

# Immunocytochemical Localization of the Receptor for Asialoglycoprotein in Rat Liver Cells

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**ABSTRACT** We used high-resolution immunocytochemistry on ultrathin frozen sections labeled with colloidal gold to study the subcellular distribution of the asialoglycoprotein receptor in rat liver. The receptor was localized along the entire hepatocyte plasma membrane, including the bile capillary membrane, but was scarce intracellularly. Sinusoidal lining (Kupffer) cells and blood cells showed no immunoreactivity.

In liver cells of rats injected with 1 to 100  $\mu\text{g}$  of asialoorosomucoid (ASOR) 2–15 min before tissue fixation, endocytotic internalization of receptors at the blood front was conspicuous. At all times in this interval, receptor was present in  $\sim 100\text{-nm}$  vesicles and larger vacuoles adjacent to the sinusoidal plasma membrane. No other significant intracellular receptor was noted during the 15-min exposure to ASOR; in particular, lysosomes and Golgi complex were not labeled. Our observations, in combination with data from the literature which demonstrate that, under these conditions, the ligand is transferred further to the Golgi complex–lysosome region, suggest that the receptor and ligand are dissociated in the vicinity of the plasma membrane, after which the receptor rapidly returns to the cell surface.

Receptor molecules in cellular membranes play an important role in the unidirectional transport of exogenous and endogenous ligands. Different receptors are capable of recognizing and binding a variety of blood plasma proteins such as insulin, IgE, Fc fragment of Ig, lipoproteins, and transferrin. These protein ligands are then selectively internalized and, generally, degraded. One such well-characterized system is the hepatocyte receptor for asialoglycoproteins, initially described by Ashwell and co-workers (1, 2). This receptor, specific for galactose-terminal glycoproteins (asialoglycoproteins [ASOR]), mediates the rapid and specific binding and internalization of asialoglycoprotein ligands. Several morphological studies with artificial asialoglycoprotein ligands have elucidated the intracellular pathway taken by the ligand once it is bound to the hepatocyte plasma membrane (3, 4, 5). The ligand enters the cell by means of endocytotic vesicles, which transfer their contents to larger vacuolar structures. These structures move towards the Golgi complex–lysosome area, a process which takes  $\sim 15$  min. There, at least a portion of the ligand molecule is degraded, presumably by lysosomal action.

The receptor molecule, however, does not necessarily undergo the same fate as the ligand. So far, direct information on the subcellular localization of the asialoglycoprotein receptor itself in intact hepatocytes is lacking.

We took an immunocytochemical approach to examine distribution of the receptor both in normal rat liver cells and in cells after exposure to experimentally administered ligand. We used the highly sensitive immunocytochemical technique in which ultrathin frozen sections are treated first with an antibody against affinity-purified receptor and, subsequently, with a protein A/gold complex (6, 7). Our findings demonstrate that the receptor normally resides in all surface membranes of the hepatocyte and that during ligand-induced endocytosis some of the receptor is internalized but remains in the cell periphery. It is not transferred in significant quantity to more central regions of the cell, such as the lysosomes and Golgi complex.

## MATERIALS AND METHODS

### Materials

Orosomucoid (American Red Cross) was desialated by treatment with neuraminidase or heating in  $\text{H}_2\text{SO}_4$  (8). Antibodies against the asialoglycoprotein receptor were raised in rabbits. The immunogen consisted of the three receptor species (mol wt 40,000, 55,000 and 65,000), all of which are derived from the same receptor polypeptide. Details of the purification procedure and the characterization of the antibody preparation are described elsewhere (9). Immunoprecipitation and SDS polyacrylamide gel analysis of  $^{125}\text{I}$ -surface-labeled rat hepatocytes demonstrated the specificity against the receptor polypeptide (9).

## Animals

After an overnight fast, male rats of ~200 g were anaesthetized intravenously with Nembutal. Livers were fixed *in situ* by a 10-min injection through the portal vein of a solution of 2% formaldehyde and 0.2% glutaraldehyde for light microscopy or with 2% formaldehyde and 0.5% glutaraldehyde for electron microscopy, both in 0.1 M phosphate buffer (PB) at pH 7.4 at room temperature. Small tissue blocks from central and peripheral parts of several liver lobes were further immersion-fixed for 1 h in the prior fixative. Liver blocks from normal rats or rats treated with ASOR were stored in 0.1 M PB with 1 M sucrose and 2% formaldehyde at 4°C until processing for immunocytochemistry.

