

Ferritin Immunoelectron Microscopic Localization of 5'-Nucleotidase on Rat Liver Cell Surface

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ABSTRACT Rat livers were prefixed by perfusion with 0.6% glutaraldehyde and briefly homogenized with a Teflon-glass homogenizer. The prefixed cells isolated by low-speed centrifugation in high yield effectively preserved the original polygonal shape and polarity. These cells were incubated with ferritin-antibody conjugates monospecific for rat liver 5'-nucleotidase, and the localization of the enzymes on the surface of hepatocytes and endothelial cells was quantitatively investigated. It was revealed that the surface density of 5'-nucleotidase is much higher on the bile canalicular surface than on the sinusoidal surface and only a few ferritin particles were detected on the lateral surface. On the bile canalicular surface ferritin particles were almost exclusively found on the microvilli in larger clusters. Similar distribution was also observed on the sinusoidal surface but the size of cluster was much smaller. On both surfaces many fewer ferritin particles were found on the intermicrovillar region, including the coated pits region, than on the microvillar region. Ferritin particles were also found on the endothelial cell surface.

5'-nucleotidase (EC 3.1.3.5) is an integral glycoprotein of plasma membrane with its active site exposed at the external cell surface (4, 14, 24, 33), and has been used as a marker enzyme of plasma membrane in biochemical studies (3). Hepatocytes are not exceptional; it has been reported that 5'-nucleotidase is highly concentrated in the plasma membrane of hepatocytes as determined by biochemical analyses of isolated membrane fractions (3) or by ultrastructural cytochemical procedures (6, 26, 30, 34). Both biochemical (7, 12, 33) and cytochemical analyses (5, 6, 26, 27) further suggested that 5'-nucleotidase activity is mainly located on the bile canalicular membranes.

Reliability of biochemical analyses, however, largely depends on the purity of the subcellular fractions prepared and exclusive localization of a marker enzyme in a particular subcellular fraction. It is usually very difficult, however, to isolate either the bile canalicular or sinusoidal membrane without contaminating the fraction of the one by the other. On the other hand, ultrastructural cytochemical analyses supply us only qualitative data. It is not always clear, therefore, whether 5'-nucleotidase exists exclusively on the bile canalicular surface or both on the sinusoidal and bile canalicular surfaces. Furthermore, cytochemical analyses cannot show the exact localization of 5'-nucleotidase, because the site of the disposition of lead phosphate does not always coincide

with the exact site of the localization of the enzymes. It has also been reported that 5'-nucleotidase activity is very sensitive to glutaraldehyde fixation (11, 27, 35), thus limiting the application of cytochemical investigation to this enzyme.

In another approach to this problem, we have used quantitative ferritin immunoelectron microscopy using monospecific antibody against 5'-nucleotidase highly purified from rat liver. This antibody was coupled to ferritin, and the ferritin-antibody conjugates with a molar ratio of ~1:1 were used for semiquantitative analyses of the localization and distribution of 5'-nucleotidase on the surface of rat hepatocytes.

Our analyses clearly indicate that 5'-nucleotidase is located on both the bile canalicular and the sinusoidal surfaces. The surface density on the former, however, was much higher than on sinusoidal surface. On each cell surface, it was exclusively localized on microvillar region in large clusters and only a few ferritin particles were found on the intermicrovillar region. The particle density on the lateral cell surface was very low. The significance of this characteristic distribution of 5'-nucleotidase on the hepatocyte cell surface is discussed.

MATERIALS AND METHODS

Purification of 5'-Nucleotidase: 5'-Nucleotidase was purified from rat livers by the procedure of Eto et al. (8, 9). The procedure will be

reported in detail elsewhere (Eto, S., and K. Kato, manuscript in preparation). Briefly, the enzyme was extracted from 10,000 g pellets of rat liver homogenate with *n*-butanol and 1% Triton X-100, and was purified about 17,500-fold over the homogenate by using a seven-step procedure: (1) DEAE-cellulose I; (2) concanavalin A-Sepharose; (3) hydroxylapatite; (4) DEAE-cellulose II; (5) Sephacryl S-300; (6) AMP-Sepharose; (7) DEAE-cellulose III (Sepharose and Sephacryl; Pharmacia, Uppsala, Sweden). The purified enzyme appeared homogeneous on electrophoresis in polyacrylamide gel in the presence of 0.1% Triton X-100 and had a molecular weight of ~316,000. In SDS PAGE, the purified enzyme showed a single protein band corresponding to an apparent molecular weight of 76,000 (data not shown).

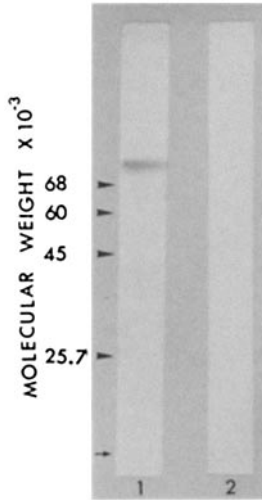


FIGURE 1 Immunoblotting of 5'-nucleotidase from rat liver plasma membranes. Plasma membrane fractions were isolated from rat livers by the method of Ray (29) and were subjected to electrophoresis in 10% slab gels containing 0.1% SDS. The proteins were transferred electrophoretically to nitrocellulose filter paper and incubated with anti-5'-nucleotidase IgG (lane 1) or control rabbit IgG (lane 2). After the first incubation, the samples were incubated with goat anti-rabbit IgG-peroxidase conjugate and were reacted with 4-chloro-1-naphthol as described by Nielsen et al. (25). The arrow indicates dye front.

Preparation and Characterization of the Antibodies: The antibodies against 5'-nucleotidase were elicited in rabbits and the IgG fraction was prepared from the pooled sera by ammonium sulfate fractionation followed by DEAE-cellulose column chromatography. Control IgG was also prepared from nonimmunized rabbit serum.

