Distribution of an Asialoglycoprotein Receptor on Rat Hepatocyte Cell Surface

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ABSTRACT Direct ferritin immunoelectron microscopy was applied to visualize the distribution of the hepatocyte cell surface of the asialoglycoprotein receptor which is responsible for the rapid clearance of serum glycoproteins and lysosomal catabolism. For this purpose, rabbit antibody against the purified hepatic binding protein specific for asialoglycoproteins was prepared and coupled to ferritin by glutaraldehyde. The specific antibody conjugates were incubated with the hepatocytes, which were isolated from rat liver homogenate after fixation by glutaraldehyde perfusion. These cells preserved well the original polygonal shape and polarity, and it was easy to identify the sinusoidal, lateral, and bile canalicular faces. The surface density of the ferritin particles bound to the sinusoidal face was about four times higher than that of particles bound to the lateral face, while the bile canalicular face was hardly labeled and almost at the control level. Using the surface area of hepatocyte measured by morphometrical analyses, it was estimated that ~90% of bound ferritin particles were at the sinusoidal face, ~10% at the lateral face, and ~1% at the bile canalicular face. Nonhepatic cells such as endothelial and Kupffer cells had no receptor specific for asialoglycoproteins.

Ashwell and co-workers found that the asialoglycoproteins (ASGPs) produced by removal of the terminal sialic acid from serum glycoproteins are recognized by a hepatic receptor, rapidly removed from the circulation, and taken up by the hepatocytes to be catabolized in lysosomes (2, 23).

Ultrastructural analyses of the carbohydrate recognition system in rat liver have been extensively carried out by Hubbard et al. (9, 10, 32) by using ^{125}I-ASGPs and two electron microscopic tracers, asialoorosomucoid (ASOR) covalently coupled to horseradish peroxidase (ASOR-HRP) and lactosaminated ferritin. According to them, the tracers injected intravenously into rat were rapidly internalized into the hepatocytes via coated pits and coated vesicles, began to accumulate in a complex arrangement of larger smooth-surfaced vesicles, and tubular structures at the sinusoidal periphery of the cells (30 sec–2 min), appeared in Golgi-lysosome regions either in small vesicles <200 nm in diameter, larger irregular vesicles or tubules (5 min), and finally, some of these vesicles fused with lysosomes (15 min). Very recently, it has been further shown by Wall and Hubbard (33) that, at low temperature or after formaldehyde prefixation, ASGP binding sites are present over much of the sinusoidal cell surface but are concentrated heavily over coated pits. Electron microscopic observations on the endocytosis of ASGP-enzyme conjugates by hepatocytes also have been reported by Stockert et al. (30).

We are interested in the distribution of the ASGP receptor on the cell surface of rat hepatocytes *in situ*. We asked whether the receptor exists homogeneously on all the cell surfaces or exclusively on the sinusoidal face, and, if the latter is true, whether it exists homogeneously on the sinusoidal face, both in the microvillar and intermicrovillar region, or exclusively in the latter region. To answer these questions, rat liver was first fixed by perfusion with glutaraldehyde, immobilizing the receptor on the cell surface, and then homogenized. The unbroken hepatocytes were prepared together with numerous nuclei by centrifugation and were incubated with ferritin antibody conjugates.

These hepatocytes preserved well the original polygonal shape and polarity. Thus, it was easy to identify the various cell surfaces such as sinusoidal, lateral, and bile canalicular faces. By counting ferritin particles on each surface, we found that the receptor is localized mainly on the sinusoidal face, the distribution on the lateral face being only one-fourth of that on the sinusoidal face, and hardly any ferritin particles were detectable on the bile canalicular face. On the sinusoidal face, they are distributed rather evenly in the microvillar and inter-

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microvillar region. In the latter region some of the ferritin particles were found clustered in the coated pits.

MATERIALS AND METHODS

Purification of Hepatic Binding Protein (HBP) and Preparation of Anti HBP Antibodies

HBP, specific for ASGPs, was purified from rat liver according to the procedure of Kawasaki and Ashwell (11) with slight modification (25). The antibody against purified HBP was raised in rabbits and prepared by ammonium sulfate fractionation followed by DEAE-cellulose column chromatography.

The immunological specificity was tested by Ouchterlony immunodiffusion and by specific inhibition of the binding of ^{125}I-ASOR to microsomes by the addition of antibody (25). SDS-PAGE analyses and subsequent fluorographic visualization of the immunoprecipitates have shown that the antibody immunoprecipitated a single component (~50,000 daltons) from a Golgi preparation that was labeled with [^{3}H]-$NaBH_4$ in vitro (22). When various subcellular fractions were immunoprecipitated from livers pulsed-labeled in vivo for 20 min with [^{35}S]methionine, again a single component of ~50,000 daltons was found in the immunoprecipitates (22).

As an additional experiment, viable liver cells were isolated by collagenase digestion (4, 27) and the surface proteins of the liver cells were labeled with ^{125}I by enzymatic iodination (8) and then immunoprecipitated for analysis by gel electrophoresis and fluorography. Again, a single component of ~50,000 daltons was detected (Fig. 1).

Preparation of rabbit antibody against purified cytochrome P-450 and control immunoglobulin G (IgG) from nonimmunized rabbit serum have been described previously (14).

Preparation of Ferritin Antibody Conjugates

Ferritin and IgG were coupled together according to the procedure of Kishida et al. (12) using glutaraldehyde as a coupling agent. The ferritin antibody conjugates with the molar ratio of IgG to ferritin of approximately 1:1 were isolated by gel filtration on Bio-Gel A 1.5 M (Bio-Rad Laboratories, Richmond, CA) as described previously (14). The conjugates thus prepared were concentrated to ~2 mg of ferritin and 0.5 mg of IgG per ml. The immunological activity of the antibody conjugates was tested by Ouchterlony double-diffusion analysis (14).

Isolation of Prefixed Liver Cells

Since we are interested in the quantitative analyses of the distribution of the receptor for ASGPs on all of the surface of hepatocytes, it was necessary to isolate the cells. Conventionally, perfusion with crude collagenase has been used for the dissociation of liver cells (4, 27). By this procedure, however, the polarity of the liver cells is lost (4) and some, if not all, of the receptors may be impaired (31) and may change their localization during the isolation procedures (24).

These considerations led us to develop a new procedure for the isolation of prefixed liver cells. That is, rat liver was prefixed by perfusion with dilute glutaraldehyde and then homogenized briefly with a homogenizer. Unbroken cells were then recovered in the nuclear fraction by the conventional cell fractionation procedure. The concentration of glutaraldehyde was selected so as to prepare unbroken cells with a maximal yield. Fixation with higher concentrations of glutaraldehyde preserved the cells better but with much less yield, while fixation with lower concentrations of glutaraldehyde could not preserve the ultrastructures. After a number of trials, 0.7% glutaraldehyde was selected and used throughout the present experiment. We also tried formaldehyde fixation, but with much less satisfactory results.

Practically, rat liver was perfused *in situ* through the portal vein, first with cold 0.9% saline briefly, and then with cold 0.25 M sucrose containing 0.7% glutaraldehyde until the liver was bleached and slightly hardened, and finally briefly with cold 0.25 M sucrose to wash out the glutaraldehyde. The perfused liver was homogenized by a 4-times up and down motion at a low speed in 4 vol of 0.25 M sucrose containing 0.05 M Tris-HCl, pH 7.4, 0.025 M KCl, 0.005 M $MgCl_2$ (0.25 M STKM) in a loosely fitted Teflon-glass homogenizer. The homogenate was centrifuged at 1,000 *g* for 5 min. The nuclear pellets were suspended in 9 vol of 2.2 M STKM and passed through one layer of gauze and centrifuged at 100,000 *g* for 60 min in a Hitachi RP 40 rotor.

The reddish brown pellets were suspended in 0.25 M STKM and washed by centrifugation at 1,000 *g* for 5 min and the pellets were resuspended in the above buffer and used as nuclear fraction for immunoelectron microscopy. The recovery of total nuclei in this fraction was more than 50%. This fraction was mainly composed of naked nuclei (80–90% in number), and the rest consisted of unbroken cells (10–20%) including hepatocytes, endothelial cells, and Kupffer cells. Occasionally, however, ~60% of nuclei in the nuclear fraction were in the unbroken cells. All the hepatocytes preserved well in the original polygonal shape, and the polarity of the hepatocyte cell surface was easily identified as shown later.