Before fixation, 0.5 ml of phosphate-buffered saline (PBS) containing 1, 10, 25, or 100 µg of ASOR was injected into a major intestinal vein. Control rats either were not injected or received 0.5 ml of PBS alone or with 25 µg of orosomucoid. Injection was performed gradually over 20 s. Its effectiveness was appreciated by the faint waves of blanching traveling through the liver. In this way, small amounts of ASOR could be offered to the liver in definite pulses. 2, 5, 10, and 15 min after the start of the injection, perfusion-fixation via the portal vein was performed as described above. Livers of two rats injected with 25 µg of ASOR 5 min previously were perfusion-fixed with a mixture of 2% formaldehyde, 0.5% glutaraldehyde, and 0.4% ruthenium red (10). These tissue blocks were dehydrated and embedded in Epon.

## Immunocytochemistry

Details of the procedure have been presented previously (6). In short, cryosections of the tissue blocks were prepared according to Tokuyasu (11). For light microscopy, ~200-nm sections were immunostained with rabbit anti-receptor followed by goat anti-rabbit antibody conjugated to rhodamine (12). The pattern of fluorescence was related to tissue structure by comparing phase-contrast and fluorescent micrographs of the same section field. For electron microscopy, ~100-nm cryosections were indirectly labeled with protein-A complexed to 5- or 8-nm colloidal gold particles, prepared as described before (7). Control sections were treated with rabbit pre-immune serum in place of anti-receptor serum before labeling with rhodamine or gold. The sections were then stained with uranyl acetate and embedded in methyl cellulose (11).

## RESULTS

### Normal Livers

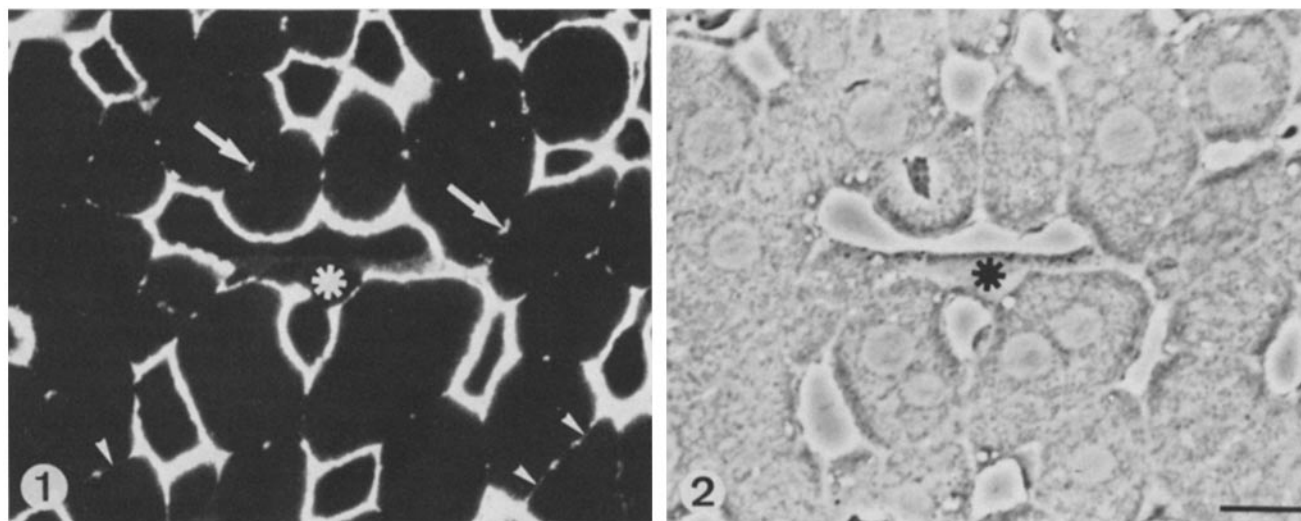
Surveys of fluorescent sections showed that the anti-receptor antibody staining was limited to hepatocytes (Figs. 1 and 2). Sinusoidal lining (Kupffer) cells (Figs. 1 and 2) were negative as were all blood cells. Reaction occurred with all hepatocytes of all liver lobes. Fig. 1 shows that only the cell surface membrane of the hepatocyte contains immunoreactive receptor.