The monospecificity of the antibody was tested by immunoblotting of 5'-nucleotidase from rat liver plasma membranes. For this purpose, plasma membrane fractions were prepared from rat livers by the method of Ray (29). The plasma membrane proteins were analyzed by SDS PAGE, and transferred electrophoretically to a nitrocellulose filter paper (Western blotting). The papers were incubated with either anti-5'-nucleotidase IgG or control rabbit IgG and subsequently with goat anti-rabbit IgG-peroxidase conjugates and reacted with 4-chloro-1-naphthol (25). A single component ~76,000 in apparent molecular weight was detected only when incubated with anti-5'-nucleotidase IgG as shown in Fig. 1, lane 1.

The immunological specificity of the antibody was also shown by the complete precipitation of 5'-nucleotidase activity by the antibody (Eto, S., and K. Kato, manuscript in preparation).

Preparation of Ferritin-Antibody Conjugates: Ferritin was purified from horse spleen according to the procedures of Granick (13) with a slight modification by Morimoto et al. (21). Ferritin and the antibodies were coupled together by using glutaraldehyde as a coupling agent (16), and the ferritin-antibody conjugates with the molar ratio of IgG to ferritin of ~1:1 were isolated by gel filtration on Bio-Gel A 1.5 M (Bio-Rad Laboratories, Richmond, CA) as described previously (20). The conjugates were concentrated to ~2 mg of ferritin and 0.5 mg of IgG per milliliter. The immunological activity of the conjugates was tested by Ouchterlony double-diffusion analysis.

Isolation of Prefixed Liver Cells: Prefixed liver cells were prepared from male Sprague-Dawley rats (~150 g) according to the improved procedure of Matsuura et al. (20) with a slight modification. Inasmuch as 5'-nucleotidase is very sensitive to glutaraldehyde, the concentration of glutaraldehyde used for perfusion prefixation was decreased from 0.7 to 0.6%. By practice, we could prepare prefixed hepatocytes from liver homogenate with almost 100% recovery. Fig. 2 shows a light micrograph of thick sections of the epoxy resin-

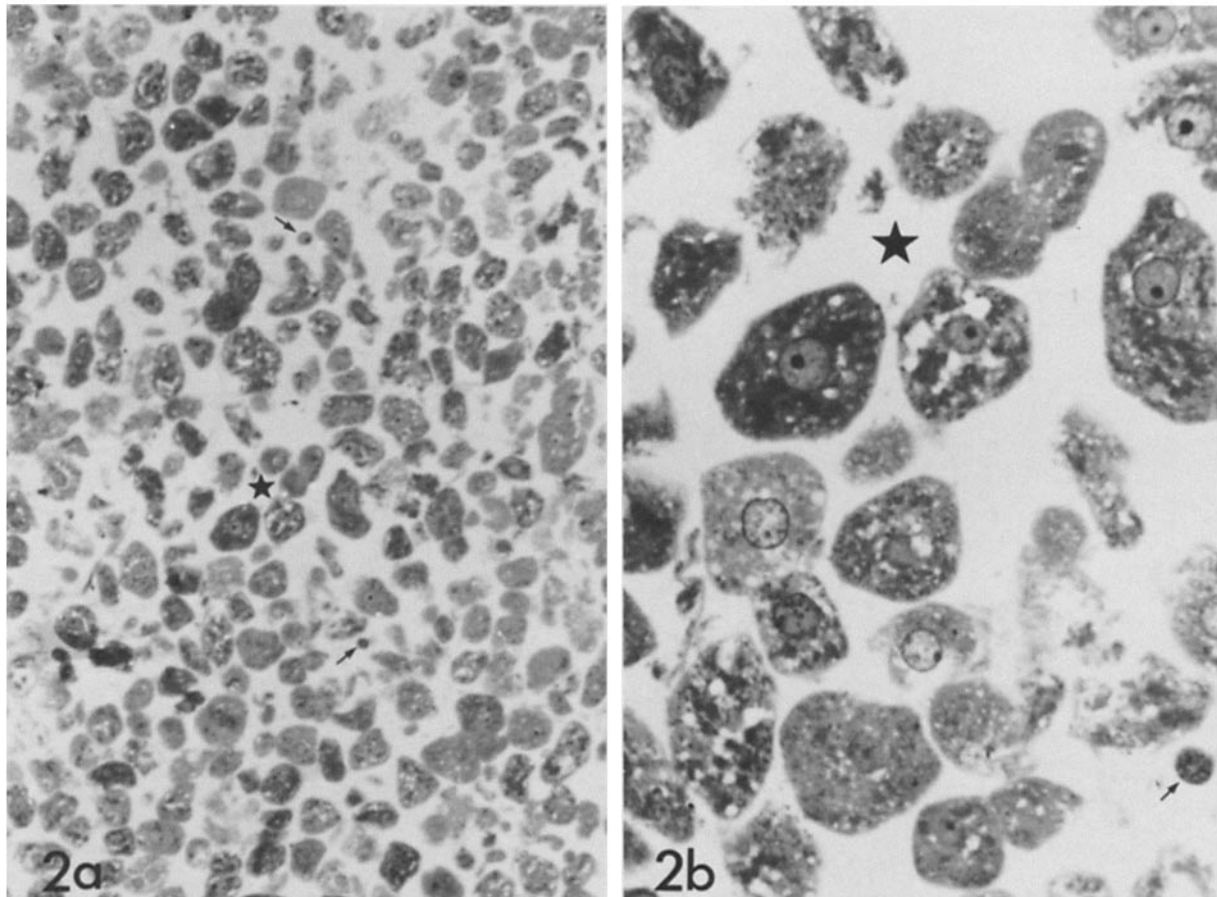


FIGURE 2 Light micrograph of the isolated hepatocytes prefixed by perfusion with 0.6% glutaraldehyde. Thick sections (~1 μ m) were cut from Epoxide-embedded specimens for electron microscopy and stained with toudine blue. Note that all the hepatocytes and endothelial cells are polygonal in shape and that few naked nuclei were detected. (a) \times 500; (b) \times 1,600. The corresponding point of the two micrographs is shown by a star. Arrows indicate naked nuclei.

embedded materials that were stained with toluidine blue. It is evident that naked nuclei are hardly detectable and the hepatocytes and endothelial cells effectively preserve their original polygonal shape.