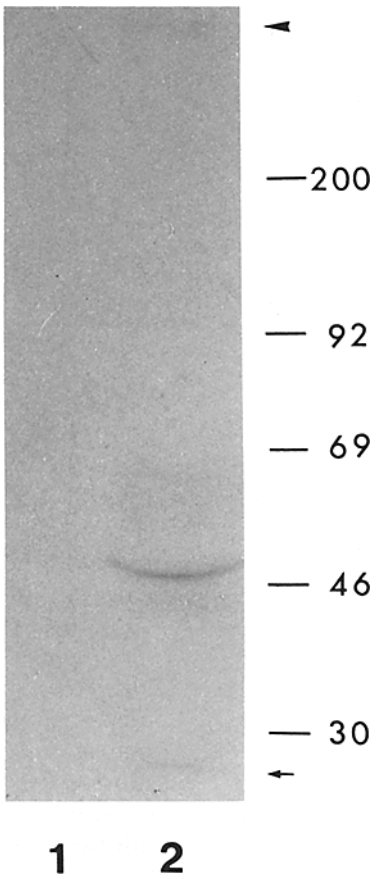


FIGURE 1 Fluorographic analysis of the hepatic binding protein immunoprecipitated from viable liver cells labeled in vitro by enzymatic iodination. Viable liver cells were isolated by collagenase digestion, and the surface proteins of the liver cells were labeled with ^{125}I and immunoprecipitated. The precipitates were subjected to SDS PAGE followed by fluorography. As a control, immunoprecipitation was done using IgG from control rabbit serum. Lane *1*: control; Lane *2*: Experiment. Arrowhead and arrow indicate starting point and dye front, respectively. Molecular weight markers at right are $\times 10^3$.

Very recently, the above procedure has been improved further; the liver homogenate was centrifuged at 1,000 *g* for 5 min and the pellets were resuspended in 0.25 M STKM, washed twice by centrifugation as described above, and the final suspension was passed through one layer of gauze. The recovery of hepatocytes in the liver homogenate was almost 100%.

The binding experiment on perfusion with ^{125}I-antibody or ^{125}I-ligands indicated that prefixation of liver by perfusion with 0.7% glutaraldehyde decreased the binding capacity of rat liver to ^{125}I-IgG against HBP only slightly (17%) but decreased the binding to ^{125}I-ligands such as ASOR and galactosylated human serum albumin (Gal_{40}-HSA) markedly (~75%).

Labeling of Isolated Liver Cells with Antibody Conjugates and Electron Microscopy

The nuclear fraction was incubated for 2–3 h at 0°–4°C with either antibody conjugates or control conjugates. To do quantitative immunoelectron microscopic analysis, it is essential to incubate hepatocytes with antibody conjugates at the saturation level of antibody (15). For this purpose, the molar ratio of antibody conjugates to HBP was determined as follows. First, the hepatocyte cell number per μl of the nuclear fraction was determined with a haemocytometer, and the total cell number in the incubation mixture was calculated. The number of the ASGP receptors per isolated hepatocyte has been reported to be 7×10^4–5×10^5 (26, 29, 36). Using the maximum number of 1×10^6 receptors per hepatocyte, the total number of the receptors in the incubation mixture was calculated and excess ferritin antibody conjugates were added to label all the receptor at the saturation

level (molar ratio of the receptor to the specific antibody was 1:10–20). After incubation, the unbound conjugates were washed out three times with 0.25 M STKM by centrifugation at 1,000 *g* for 5 min.

Three different control experiments were done: (*a*) antibody conjugates were replaced by the same amount of rabbit control conjugates which were prepared by the conjugation of ferritin with IgG from nonimmunized rabbit serum; (*b*) as a blocking test, the nuclear fraction was preincubated with a 10- to 20-fold excess of anti HBP antibody, and then the antibody conjugates were applied; (*c*) the nuclear fraction was incubated with anti cytochrome P-450 antibody conjugates. After incubation, the nuclear fraction was washed as described above.

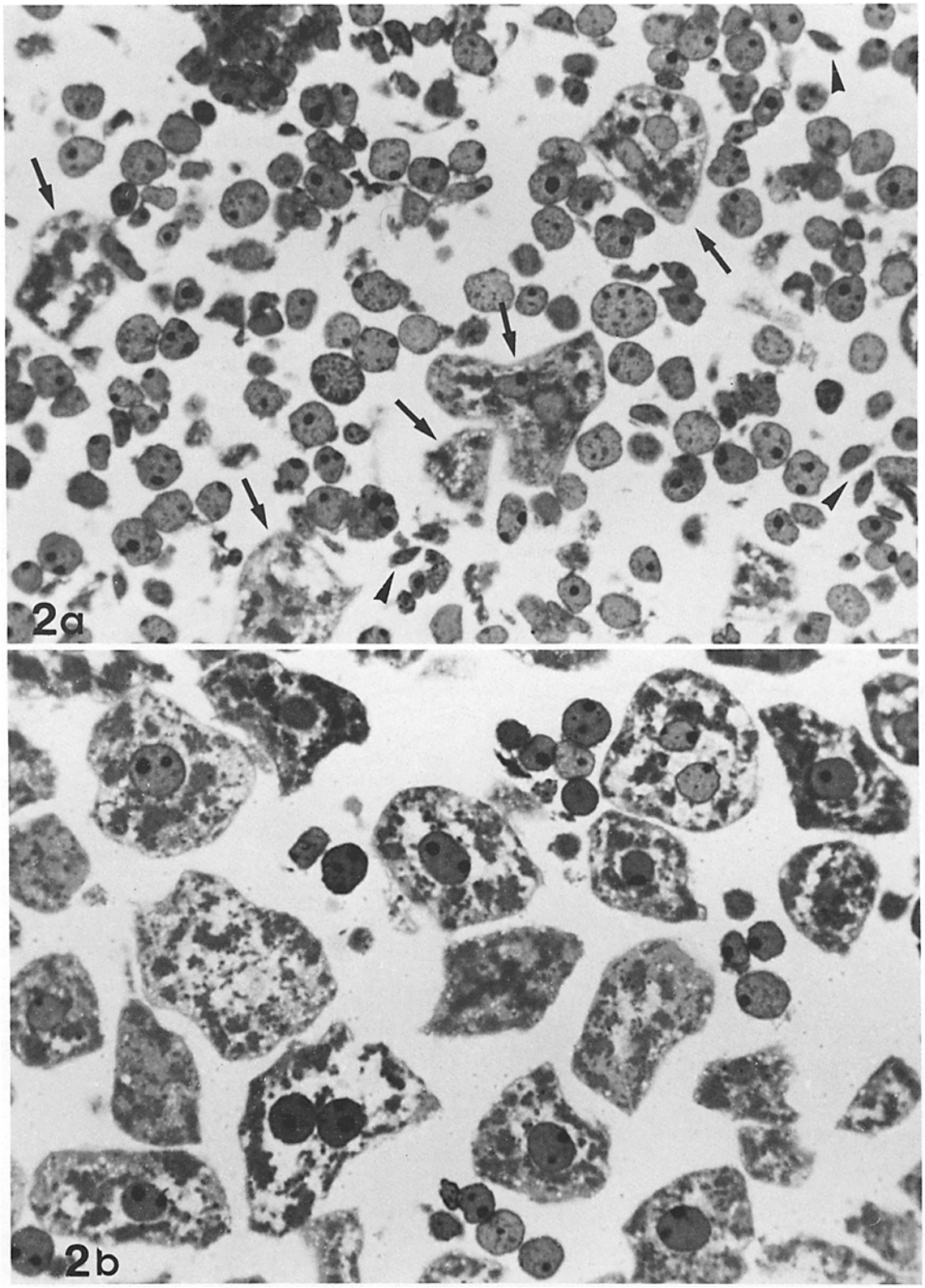


FIGURE 2 Light microscopic view of the nuclear fraction. The specimens embedded in Epon for electron microscopy were sectioned for light microscopy and stained with toluidine blue. Arrows and arrow heads indicate hepatocyte and endothelial cell, respectively. Note polygonal shape of the hepatocytes. (a) Conventional case where only 10–20% of the nuclei are in unbroken cells. (*b*) An exceptional case where ~60% of the nuclei are in unbroken cells. × 1,000.