No intracellular staining could be detected. Anti-receptor staining was present at the entire cell membrane. Fluorescence of the sinusoidal membrane was often much more intense than that of the rest of the plasma membrane, a phenomenon partly caused by the more irregular outline of the sinusoidal membrane. Fluorescence of the bile capillary varied. There was no demonstrable fluorescent staining in sections treated with pre-immune rabbit serum.

Electron microscopy observation demonstrated gold particles (linked to protein A bound to anti-receptor antibody) associated with the external surface of the entire plasma membrane, including the bile capillary (Figs. 3 and 4). Receptor was also found on the luminal surface of small (~100-nm) vesicular profiles (further referred to as vesicles) present in small numbers just below the sinusoidal membrane. The gold labeling appeared more intense at the sinusoidal membrane than at the lateral and bile capillary membrane (Fig. 3). Top, sides, and base of the microvilli as well as the intermicrovillar membrane regions with pits were uniformly labeled. No labeling occurred over cell junctions and intracellular structures other than the vesicles mentioned. Some Golgi apparatus labeling was irregularly seen. Sinusoidal lining (Kupffer) cells were negative (Fig. 4).

### ASOR-treated Livers

The effects of the various doses of ASOR (10–100 µg) used were qualitatively similar and will be demonstrated in the figures for 25 µg only. Injection of ASOR, in contrast to orosomucoid or PBS (not shown), greatly intensified endocytosis at the sinusoidal membrane and at the lateral plasma membrane close to the sinusoids. Endocytotic activity could clearly be seen only by electron microscopy due to the small dimensions of the structures involved. By light microscopy, at best some punctuate fluorescence could be discerned in the vicinity of the sinusoids (Fig. 5). Apart from this, no apparent change in fluorescence was noticed up to 15 min after ASOR administration (compare Figs. 5 and 6 with Fig. 1). (The absolute fluorescence intensity varies from one preparation to another, accounting for the quantitative differences in Figs. 1, 5, and 6.)



FIGURES 1 and 2 Twin of fluorescence and phase-contrast micrographs of ~200-nm frozen section of normal rat liver. The section was stained with rabbit anti-asialoglycoprotein receptor antibody followed by fluorescent goat anti-rabbit serum. Fig. 1 shows intense fluorescence along the sinusoidal cell borders. Staining is less intense at the bile capillaries (arrows) and lateral cell surface (arrowheads). Sinusoidal lining (Kupffer) cells (asterisk) are unreactive. Bar 10 µm. × 1,100.

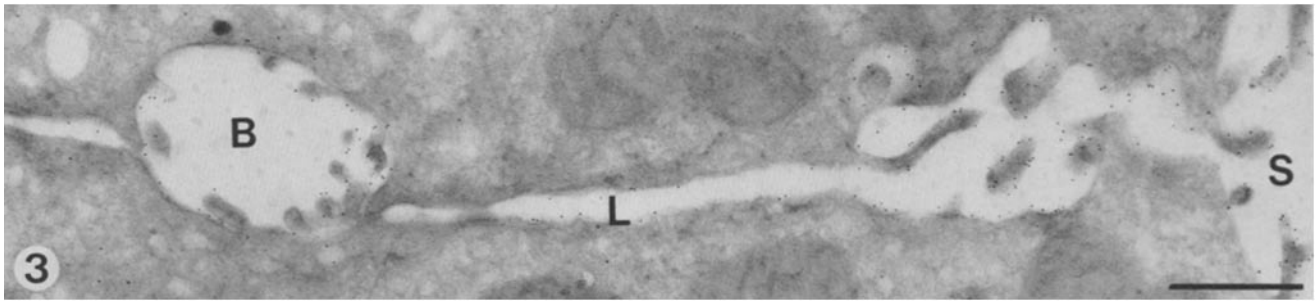


FIGURE 3 Electron micrograph of ultrathin (~100-nm) frozen section of normal rat liver. Anti-asialoglycoprotein receptor—8 nm gold. Gold particles are associated with the outer surfaces of the sinusoidal (S), lateral (L), and bile capillary (B) plasma membrane. Bar 0.5  $\mu\text{m}$ .  $\times 34,000$ .