Labeling of Isolated Liver Cells with Antibody Conjugates and Electron Microscopy: Prefixed rat liver cells were incubated for 2–3 h at 0–4°C with either antibody conjugates or control conjugates. The incubation with antibody conjugates was always carried out at the saturation level of the antibody as described previously (20), and the corresponding concentration of control conjugates was used for the control experiments. After incubation, the unbound conjugates were washed out three times with 0.25 M sucrose containing 0.05 M Tris-HCl, pH 7.4, 0.025 M KCl, and 0.005 M MgCl₂ by centrifugation at 1,000 g for 5 min.

Two kinds of control experiments were carried out; the first, in which the control conjugates or antibody conjugates specific for the other proteins were used in place of the specific antibody conjugates; and the second, in which liver cells were incubated with a 10-fold excess of free antibody and then with the antibody conjugates (blocking test).

The washed pellets were postfixed successively with 2.5% glutaraldehyde and 1% osmium tetroxide, dehydrated, and embedded in Epon. The thin sections ~70 nm in thickness were cut and observed under a Hitachi electron microscope HU-12 (Hitachi Ltd., Tokyo, Japan). Electron micrographs of liver cells were taken at random, and counts were made from micrographs printed at × ~100,000. The number of ferritin particles on the membranes strictly perpendicular to the section were counted, the length of the membranes 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), and the average number of ferritin particles per micrometer of hepatocyte or endothelial cell surface was calculated as described previously (19).

RESULTS

Identification of Bile Canalicular, Lateral, and Sinusoidal Surface of Hepatocytes

Fig. 3 is a low-magnification electron micrograph of a hepatocyte. The bile canalicular surface is easily identified by the presence of characteristic microvilli, usually vacuolated, and by a pair of the junctional complexes (small arrows), the

lateral surface by the smooth surface profile, and the sinusoidal surface by the presence of a number of microvilli, some of which were occasionally vacuolated.

Bile Canalicular Cell Surface of Hepatocytes

Figs. 4 and 5 illustrate the high-magnification views of the bile canalicular face of the hepatocytes that were incubated with ferritin-antibody conjugates specific for 5'-nucleotidase. As pointed out above, this face can be easily identified by the presence of junctional complexes and characteristic microvilli. The latter are usually markedly vesiculated or ballooned even after perfusion fixation with glutaraldehyde.

As clearly shown in Figs. 4 and 5, ferritin particles are exclusively observed on the microvilli in large clusters. This is in marked contrast to the intermicrovillar regions, where many fewer ferritin particles were observed. Especially on the coated pits that are occasionally observed at the intermicrovillar region on the bile canalicular face, hardly any ferritin particles were observed.

According to our method, hepatocytes are usually dissociated mechanically at the site of the junctional complexes; thus the bile canalicular face of each hepatocyte is opened up (Fig. 4). Occasionally, however, hepatocytes with almost intact bile canalicular profiles were found as shown in Fig. 5. Even in such a case, the characteristic distribution of ferritin particles were observed as in the usual case.

In accordance with the previous reports (20, 27, 31), the bile canalicular microvilli are usually blistered as shown in Figs. 4 and 5. Occasionally, however, bile canaliculi with nonvesiculated microvilli are found as shown in Fig. 6. Clustering of ferritin particles is also observed on such microvilli.