The final pellets were fixed successively with 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated, embedded in Epon, and sectioned as described previously (14, 15). The thin sections, ~70 nm, were observed under a Hitachi electron microscope HU-12. Electron micrographs of liver cells were taken at random, and counts were made from micrographs printed at a final magnification of × 200,000. The morphometry of electron micrographs was done according to Weibel et al. (5, 35). The surface enlargement by formation of microvilli in the sinusoidal and bile canalicular space was measured by a Mutoh digitizer model G-2 (Mutoh Industrial Inc., Tokyo, Japan) connected to a Sord microcomputer model 223 Mark III (Sord Inc., Tokyo, Japan). The surface length of each cell surface domain was thus determined, and the average number of ferritin particles per micrometer of hepatocyte, endothelial cell, or Kupffer cell surface was calculated as described previously (15). Ferritin particles bound on the three surface domains of hepatocytes were counted separately.

RESULTS

Morphology of Isolated Hepatocytes

To visualize the *in situ* distribution of HBP on isolated hepatocyte cell surface, it was necessary to immobilize the membrane proteins and maintain the original shape of the cells. Figs. 2 and 3 show light micrographs and an electron micrograph, respectively, of liver cells which were isolated successfully in the nuclear fraction by mild homogenization after perfusion with 0.7% glutaraldehyde. Fig. 2 *a* shows a usual case where 10–20% of the nuclei were in unbroken cells, while Fig. 2 *b* is an exceptional case where ~60% of the nuclei were in unbroken cells. Three kinds of liver cells were identified: hepatocytes, endothelial cells, and Kupffer cells.

It is to be noted here that all the hepatocytes preserved well the original polygonal shape (Figs. 2 and 3), and the sinusoidal, lateral, and bile canalicular domains of hepatocyte cell surface were easily identified (Fig. 3). It is further noted that the present method of preparation can preserve the cellular structures such as plasma membranes, nuclei including nuclear envelopes, rough endoplasmic reticulum, and mitochondria, but the other structures such as Golgi apparatus and smooth endoplasmic reticulum are poorly preserved and sometimes lost in the course of the preparation of the specimens.

Immunoelectron Microscopic Observation of ASGP Receptor on Hepatocyte Cell Surface

The distribution of the asialoglycoprotein receptor on the rat liver cell surface was studied by incubating isolated cells with excess antibody conjugates at 0°–4° C for 2 h as described in Materials and Methods. Figs. 4, 5, 8, 9 illustrate the distribution of ferritin particles bound to the three different domains of the hepatocyte cell surface. As shown in Figs. 4 and 5, the sinusoidal face was heavily labeled with ferritin particles. This is in marked contrast with the control electron micrographs, in which the nuclear fraction was incubated with either control conjugates (Fig. 6), or anti P-450 antibody conjugates (Fig. 7) in place of anti-HBP antibody conjugates, or preincubated with excess anti HBP antibody before incubation with the ferritin antibody conjugates (blocking test). In Fig. 6, all the hepatocyte surfaces, including the sinusoidal cell surface, were hardly labeled with ferritin. The electron micrographs of the blocking test were similar to Fig. 6 (data not shown). In Fig. 7, very few ferritin particles were found on the sinusoidal surface of the hepatocyte, while contaminating microsomes shown by arrow

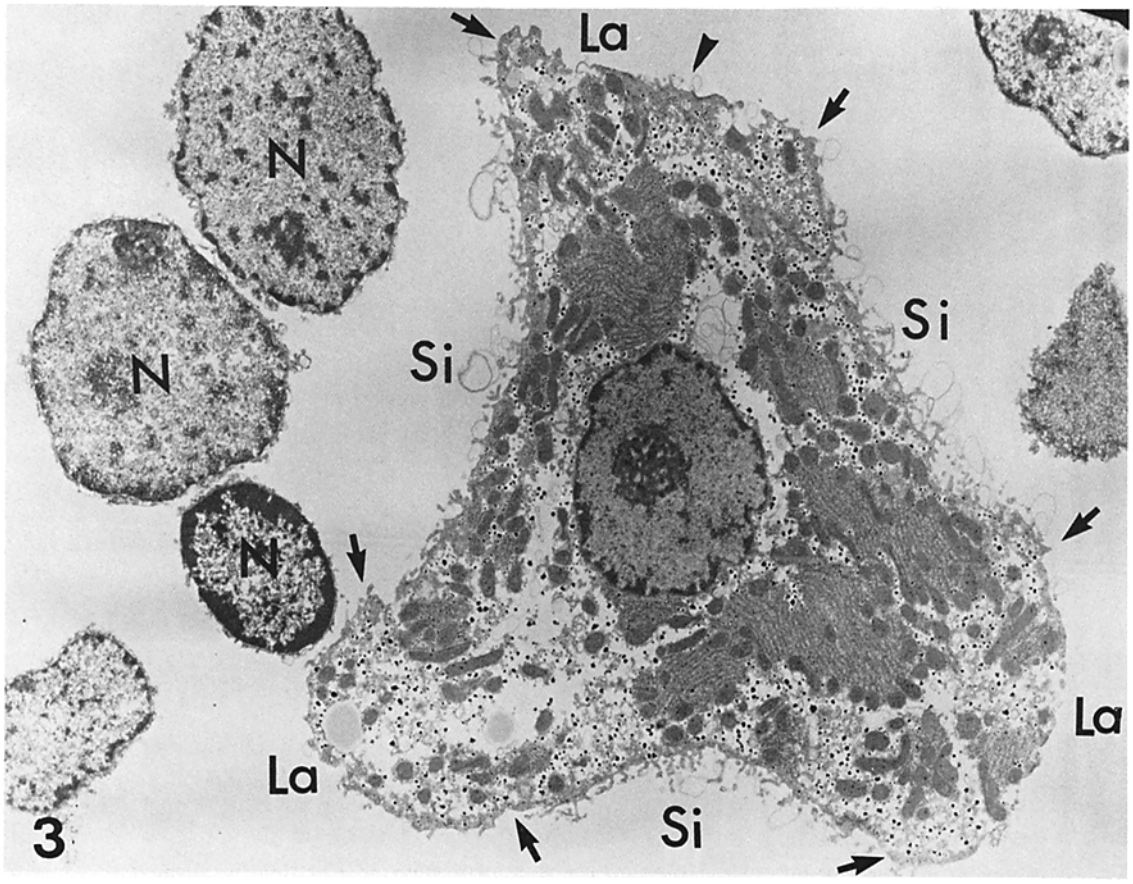


FIGURE 3 Electron micrograph at a low magnification of a hepatocyte in the nuclear fraction. Original polygonal shape of the cell and the cell surface were well preserved. Arrows indicate the boundary between the lateral and sinusoidal domains, while arrowhead indicates bile canalicular region. *Si*: sinusoidal face, *La*: lateral face, *N*: nuclei. × 5,800.

heads were heavily labeled with ferritin anti P-450 antibody conjugates. The latter finding is to be expected because all the microsomal vesicles derived from endoplasmic reticulum membranes were heavily labeled with ferritin anti P-450 antibody conjugates as reported in the previous papers (14, 15).

Figs. 4 and 5 show that microvillar and intermicrovillar regions of the sinusoidal face were almost equally labeled with ferritin particles. It is also noted that ferritin particles were not always uniformly distributed throughout the two regions but were occasionally aggregated in large clusters of 7–30 or more ferritin particles (25 in average). Coated pit regions were most heavily labeled (Fig. 4, *insets*).