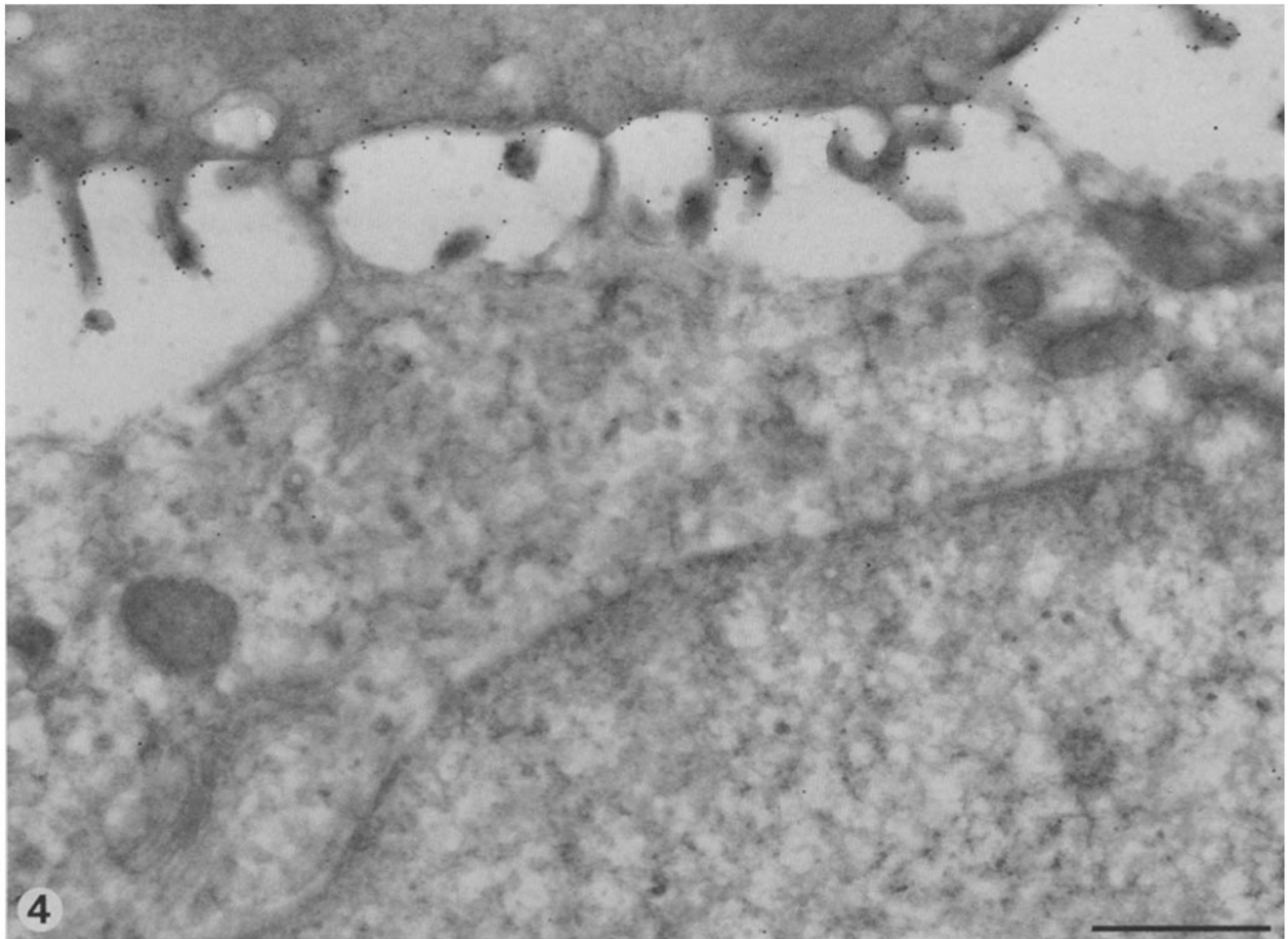


FIGURE 4 As Fig. 3. Gold particles are restricted to the plasma membrane of the hepatocyte in the upper part of the figure whereas the Kupffer cell below is unreactive. Bar 0.5  $\mu\text{m}$ .  $\times 48,000$ .