Fig. 7 shows a control experiment, in which liver cells were

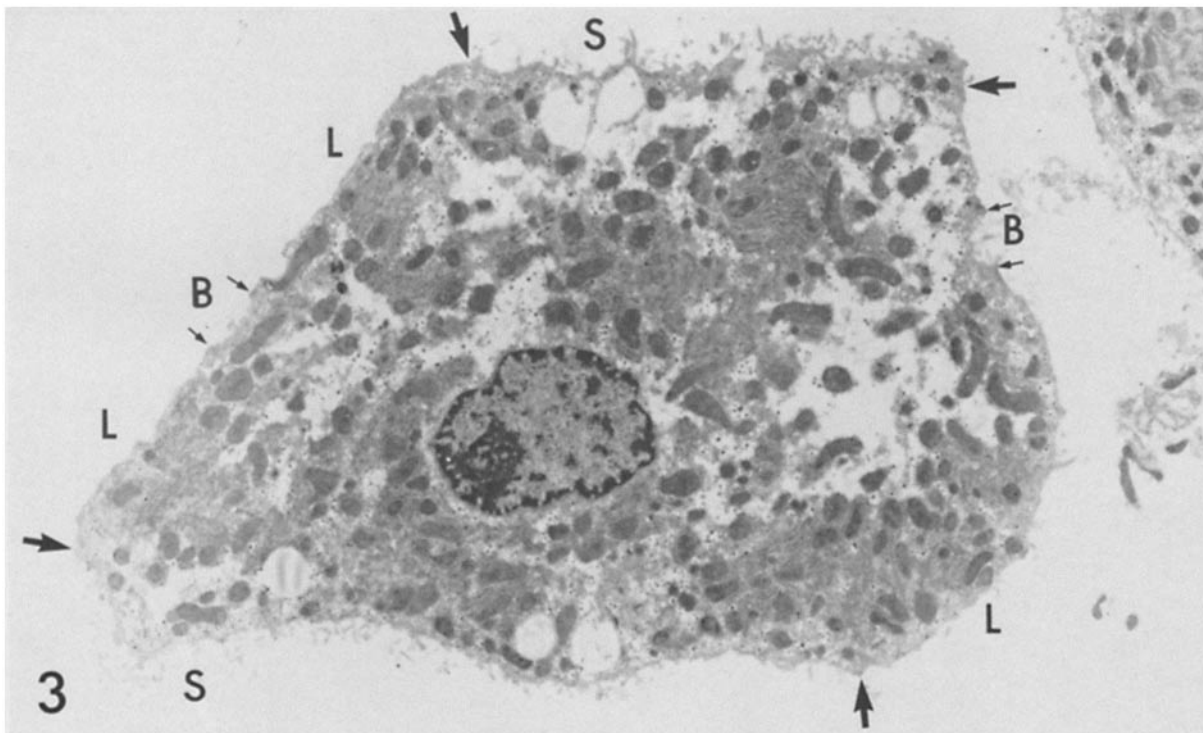
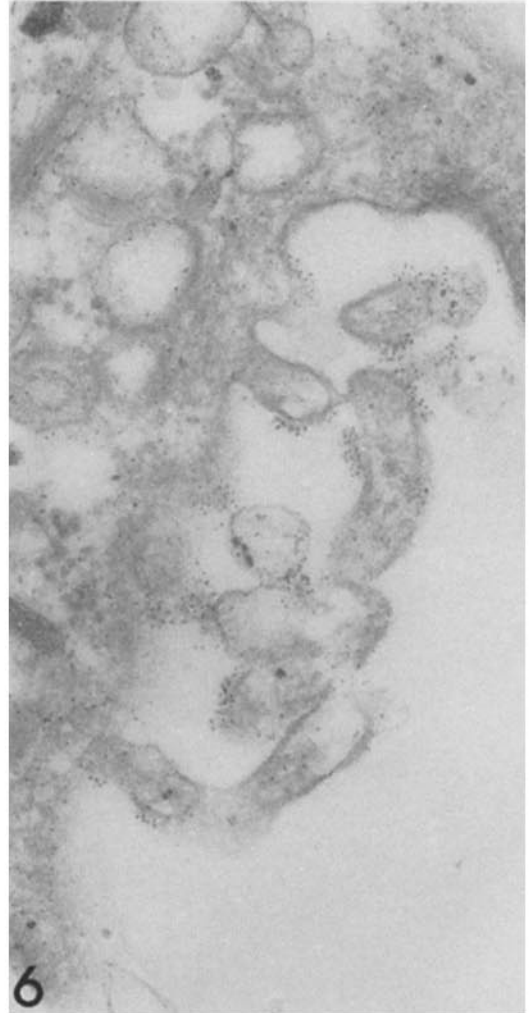
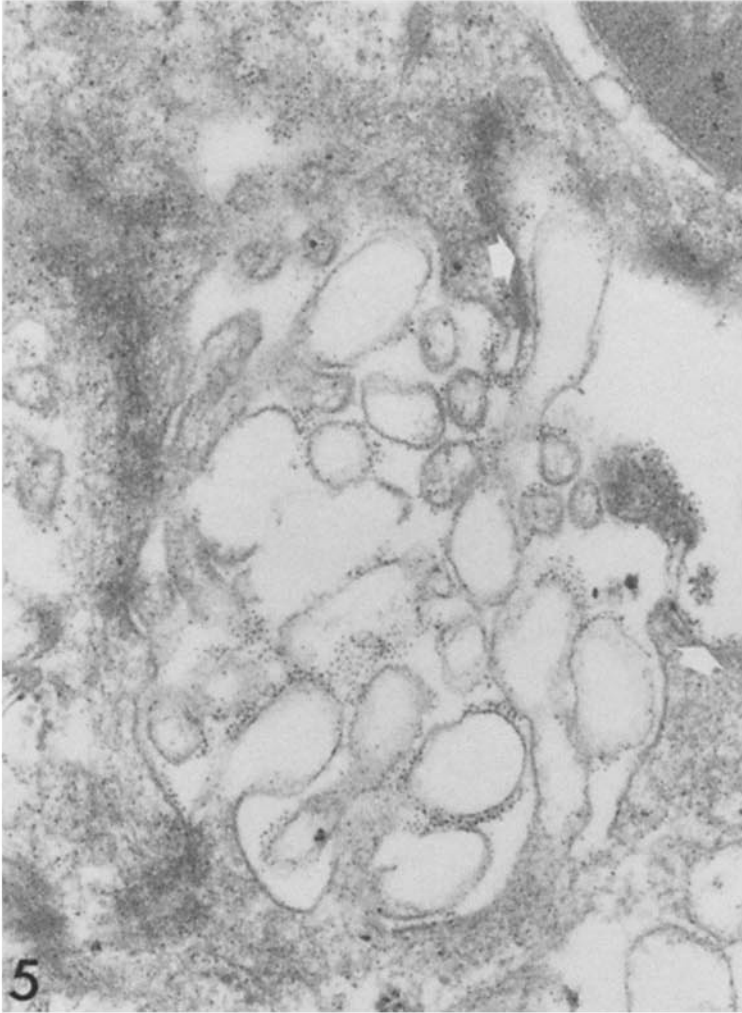
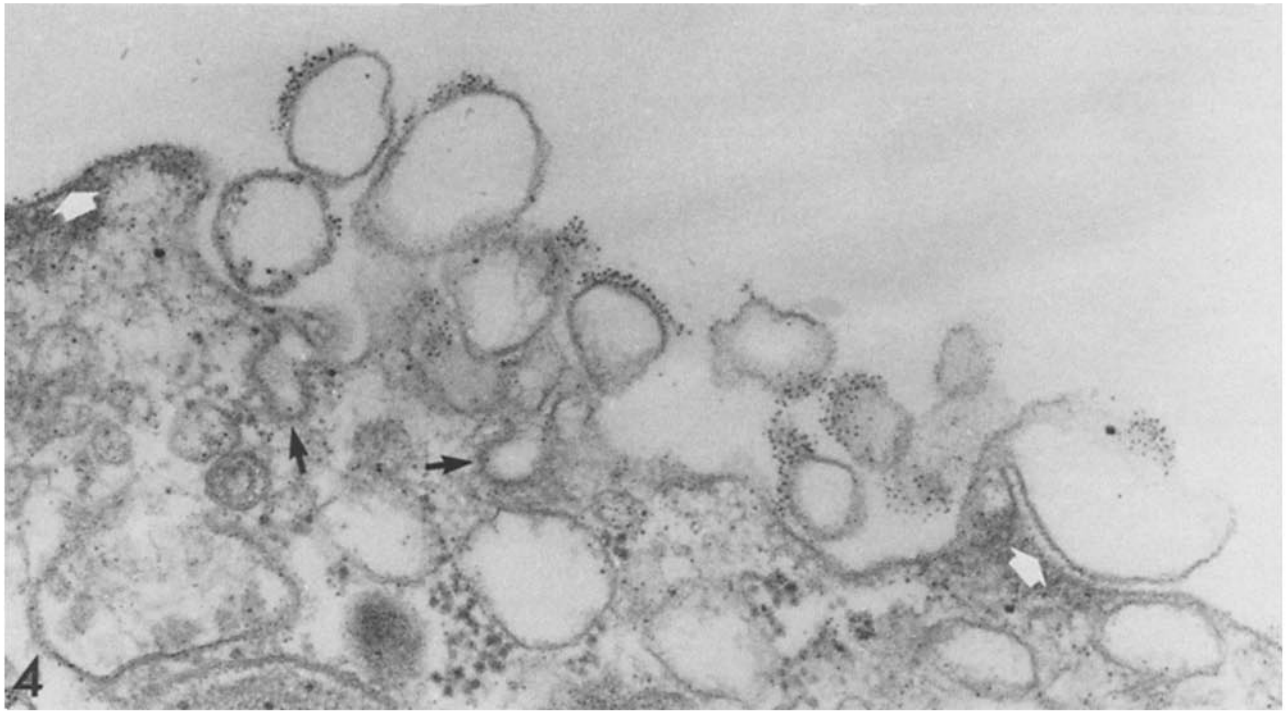