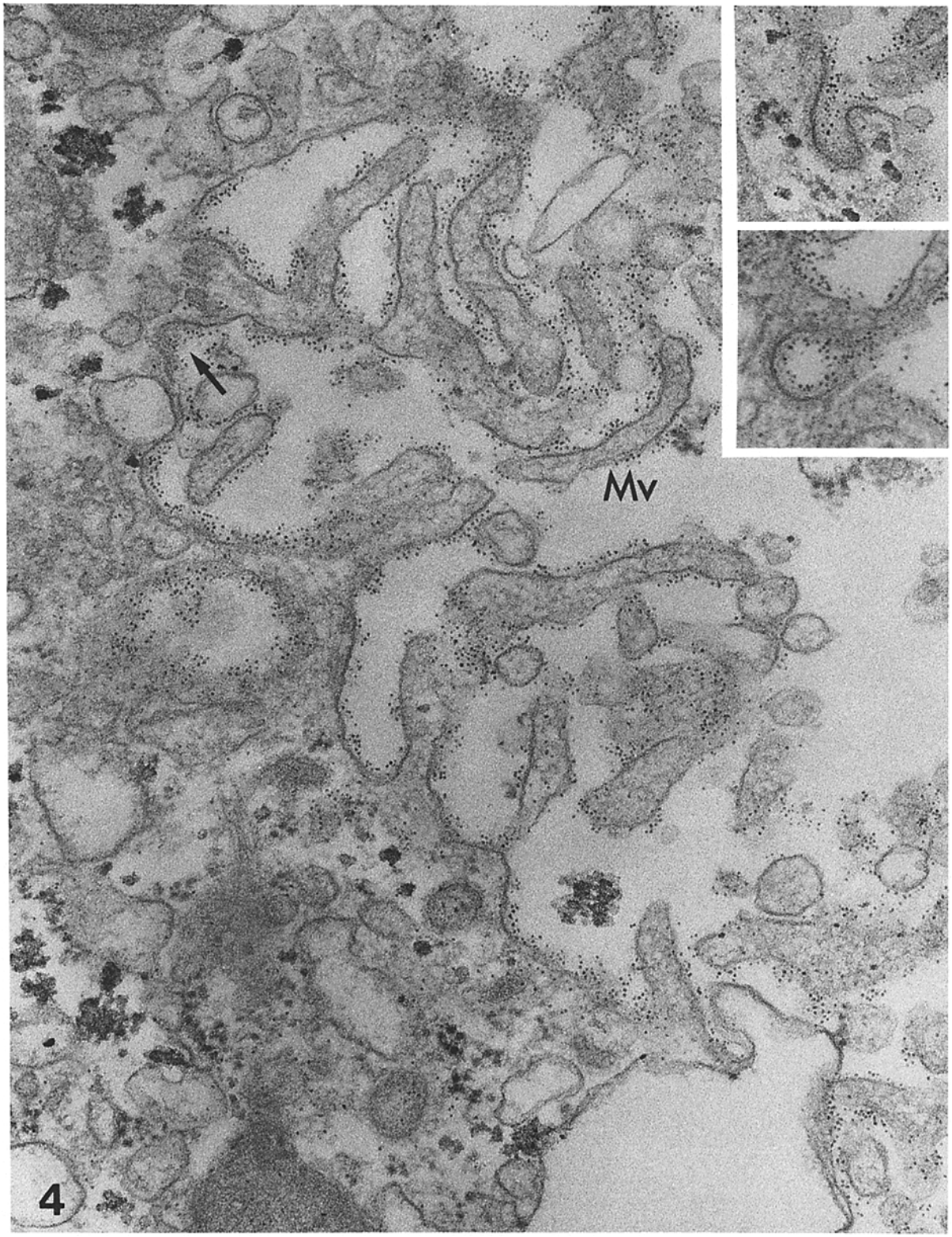


FIGURE 4 Sinusoidal face of a hepatocyte incubated with ferritin antibody conjugates specific for hepatic binding protein. Microvillar (*Mv*) and intermicrovillar regions were heavily labeled. Arrow shows coated pits. × 77,500. Insets shows coated pits heavily labeled with ferritin particles. × 87,500.

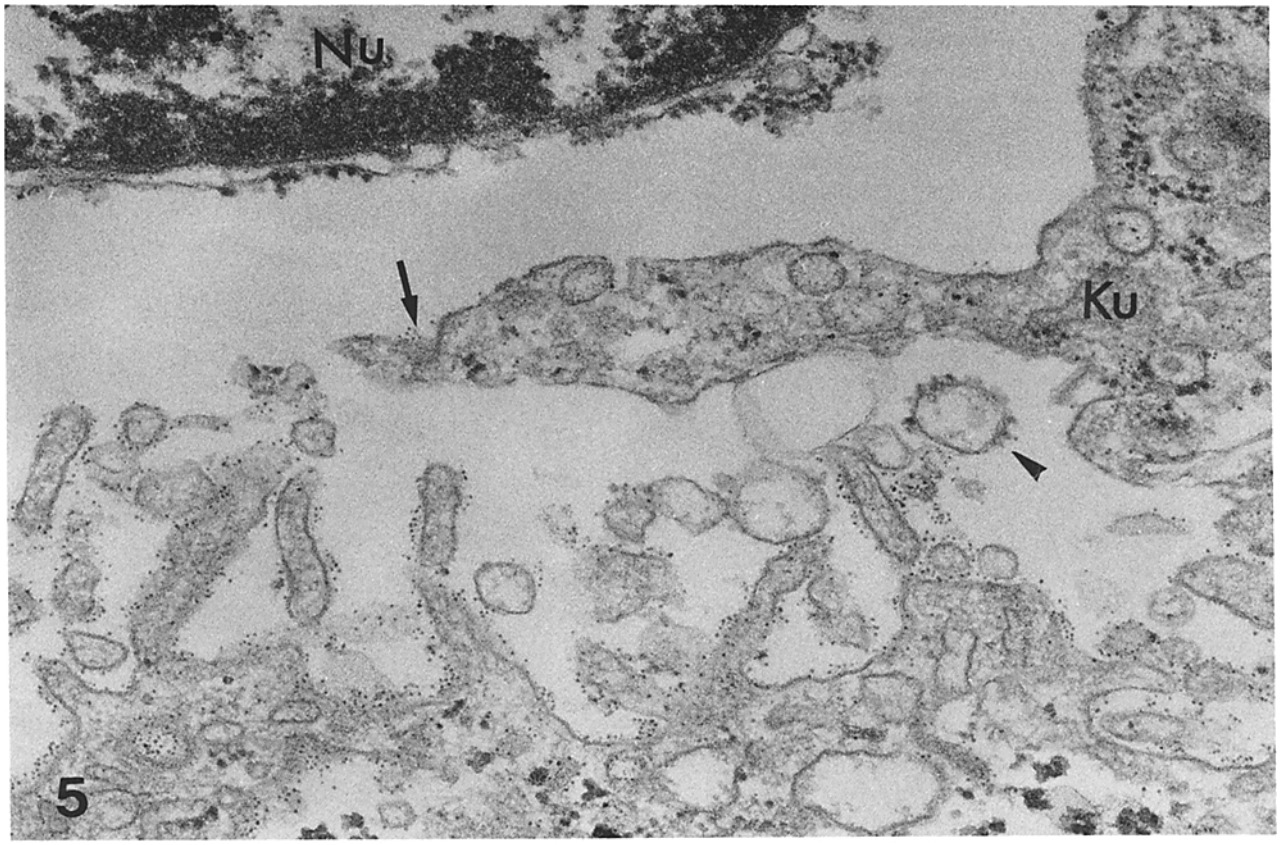


FIGURE 5 Nuclear fraction incubated with ferritin antibody conjugates. Sinusoidal face of a hepatocyte was heavily labeled with ferritin particles. Nuclear (*Nu*) membrane and Kupffer (*Ku*) cell surface were not labeled with ferritin particles, except for coated pit region of Kupffer cell (arrow). Contaminating microsomes (arrow head) were not labeled. × 65,000.