Electron microscopy demonstrated that by 2 min after ASOR administration, in addition to invaginations and pits of the plasma membrane, vesicles (~100 nm) suggestive of endocytosis had appeared at the sinusoidal surface (Figs. 7 and 8). Ruthenium red, when included in the fixative, stained only that portion of the plasma membrane containing invaginations and pits (Fig. 7). Possible coats on these structures (5) were indiscernible in the frozen sections. The membranes of pits and vesicles were labeled at their extracellular and luminal surface, respectively (Figs. 10–15). From 5 min onward, numerous vesicles containing receptor were present close to the sinusoidal membrane (Figs. 12–15). These vesicles most abundant at the

earliest times of observation were still present at 15 min after ASOR administration. Many vesicles were continuous with or near to vacuoles with various labeling patterns and intensity (Figs. 12–14). Within the entire 15-min period, immunoreactive receptor was confined to the cell periphery. At no time-point up to 15 min was there any significant decrease of receptor immunoreactivity of the plasma membrane as a whole. At 15 min, some gold particles were occasionally seen over Golgi complex structures, but their numbers were insignificant with respect to the labeling of the peripheral structures. Endoplasmic reticulum, lysosomes, and the majority of Golgi complexes contained no detectable receptor (Fig. 9). Labeling of the bile

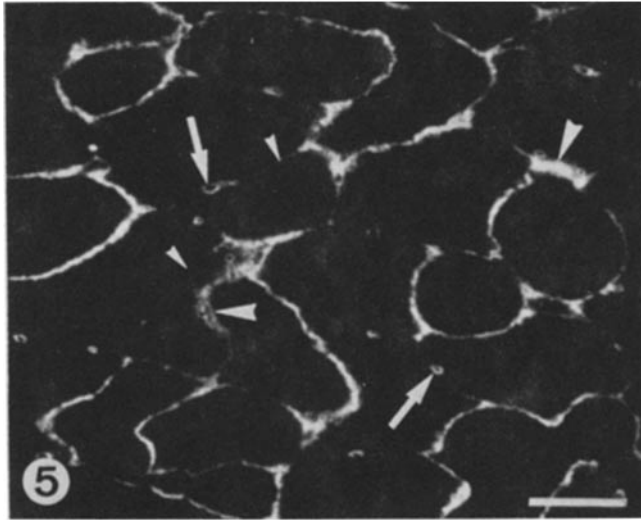


FIGURE 5 Fluorescence micrograph showing receptor immunoreactivity in a section of a liver fixed 2 min after injection of 25  $\mu$ g of ASOR. Sinusoidal, lateral (small arrowheads), and bile capillary (arrows) plasma membranes show immunofluorescence. Some punctuate reactivity can be seen just below the sinusoidal membrane (large arrowheads), possibly representing endocytotic structures. Bar 10  $\mu$ m.  $\times$  1,400.

capillary membrane was unaffected after ASOR administration; there was no increase of endocytotic structures in this area (Fig. 9).

## DISCUSSION

The anti-asialoglycoprotein receptor antibody used was raised against the purified soluble receptor isolated from rat liver (9). The antibody specifically precipitated only the receptor protein from solubilized surface proteins from iodinated rat hepatocytes. During our study, this polyclonal antibody reacted almost exclusively with residues at the extracellular surface of the hepatocyte membrane. It is possible that potential recognition by the antibody of receptor molecule sites within the membrane or on the cytoplasmic surface of the plasma membrane did not occur due to diminished accessibility to the immunoreagents. We and others have demonstrated that there are  $\sim$ 500,000 ligand-binding sites on the surface of each hepatocyte (8, 13), although other laboratories have reported lower numbers of surface ligand-binding sites (see reference 8 for discussion). Pricer and Ashwell (14) have determined substantial ligand-binding activity associated with a variety of subcellular fractions from normal rat liver. Using Triton X-100 solubilized hepatocytes, ligand binding, and ammonium sulfate precipitation, Steer and Ashwell (15) estimated that there are  $\sim$ 10<sup>6</sup> total receptor binding sites per cell, a figure twenty times that detected on the cell surface in the same study. However, because different assays were employed in many of the experiments, it is not possible to compare directly any of these determinations. We feel that all of the ligand binding data are consistent with the notion that the vast majority of functional receptors are on the cell surface. Our immunocytochemical data are consistent with the estimation that only a minor part of the total rat liver receptor is intracellular. Recently, we have quantified functional receptor distribution in a hepatoma cell line by examining surface binding and uptake of ligand before and after destruction of surface receptors with trypsin (A. L. Schwartz, S. E. Fridovich, H. F. Lodish, unpublished obser-

