated in the ASGP receptor recycling pathway by three observations. First, ASGP binding activity has been detected in lysosomal fractions from rat hepatocytes, although the receptor was apparently oriented with its binding site toward the cytoplasm rather than facing the lysosomal lumen (22). Second, Tolleshaug and Berg (26) have reported that exposure of isolated hepatocytes to chloroquine, a drug that inhibits degradation of proteins in lysosomes, results in a decrease in the binding capacity of hepatocyte plasma membranes to 15% of control levels. However, chloroquine reduced cell viability, its effect was not reversible, the

concentration necessary to reduce binding capacity was higher than the level needed to block lysosomal degradation, and the postulated intracellular accumulation of ASGP binding activity was not substantiated. Third, ligand-receptor dissociation in vitro occurs at low pH (<6) and the lysosome is the only known organelle with such an acidic environment (27). Two of our present findings argue against such suggestions of lysosomal involvement in the receptor salvage pathway: (*a*) the inhibition of fusion of endocytic vesicles with lysosomes at 16°C had no effect on the number of cell surface receptors, and (*b*) the lack of a measurable decrease in receptor number or change in their distribution in the temperature-shift experiments. Taken together, our observations and the results of others discussed above indicate that receptor reutilization occurs by a very fast mechanism capable of maintaining a constant population of cell surface receptors that does not interchange rapidly with a much larger intracellular pool.

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