FIGURE 3 Identification of the sinusoidal, lateral, and bile canalicular surfaces of a hepatocyte in a low-magnification electron micrograph. The boundary between the sinusoidal and lateral surface was indicated by large arrows, while that between the bile canalicular and lateral surface by small arrows. The specimens for electron microscopy were prepared after incubation and washing for antibody staining. Note preservation of the morphology of hepatocytes. S, Sinusoidal surface; L, lateral surface; B, bile canalicular surface. × 5,000.



FIGURES 4-6 Bile canicular surface of isolated hepatocytes incubated with ferritin anti-5'-nucleotidase antibody conjugates. In Figs. 4 and 5, junctional complexes are indicated by white arrows. Ferritin particles were observed in large clusters on the microvillar regions, whereas hardly any particles were observed on the intermicrovillar regions. Fig. 5 shows a hepatocyte with almost intact bile canaliculi. In Figs. 4 and 5, almost all the microvilli are blistered, whereas in Fig. 6, microvilli are not blistered. The clustering of ferritin particles in Fig. 6 was similar to that in Figs. 4 and 5. Figs. 4 and 5, $\times 70,000$; Fig. 6, $\times 60,000$.

incubated with control conjugates. Ferritin particles were hardly observed. Similar results were obtained when liver cells were incubated with an excess amount of the antibody against 5'-nucleotidase before the incubation with the antibody conjugates (data not shown).

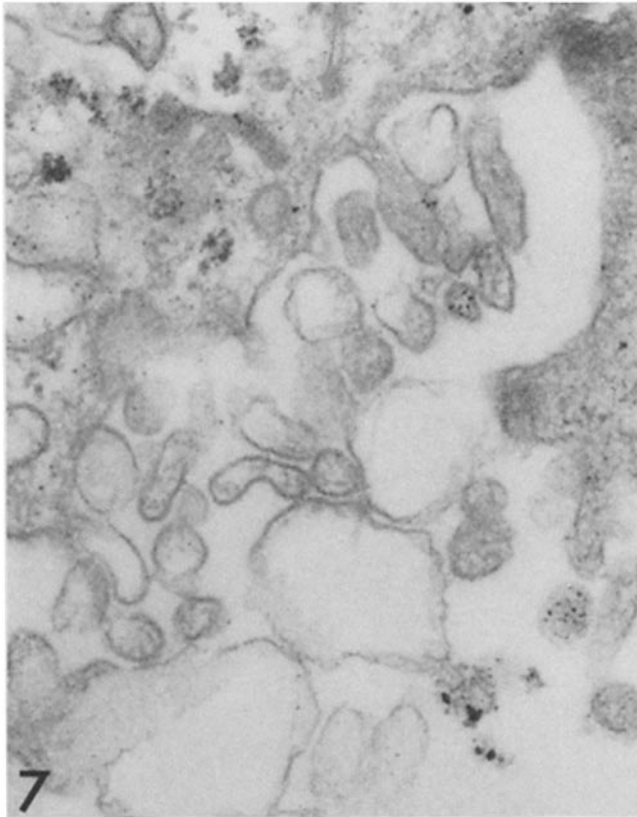


FIGURE 7 Bile canalicular region of isolated hepatocyte incubated with control conjugates. Few ferritin particles were attached to the cell surface. $\times 60,000$.