vations). These results demonstrate that 86% of functional receptors are on the cell surface. Nonetheless, further biochemical dissection of receptor localization in normal cells will be necessary.

We have localized the receptor to the entire cell surface membrane, except for the cell junctions. Immunoreactivity of the bile capillary membrane, although unexpected, may result either from uniform insertion of newly made receptor to all regions of the plasma membrane or from uniform recycling of internalized receptor to all regions of the cell surface. Furthermore, the absence of receptor endocytosis in areas near the bile canaliculi, after ASOR administration, is consistent with the inability of ASOR ligand to be presented to these receptor molecules. The presence of asialoglycoprotein receptor at the lateral cell surface is more comprehensible, as asialoglycoprotein uptake in this region has been described previously (3).

The receptor in normal liver cells was uniformly distributed along the sinusoidal cell membrane. This is consistent with observations on the localization of ligand binding sites (16). Both in normal liver and in liver after ASOR administration, the sinusoidal membrane contained pits which were receptor

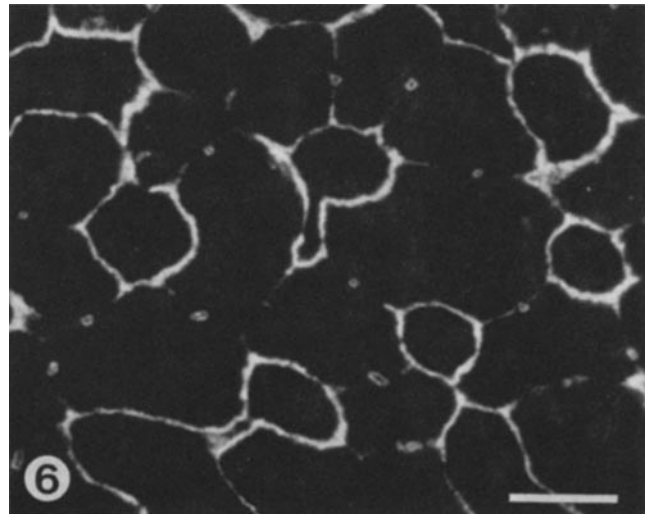


FIGURE 6 The same as Fig. 5, but 15 min after ASOR administration. Distribution of fluorescence is similar to that in normal livers (compare with Fig. 1). Bar 10  $\mu$ m.  $\times$  1,400.