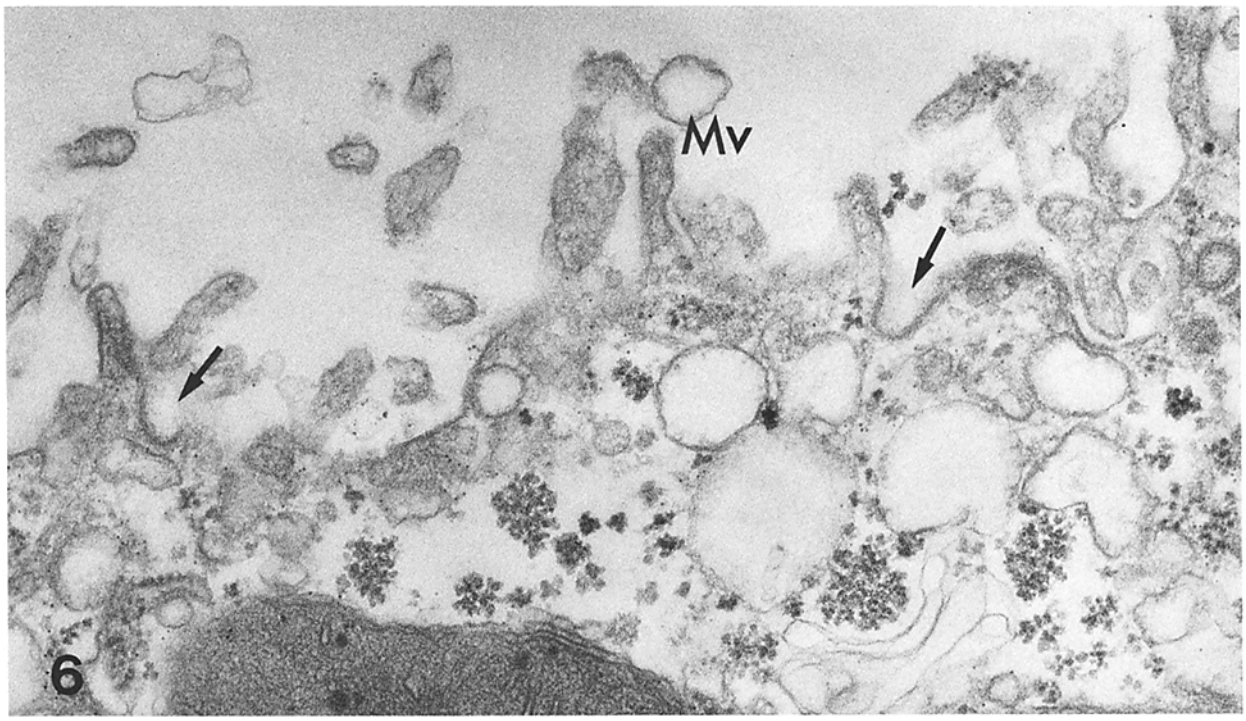


FIGURE 6 Sinusoidal domain of isolated hepatocyte incubated with control conjugates. The control conjugates were prepared by the conjugation of ferritin with IgG from nonimmunized rabbit serum. Only a very few ferritin particles were found on the cell surface. Coated pits are indicated by arrows. *Mv*: microvilli. × 62,000.

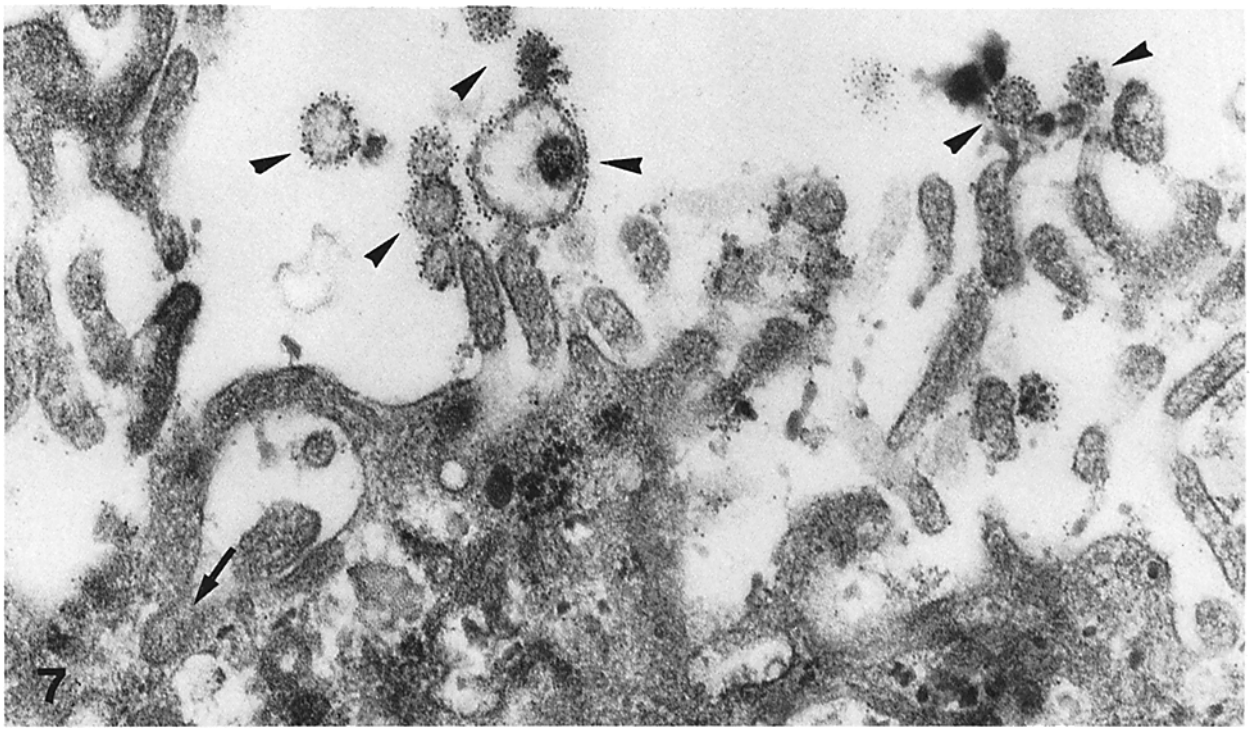


FIGURE 7 Sinusoidal domain of isolated hepatocyte incubated with ferritin anti P-450 antibody conjugates. Nuclear fraction was prepared from rats previously treated with phenobarbital for 3 d as described before (14). Contaminating microsomes (arrow heads), one of which is loaded with a lipoprotein particle, were heavily labeled with ferritin particles, while the hepatocyte cell surface was hardly labeled. This micrograph serves as another control. Coated pits are indicated by an arrow. × 63,000.

It is difficult to indicate clearly the boundary between the sinusoidal region and the lateral membrane region (contiguous plasma membrane regions). We can roughly estimate, however, the beginning of the lateral surface as shown by arrows in Fig. 3. That is, the lateral surface is characterized as a flat surface without microvillar extrusions. This lateral region is only slightly labeled with ferritin when compared with the sinusoidal region (Fig. 8). Labeling of the lateral region towards the sinusoidal region, however, was more marked than the rest of the region, and sometimes we observed coated pits labeled with ferritin particles (Fig. 8 *b*).

The bile canalicular region was clearly identified by the presence of characteristic microvilli and adjacent tight junctional complexes (Fig. 9, *T*) as reported by Song et al. (28). Only a few ferritin particles were found on this region, and the degree of labeling was almost at the control level.

It is to be pointed out here that all the hepatocytes in the nuclear fraction, even in the exceptional case when ~60% of the nuclei were in unbroken hepatocytes, were heterogeneously labeled with ferritin, and thus this heterogeneous labeling with ferritin antibody conjugates is certainly a general property of the hepatocytes.

Nonhepatic cells such as endothelial and Kupffer cells were also found in the nuclear fraction. As shown in Figs. 5, 10 and 11, labeling of the cell surfaces with ferritin particles was at a control level except for the coated pit regions. The latter regions were labeled not only with antibody conjugates but also with the control conjugates. This is in marked contrast to the labeling of the coated pit regions on the sinusoidal-lateral region of hepatocytes which were labeled only with the anti HBP antibody conjugates.

Quantitative Analysis of the Immunoelectron Micrographs

To compare quantitatively the degree of labeling, the particle density or number of ferritin particles bound per micrometer of the cell surface of the different domains of hepatocytes and the other liver cells was calculated and is shown in Table I. For this calculation, the total number of ferritin particles counted was ~16,000 and the total cell surface measured was ~76 μm^2.

The particle density of the sinusoidal face of hepatocytes was about four times higher than that of the lateral face, and the particle density of bile canalicular face was almost at the control level. On the sinusoidal face, the particle density of the intermicrovillar region was only slightly higher than that of the microvillar region. Coated pit regions were most heavily labeled, being 1.7 times denser than the average sinusoidal region.

The particle densities of the nonhepatic cells such as endothelial and Kupffer cells were almost at a control level except for the coated pit regions which were labeled heavily with ferritin particles.

The average surface areas of the sinusoidal, lateral, and bile canalicular domains of the hepatocyte cell surface were determined by morphometry as 1,756 μm^2 (60%), 785 μm^2 (27%), and 407 μm^2 (13%), respectively. These values are in agreement with the corresponding values estimated from the data reported by Weibel et al. (35).