Sinusoidal and Lateral Surfaces of Hepatocytes

Fig. 8 shows the sinusoidal surface of hepatocytes. Essentially similar distribution of ferritin particles was observed on the sinusoidal surface; ferritin particles were exclusively found on the microvilli in clusters, and were hardly observed on the intermicrovillar regions. The microvilli on this face were usually not blistered, the number of ferritin clusters and of ferritin particles per cluster were many fewer than those on the bile canalicular face. Again few ferritin particles were observed on the coated pit regions as shown by an arrow.

Fig. 9, *a* and *b* shows the lateral face of hepatocytes. On this surface no microvilli are observed. Ferritin particles were located only sporadically in small clusters on the smooth lateral surface of hepatocytes.

Endothelial Cell Surface

Fig. 10 shows the localization of ferritin particles on the endothelial cell surface. In marked contrast to the heterogeneous distribution on hepatocyte surface, ferritin particles on the endothelial cell surface are distributed rather evenly.

Particle Density on the Various Hepatocyte Cell Surfaces

Table I shows the number of ferritin particles found per micrometer of the various hepatocyte cell surfaces and of endothelial cell surface. The total number of ferritin particles counted was 12,000 and the total cell surface measured was $60 \mu\text{m}^2$. The average thickness of the thin sections was assumed to be 70 nm.

The particle density of the bile canalicular surface of hepatocytes was about four times higher than that of the sinusoidal surface and that of lateral surface was only about one-tenth of that of bile canalicular surface.

The average particle density of the total hepatocyte cell surface, when incubated with control conjugates, was 0.3

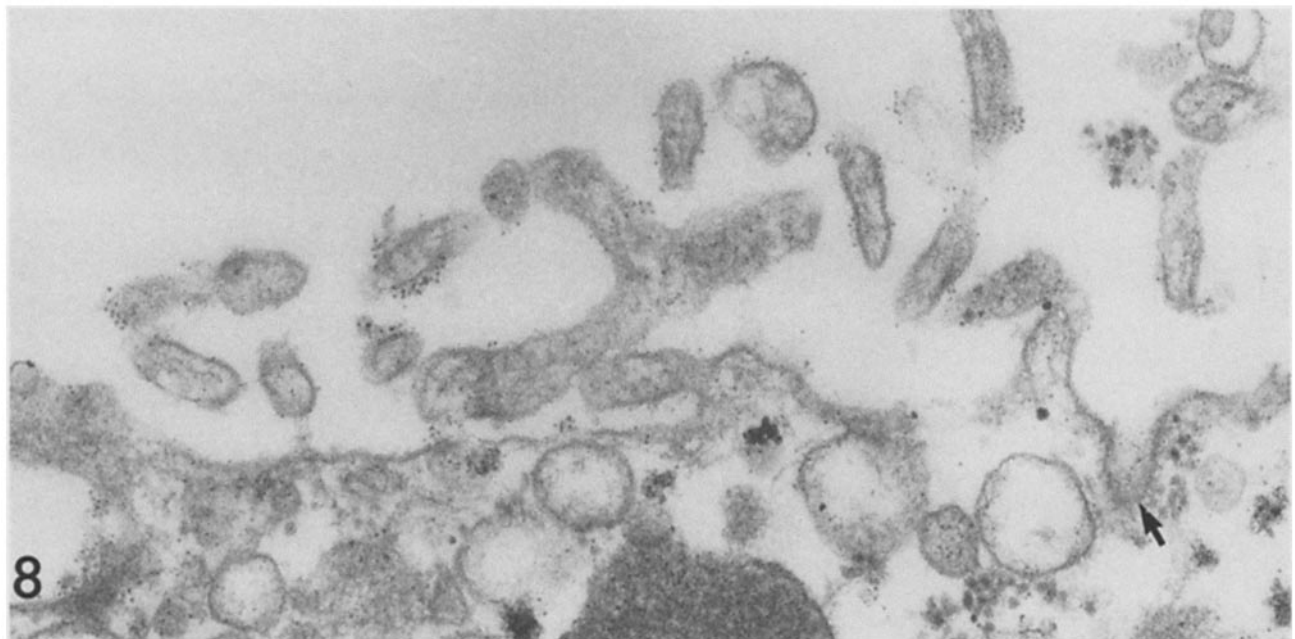


FIGURE 8 Sinusoidal region of isolated hepatocyte incubated with anti-5'-nucleotidase antibody conjugates. Note almost exclusive localization of ferritin particles on the microvilli in cluster. The number of ferritin particles on the microvilli of sinusoidal face was many fewer than that on the bile canalicular face. Few ferritin particles were present on the coated pit (arrow). $\times 70,000$.