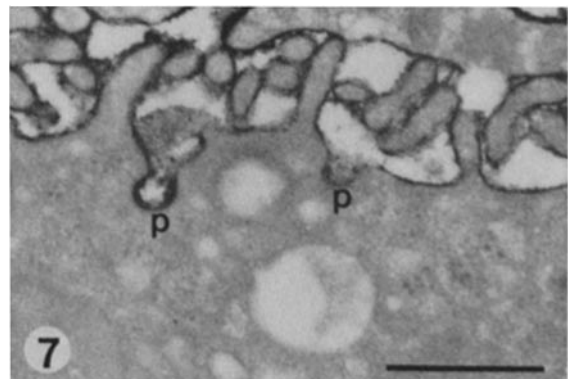
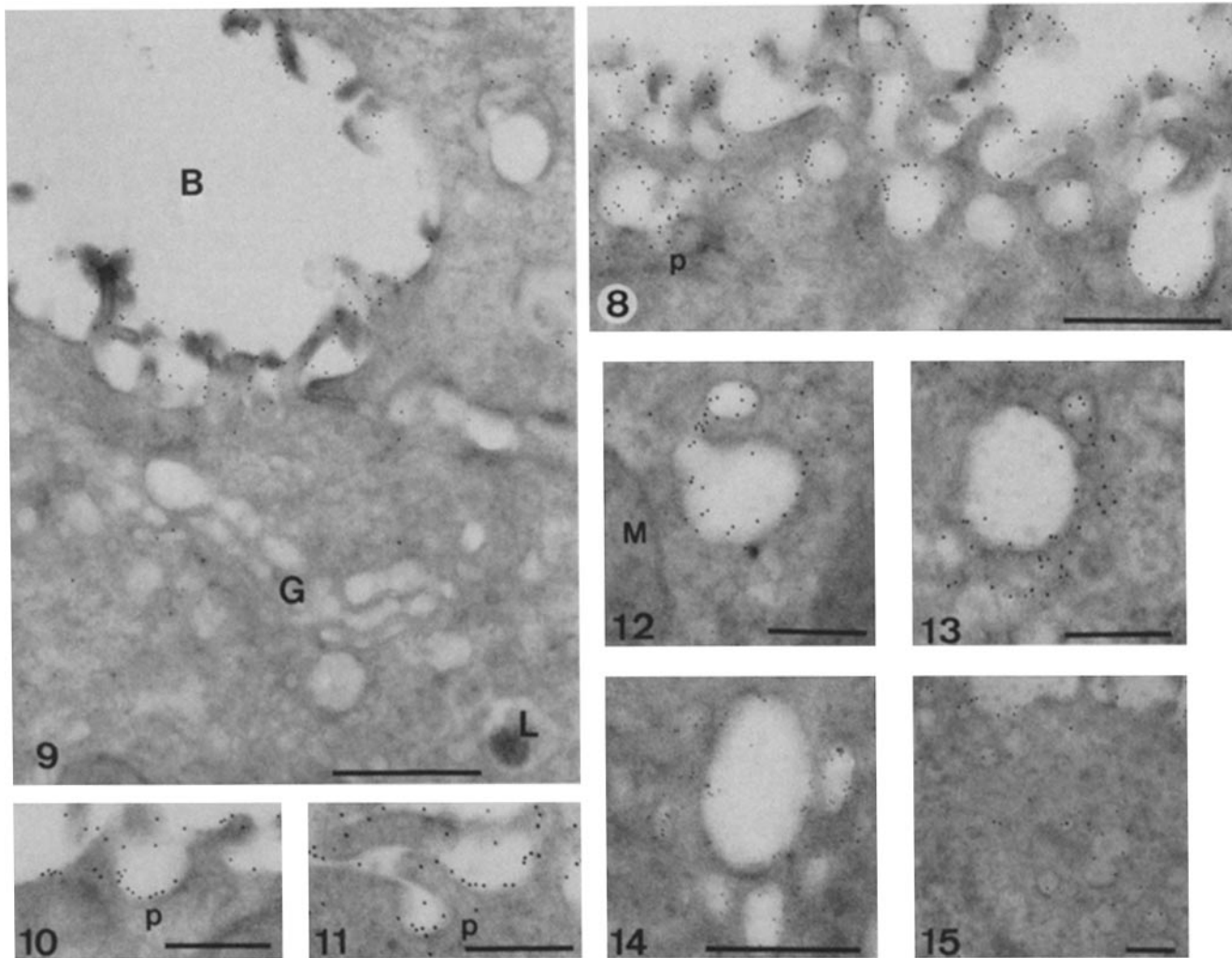


FIGURE 7 Plastic section ( $\sim$ 200 nm) of liver fixed in the presence of ruthenium red 5 min after injection of 25  $\mu$ g of ASOR. Ruthenium red staining is present along the sinusoidal plasma membrane including two pits (P) but is absent from intracellular vesicles and vacuoles. Bar 0.5  $\mu$ m.  $\times$  42,000.