By using these data, the total number of ferritin particles and their relative distribution on the various cell surface domains of hepatocyte were estimated. As shown in Table II, ~90% of

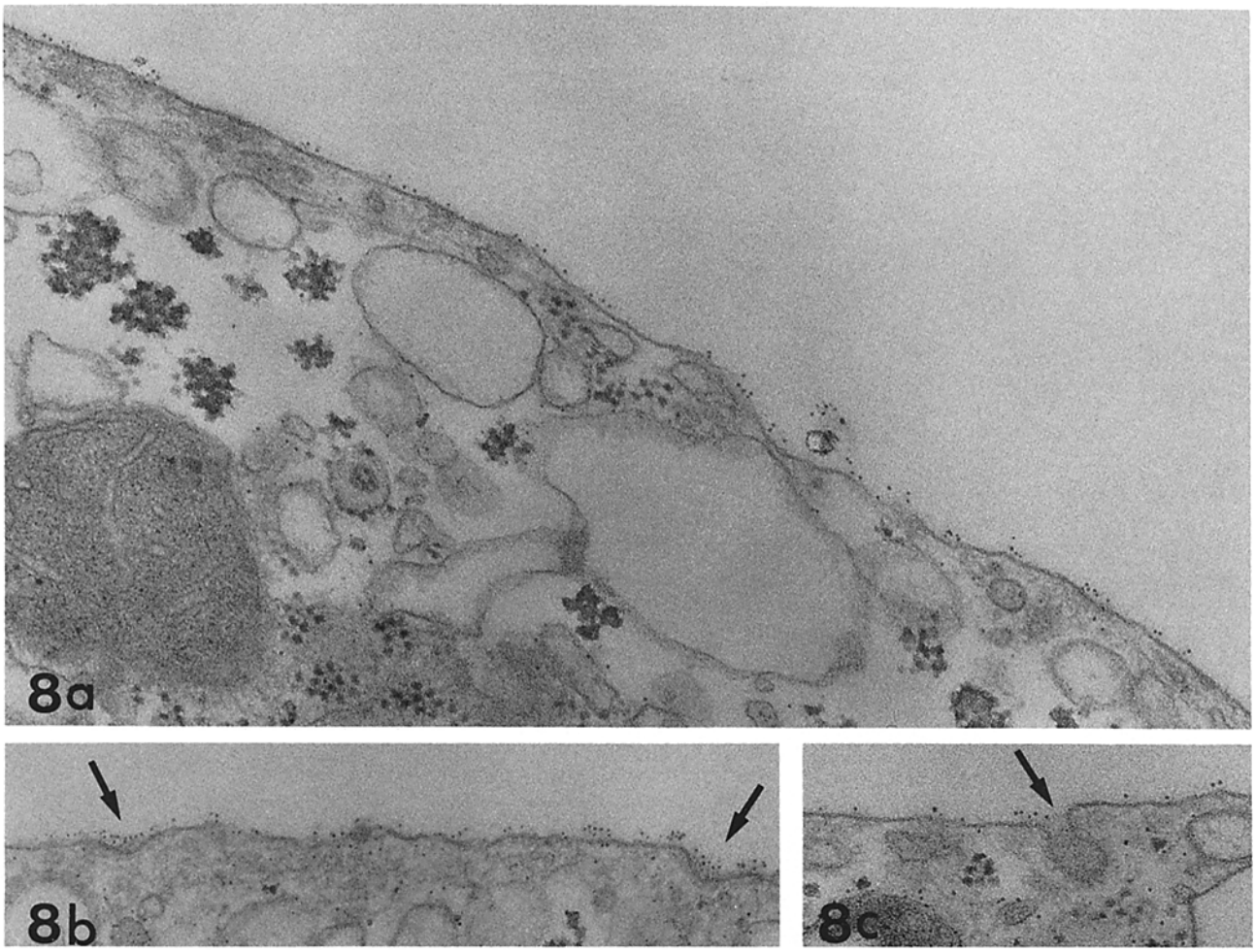


FIGURE 8 The lateral face of the hepatocyte, when incubated with ferritin antibody conjugates, was only slightly labeled compared with the sinusoidal face. In this region, coated pits (arrows) are labeled as shown in *b* and *c*. *a* and *c*, × 72,000. *b*, × 63,000.

the ferritin particles were on the sinusoidal face, and ~10% on the lateral face. In this calculation, the section thickness was assumed to be 70 nm. The total number of ferritin particles on the hepatocyte cell surface was calculated as $\sim 1.1 \times 10^6$ per cell.

Table III shows semiquantitative analyses of coated pit regions of the hepatocyte sinusoidal face. These specialized regions comprise ~3% of the surface area of the sinusoidal front. This value is in good agreement with the result reported by Wall and Hubbard (32). From these data, the number of ferritin particle per coated pit was calculated as 14–25. Since almost all the coated pits in the sinusoidal intermicrovillar region were heavily labeled with ferritin particles, these data clearly indicate the functional role of the coated pit as the route of internalization of the ligands, as reported by Wall and Hubbard (32).

DISCUSSION

Receptor-mediated endocytosis is a fascinating function of eukaryotic cells and has attracted a number of investigators. Recently, the endocytotic processes of the galactose recognition system in mammalian liver have been studied extensively (2, 23). This system appears to be preferable for studying endocytosis because of the high content (~1 mg/g liver protein) of this receptor protein in rat liver (25, 34) and the relative easiness of its purification.

Ultrastructural aspects of the carbohydrate recognition system have also been studied by several groups (7, 9, 10, 13, 30, 32) including Hubbard and co-workers, who reported in detail the endocytotic process of ^{125}I-ASGP by radioautography (9, 10) and by using ASOR covalently coupled to horseradish peroxidase and lactosaminated ferritin (32, 33).

Since we were interested in observing *in situ* distribution of the galactose specific receptor on all of the cell surface of rat hepatocytes, we had to develop a suitable procedure for the isolation of hepatocytes with definite polarity. The procedure we finally adopted was to prepare the hepatocytes from rat livers prefixed with glutaraldehyde as described in detail in Materials and Methods. It was possible to identify the sinusoidal, lateral, and bile canalicular faces of the isolated hepatocytes.

Our quantitative analyses clearly indicate that a marked heterogeneity exists in the distribution of the receptor on the hepatocyte cell surface. As shown in Table I, the surface density was highest on the sinusoidal face, intermediate on the lateral face, and almost at the control level on the bile canalicular face. It is not clear, however, whether the labeling of the bile canalicular face is simply due to nonspecific binding of the conjugates or has some biological significance. The possibility