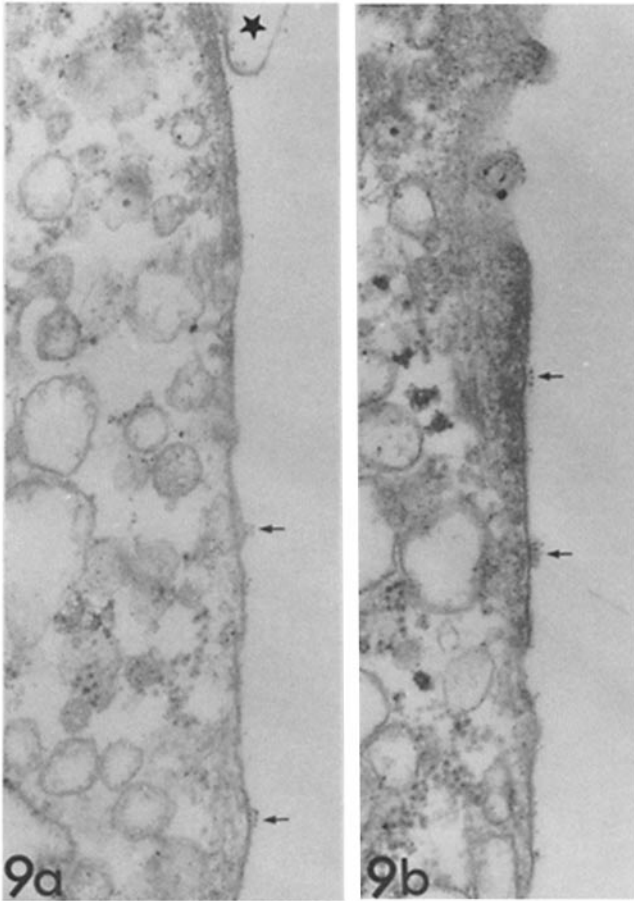


FIGURE 9 (a and b) Lateral region of isolated hepatocyte incubated with anti-5'-nucleotidase antibody conjugates. Only occasionally were small clusters of ferritin particles observed on the lateral surface (arrows). In a, the bile canaliculus region is shown by a star. $\times 50,000$.

particles/ μm . The particle density of the bile canaliculus cell surface, when previously incubated with the corresponding free antibody and then with the ferritin-antibody conjugate, was again only 0.3 particles/ μm (blocking test). These control values were subtracted from the experimental values and the corrected particle densities are listed in Table I.

DISCUSSION

Exact localization and quantitative estimation of the distribution of cytoplasmic membrane enzymes at an ultrastructural level is very important for the clear understanding of the functions of these enzymes. This is especially true when polarized epithelial cells such as hepatocytes are the subject of investigation.

5'-Nucleotidase is one of the most typical cell membrane enzymes and its distribution has been studied extensively by biochemical (7, 12, 33) and cytochemical (10, 11, 16, 17) procedures. The exact localization and quantitative estimation of 5'-nucleotidase on hepatocyte cell surface, however, have not been reported. In the present experiment prefixed liver cells were isolated according to a modification of our procedure (20) without losing their polarized structures, and the distribution of 5'-nucleotidase was investigated according to our quantitative ferritin immunoelectron microscopic technique (19).

Table I shows that the surface density of 5'-nucleotidase is almost four times higher on the bile canaliculus surface than on the sinusoidal surface and that only a few enzymes are found on the lateral surface.

In the previous paper (20), the average area per cell of sinusoidal, lateral, and bile canaliculus surfaces of hepatocyte were determined by morphometry as 1,756, 785, and 407 μm^2 , respectively. Table II shows the total number of ferritin particles on each cell surface and their relative distribution. It is shown that almost equal numbers of 5'-nucleotidase exist on the sinusoidal and bile canaliculus surface and <10% on the lateral surface. These results are consistent with the biochemical (7, 12, 33) and cytochemical findings (5, 6, 26, 27) on the overall distribution of 5'-nucleotidase.

If we assume that each 5'-nucleotidase binds to one ferritin-antibody conjugate, and that perfusion fixation with 0.6% glutaraldehyde does not impair the binding capacity of the antigen with antibody conjugates, the average number of 5'-nucleotidase on each hepatocyte is calculated to be $\sim 2.6 \times 10^5$ molecules/cell. According to Stanley et al. (32), $\sim 50\%$ of 5'-nucleotidase exists on the hepatocyte cell surface. It is estimated, therefore, that the total number of 5'-nucleotidase per cell is $\sim 5 \times 10^5$ molecules. A similar number of $\sim 5 \times 10^5$ molecules has been estimated from biochemical data by Bailes et al. (1).

It was further revealed that 5'-nucleotidase is almost exclusively localized on the microvilli in large clusters both on the bile canaliculus and sinusoidal surfaces. As to the clustering of 5'-nucleotidase, two possibilities should be considered. One is that the clustering on the microvilli faithfully reflects the existence of 5'-nucleotidase *in vivo* and another is that the clustering was produced artificially by the blistering of the microvilli and/or by incubation with ferritin-antibody conjugates containing divalent antibodies.

Clustering of ferritin particles was also observed on the sinusoidal microvilli which did not blister at all (Fig. 8), as well as on the nonblistered bile canaliculus microvilli (Fig. 6). Furthermore, distribution of 5'-nucleotidase on the endothelial cells in the same specimens was random (Fig. 10). Therefore, we favor the interpretation that clustering of 5'-nucleotidase is not due to the artificial aggregation but rather to the existence of such clusters *in vivo* on the microvillar surface. The possibility of aggregation of membrane proteins by incubation with ferritin-antibody conjugates containing divalent antibodies has been discussed previously in detail (18, 19). In spite of these considerations, the possibility of artificial aggregation of 5'-nucleotidase induced by blistering of canaliculus microvilli should not be excluded.

It has been reported that 5'-nucleotidase has an important physiological function in the production of adenosine from extracellular nucleotides (22, 23, 28). Since one of the most important functions of microvilli is to absorb nutrients from extracellular space, it is suggested that 5'-nucleotidase on microvilli of the sinusoidal surface of hepatocytes engages in the absorption of nucleosides from blood plasma. Although the surface density of the enzyme on the sinusoidal surface is much lower than that on the bile canaliculus surface, the total number of the enzymes on the former is almost equal to that on the latter because of very wide surface area of the former. This amount of 5'-nucleotidase may be enough to supply sufficient nucleosides to hepatocytes.