FIGURES 8 to 15 Immunoelectron micrographs showing the distribution of asialoglycoprotein receptor following the injection of 25  $\mu\text{g}$  ASOR. The sections were labeled with 8-nm gold particles, except for Fig. 14 showing a 5-nm gold particle. Fig. 8: 2 min after ASOR. The sinusoidal membrane shows many folds and pits (one included in the figure at *p*). Receptors are not uniformly distributed but are clustered at some places. Bar 0.5  $\mu\text{m}$ .  $\times 48,000$ . Fig. 9: 15-min ASOR. The figure shows part of a bile capillary (*B*), a Golgi complex (*G*), and a lysosome of the residual body type (*L*). Gold labeling of receptors is restricted to the bile capillary membrane. Bar 0.5  $\mu\text{m}$ .  $\times 44,000$ . Figs. 10 and 11: 2-min ASOR treatment. Label has accumulated in pits (*p*) of the sinusoidal membrane, whereas the microvilli tips show relatively little labeling. Bar 0.25  $\mu\text{m}$ .  $\times 64,000$ . Figs. 12-14: 5-min ASOR treatment. The figures show vacuoles with irregular labeling which are continuous with or lie close to labeled vesicular structures. *M*, mitochondrion. Bar 0.25  $\mu\text{m}$ . Figs. 12 and 13,  $\times 64,000$ . Fig. 14,  $\times 94,000$ . Fig. 15: 5-min ASOR treatment. Close to the sinus are numerous small vesicles with gold particles. Bar 0.25  $\mu\text{m}$ .  $\times 35,000$ .

labeled. Membrane pits have been implicated in asialoglycoprotein endocytosis and were found coated at their cytoplasmic surface (5). Membrane "coats" could not be detected in our frozen sections using the current fixation protocols. However, future studies using double-labeling of receptor and clathrin should allow more detailed quantification of receptor localization especially in regard to "coated" pits and vesicles.

By 5 min after ASOR administration there was receptor in both large intracellular vacuoles and  $\sim 100$ -nm vesicles. The vacuoles were irregularly labeled at the luminal face of their membranes and were often continuous with or near to labeled vesicular profiles. We used ruthenium red to establish that these profiles do not represent sections through pits and tubular extensions of the cell surface membrane. Ruthenium red binds negatively-charged groups and cannot pass intact membranes but is small enough to enter 3-nm channels (10). Most vesicular profiles were unstained and thus had no open connection with

the exterior at the time of fixation. The few ruthenium red-positive vesicles present in ultrathin sections most likely represented pits in surface membrane invaginations outside of the plane of section. In 250-nm sections such positive free vesicles were absent.

The following considerations lead us to suggest that the vesicles contain receptor molecules which originate from the plasma membrane and which are mobilized by ligand internalization: (a) the vesicles appear as a reaction to ASOR administration; (b) the vesicles are as strongly labeled as the sinusoidal membrane where most of the endocytosis occurred; and (c) no significant labeling of other intracellular compartments was found. This latter result indicates that there is no important source of new receptor in intracellular organelles. In this study, because no clear time-related alteration in labeling pattern was apparent, we were unable to trace the direction of vesicle movement. Undoubtedly, a portion of the vesicles trans-

port receptor-ligand complex inward. Wall et al. (5) and Stockert et al. (3) have described corresponding vesicles engaged in centripetal ligand transport. On the other hand, it can be assumed that vesicles mediate the return of receptor molecules back to the plasma membrane after having discharged their bound ASOR. Free ASOR presumably is released into vesicles destined to fuse with Golgi complex and/or lysosomal membranes (3, 5, 17). This assumption is supported by the observation that exposure of the cells to a wide range of ASOR doses for up to 15 min did not result in a significant shift of receptor reactivity from the cell periphery, even though by this time the ligand had presumably been delivered to the Golgi complex-lysosome region (3, 4, 5). Furthermore, no significant decrease in receptor labeling at the plasma membrane occurred, despite intensive endocytosis. The possibility that the receptor is spared from degradation and is restored to the plasma membrane is in agreement both with turnover data which suggest that the half-life of the receptor is considerably longer than the intracellular transit time of the ligand (17, 18) and with other data supporting receptor recycling (15).

We propose that the receptor and ligand become uncoupled in intracellular vesicles in the vicinity of the plasma membrane; this point probably is at the vesicle-vacuole junction. Experiments with the new immunodouble labeling technique (6) for the simultaneous identification of both receptor and ligand should allow us to define this key point in more detail.

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