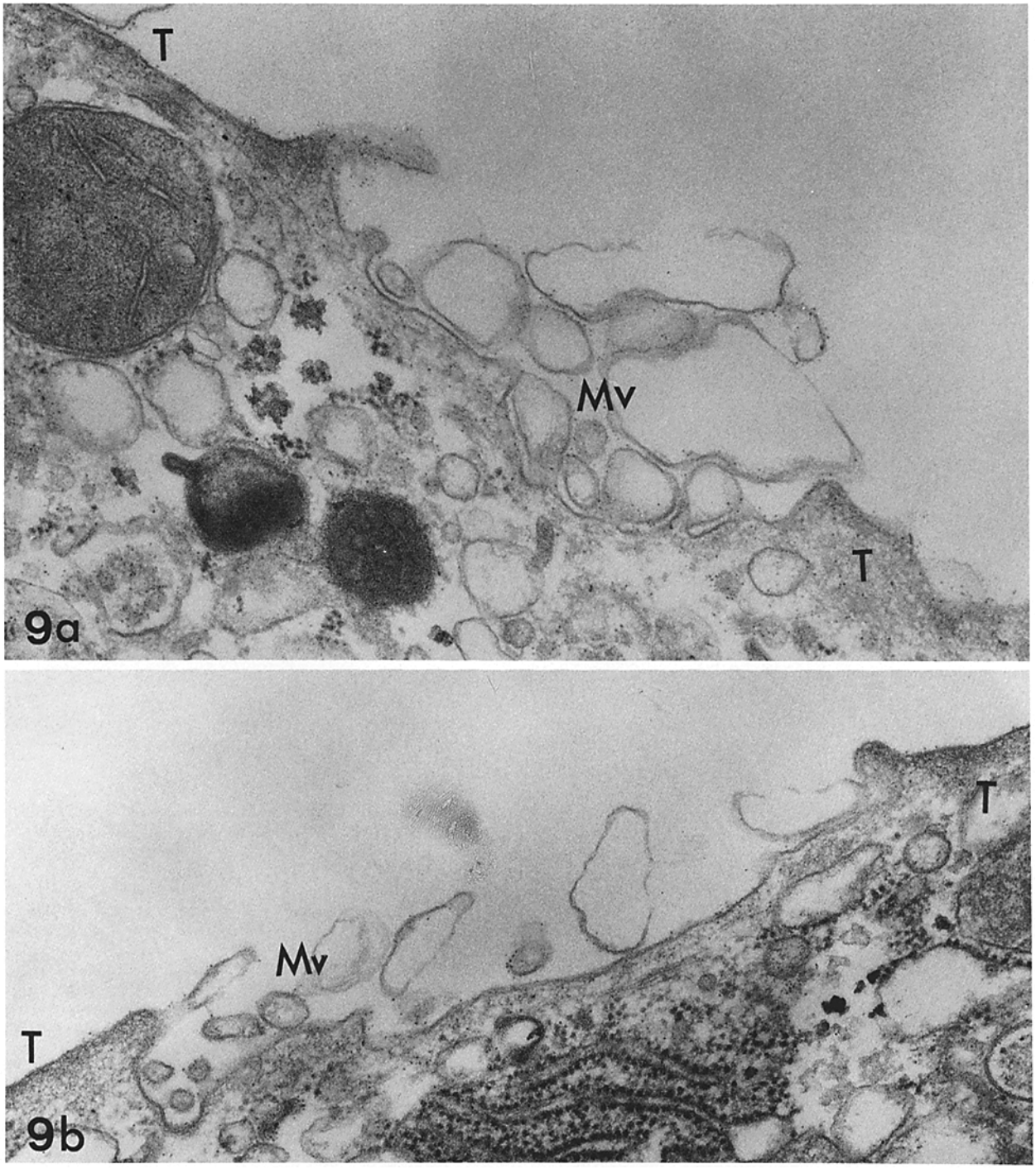


FIGURE 9 Bile canalicular region of hepatocyte incubated with ferritin antibody conjugates. Labeling with ferritin particles was slight and almost at the control level. *T*: Tight junctional complex region *Mv*: microvilli × 60,000.

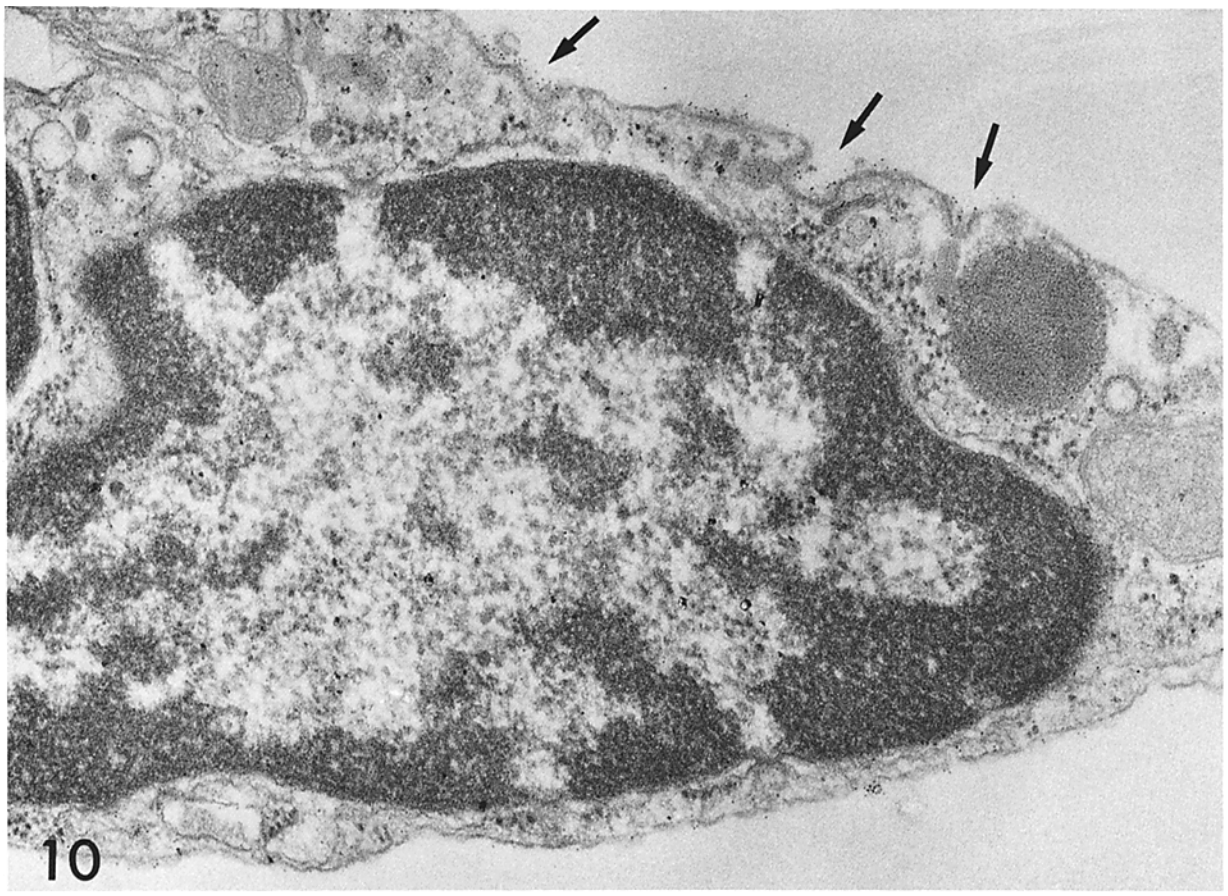
remains that some of the receptors are carried from the sinusoidal face to the bile canalicular face via the transhepatocyte route, as exemplified by IgA (6, 19, 20).

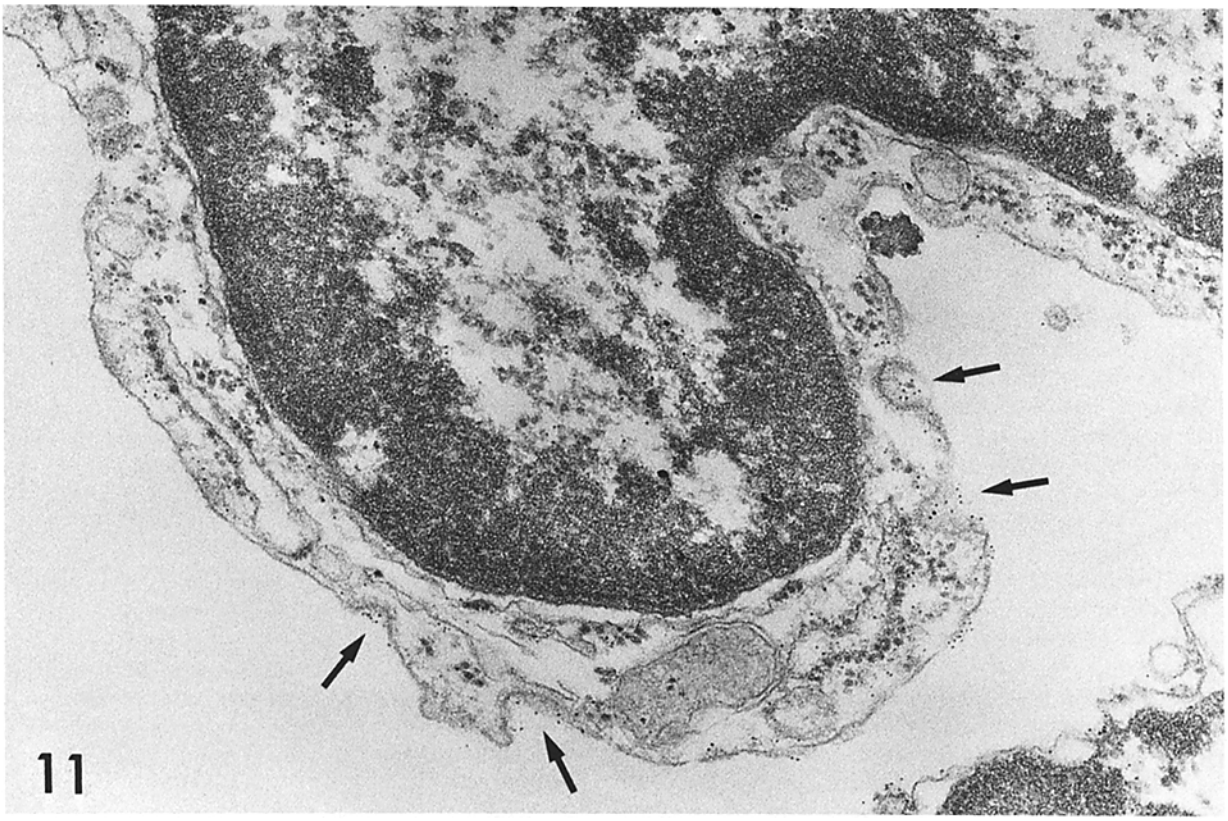
The presence of the ASGP receptor on the lateral domain has been reported previously (8, 30), but no quantitative analysis has been reported. Bergeron et al. (3) also suggested the presence of the insulin receptor on this domain.

The heterogeneous distribution of the ASGP receptor becomes more apparent if we compare the percentage distribution of the ferritin particles on each cell surface by multiplying the surface density with the corresponding surface area. As shown in Table II, ~90% of the receptors exist on the sinusoidal face, 10% on the lateral face, and 1% on the bile canalicular face.

Such a markedly heterogeneous distribution of the receptor protein indicates that tight junctions may serve as a diffusion barrier for the receptor proteins as suggested by various authors (6, 24). There is, however, no clear diffusion barrier between the sinusoidal and lateral faces, and it is not yet clear why the receptors are more numerous on the sinusoidal face than on the lateral face.

Table I also indicates that the receptors exist almost equally on the microvillar and intermicrovillar regions. This result is





FIGURES 10 and 11 Endothelial cells in nuclear fraction incubated with ferritin antibody conjugates. Endothelial cell surface was not labeled with ferritin except for the coated pit regions (arrows). Fig. 10, × 50,000. Fig. 11, × 60,000.

TABLE I

*Density of Ferritin Particles on Various Cell Surfaces**

Cells and cell surfaces	Antibody conjugates added	No. of ferritin particles bound to cell surface
		per micrometer
Hepatocyte		
Sinusoidal intermicrovillar	Anti HBP antibody	44.0
microvillar	"	38.5
Lateral	"	12.8
Bile canalicular	"	3.2
Coated pits on sinusoidal surface	"	69.4
Nonhepatocyte		
Endothelial	"	2.2
Kupffer	"	2.1
Coated pits on nonhepatocytes	"	37.1
Control experiments		
Hepatocyte sinusoidal	Anti P-450 antibody (or control conjugates)	1.2
Nuclei	Anti HBP antibody	1.6

* Values are average of two experiments.

TABLE II

Number and Percentage of Ferritin Particles on Different Hepatocyte Cell Surfaces

	Ferritin particles	% of total number
Sinusoidal	9.99×10^5	87.6
Lateral	1.27×10^5	11.1
Billiary	1.05×10^4	1.3

In this calculation, the average thickness of the sections was 70 nm. The sinusoidal, lateral, and bile canalicular surface areas were determined as 1,756, 785, and 407 μm^2, respectively. The percentage of the sinusoidal area was thus calculated to be 59.6% of the total surface area of the hepatocytes.

not consistent with the data of Wall and Hubbard (32) who reported that, at the earlier times examined (15–60 s), only 5–7% of lactosaminated ferritin particles were found on the microvilli and that most of the particles were localized either on the base of microvillar projections or in the coated or uncoated vesicles in the apical cytoplasm. The endocytotic process is very rapid, as described by those authors (32). Since lactosaminated ferritin was injected intravenously and then the liver was fixed by perfusion with glutaraldehyde in their experiments, it might be difficult to stop, instantaneously, the endocytosis of lactosaminated ferritin. In our experiment, however, the receptor was first immobilized by prefixation with glutaraldehyde, and then the isolated hepatocytes were incubated with ferritin antibody conjugates. The fact that the ferritin particle does not exist intracellularly in the coated vesicles (data not shown) strongly indicates the effectiveness of the prefixation procedure for the immobilization of the receptor proteins. Some apparent coated vesicles loaded with ferritin particles may represent coated pits connected with the extracellular space in a plane other than that of this thin section. We believe that our electron micrographs show a more real

TABLE III

Quantitative Analysis of the Coated Pit Regions of Hepatocyte

Coated region of sinusoidal face	Ferritin particles bound to coated region	Coated region labeled with ferritin	Ferritin particles per coated pit
%	%	%	
~3	~4.5	>90	14–25

distribution of the ASGP receptors *in situ* when the clustering of the receptors is not induced by the addition of the corresponding ligands. Comparing our results with those of Wall and Hubbard (32), it is apparent that clustering of receptors and formation of coated pits should be very rapid processes as has been suggested in the process of endocytosis of various other ligands (1, 17). Very recently, Wall and Hubbard have shown that, at low temperature or after immobilization by formaldehyde fixation, ASGP binding sites are present over much of the sinusoidal cell surface (33).

Table III clearly shows that almost all the coated pits in the sinusoidal region are heavily labeled with ferritin particles. Since rat liver was prefixed by perfusion with glutaraldehyde and since the liver cells were prepared from the prefixed liver, the coated pits in our electron micrographs may be in the process of naturally occurring endocytosis. It is not surprising that most of the coated pits are labeled with ferritin particles, because the ASGP receptor is certainly one of the most abundant receptors on the hepatocyte cell surfaces. It is also possible that several receptors, specific for different ligands, are collected in the same patches as reported by Maxfield et al. (16).

It has been reported that each hepatocyte contains 5×10^4–5×10^5 surface receptors for ASGPs (26, 29, 33, 36). Recently, Schwartz et al. (26) have reported that the presence of serum or albumin in the incubation medium markedly reduced the number and that, in the absence of serum or albumin, the absolute number of ASGP receptors on the surface of rat hepatocytes was about 500,000/cell.

Similar values of 2.7×10^5 and 6.4×10^5 surface receptors per cell were obtained in a preliminary perfusion experiment using ^{125}I-ASOR and ^{125}I-Gal$_{40}$-HSA, respectively. These numbers are, however, definitely smaller than the number of ferritin particles bound per hepatocyte (1.1×10^6 per cell, Table II). To estimate the number of receptor molecules on the cell surface from the latter value, we have to know how many ferritin antibody conjugates can bind to each receptor molecule. Unfortunately, it is very difficult, if not impossible, to prepare a large amount of equimolecular ferritin antibody conjugates to carry out the perfusion experiment at a saturation level of the antibody. Our preliminary perfusion experiment using ^{125}I-IgG against HBP, however, indicated that 1.8×10^6 IgG molecules bind per cell. This means that ~3 or more molecules of IgG can bind to each receptor molecule. If we assume that ~3 ferritin antibody conjugates can bind to each receptor molecule, the surface receptor number is calculated as $\sim 3.7 \times 10^5$ per cell. This value would be a minimum estimate because of the steric hindrance due to the large ferritin molecule in the conjugates. From the data of other authors and also from our results, we could safely estimate that $\sim 5 \times 10^5$ receptors exist on the cell surface of each hepatocyte.

Steer and Ashwell have suggested a total of 1.2×10^6 receptors per hepatocyte (29). This means that a large proportion of the receptor molecules may be on the hepatocyte cell surface as suggested by Schwartz et al. (26).

The nuclear fraction contains nonhepatocytic cells such as

endothelial cells and Kupffer cells, and they were incubated with ferritin antibody conjugates together with hepatocytes. It was clearly shown that only hepatocytes are heavily labeled with ferritin particles. This result confirms the conclusion that the ASGP receptors specific for the terminal galactose residue exist exclusively on hepatocytes (9, 10, 18). The only exception was that the coated pit regions of these nonhepatocytic cells are labeled with ferritin particles. This labeling was not specific for the ferritin anti HBP antibody conjugates, as described previously. Similar results have been reported by Wall and Hubbard (32) after administration of ASOR-HRP conjugates. Some HRP activity was detected in coated pits and coated vesicles of Kupffer and endothelial cells, and this result was attributed to either a small percentage of the conjugates being recognized by the *N*-acetylglucosamine/mannose system present in nonhepatocytic cells (10, 32) or to fluid pinocytosis by these cells. This problem was not investigated further.

Recently, we have shown that rat HBP specific for ASGPs is synthesized on rough endoplasmic reticulum and spans the membrane, probably exposing the carboxyl-terminal segment on the cytoplasmic surface and the amino-terminal segment charged with carbohydrate moieties on the luminal surface (21). Since the binding protein exists exclusively on the sinusoid-lateral surface, and since the intrinsic membrane proteins probably are not allowed to diffuse freely through the tight junction (6, 24), it is strongly suggested that the intracellular route of transport from rough endoplasmic reticulum to the cell surface is also quite polarized, allowing only the sinusoid-lateral insertion of the binding protein.

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