What is the function of 5'-nucleotidase on the bile canaliculus surface? It has been reported that bile contains various

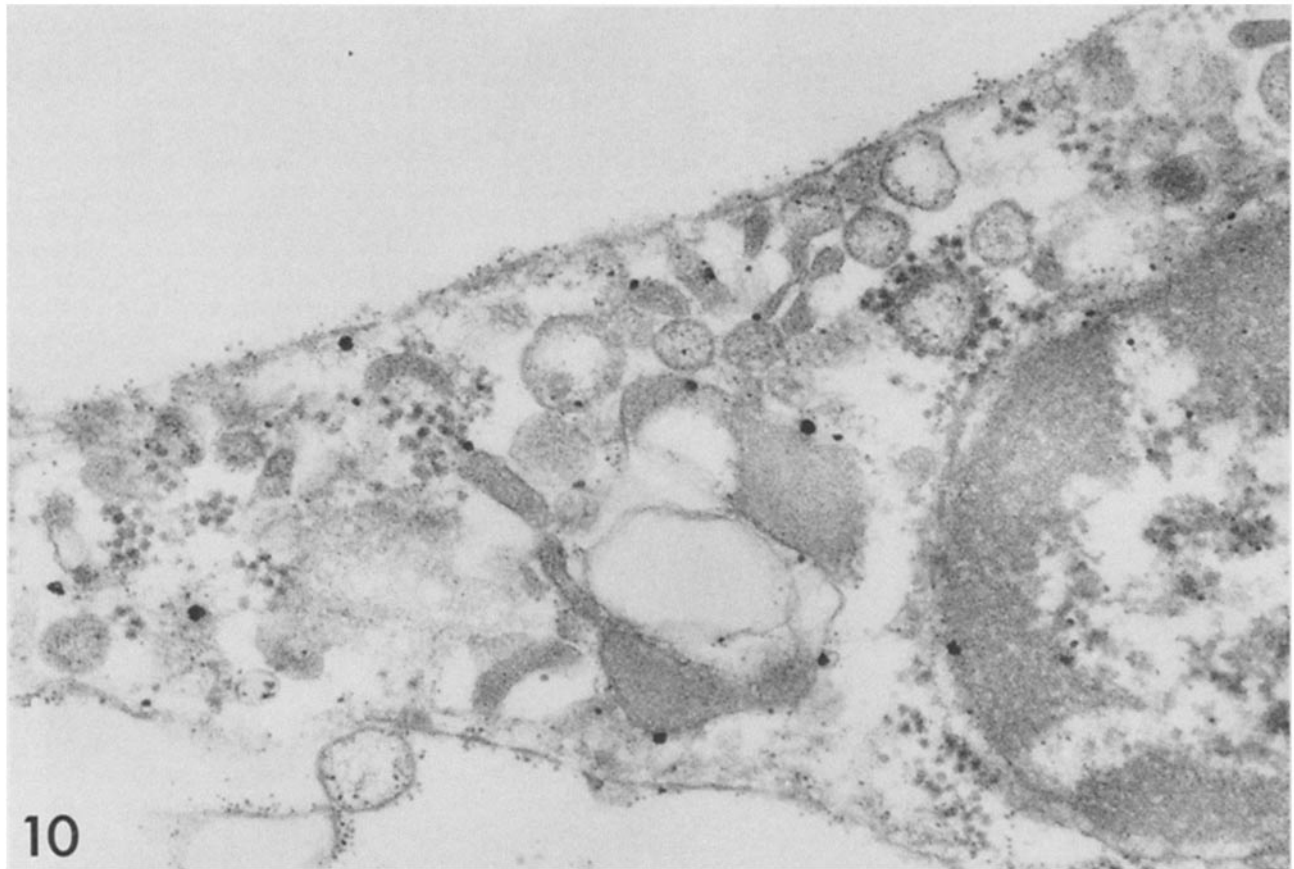


FIGURE 10 Endothelial cells incubated with ferritin antibody conjugates. The endothelial cell surface was rather evenly stained with ferritin-antibody conjugates. $\times 75,000$.

TABLE I
Density of Ferritin Particles on Various Cell Surfaces

Cells and cell surfaces	Antibody conjugate	No. of ferritin particles per micrometer of cell surface
Hepatocytes		
Sinusoidal	Anti-5'-nucleotidase	5.6
Lateral	Anti-5'-nucleotidase	2.1
Bile canalicular	Anti-5'-nucleotidase	20.1
Coated pit*	Anti-5'-nucleotidase	~0
Endothelial cells	Anti-5'-nucleotidase	12.2
Control experiments		
Bile canalicular	Control-IgG	0.2
	Block test	0.3
Total hepatocyte cell surface	Control-IgG	0.3

* Sinusoidal and bile canalicular.

enzymes such as 5'-nucleotidase, alkaline phosphatase, and γ -glutamyl transpeptidase, and that the activities of these enzymes in the serum from patients with hepatobiliary disorders markedly increase (2, 10, 15). As described previously, only a small amount of 5'-nucleotidase exists on the intervillar regions where secretion by exocytosis may occur exclusively. On the contrary, 5'-nucleotidase was mostly found on the microvillar region in large clusters on both the bile canalicular

TABLE II
Number of Ferritin Particles on Different Hepatocyte Cell Surfaces

Surface	No. of particles $\times 10^5$	Distribution* %
Hepatocytes		
Sinusoidal	1.28	49.2
Lateral	0.18	6.9
Bile	1.04	43.8
Total	2.60	100
Nonhepatocytes	~0.9	

* Values represent percent of total hepatocyte surface particle density.

and sinusoidal surfaces. It is strongly suggested, therefore, that these enzymes are released into bile or blood serum by pinching off of the tips of the microvilli. This mode of extrusion corresponds to microapocrine secretion or extrusion type III of Kurosumi (17). A small amount of ectoenzymes on the bile canalicular surface such as 5'-nucleotidase, alkaline phosphatase, leucine aminopeptidase, and γ -glutamyl transpeptidase may be released into bile when bile salt is secreted from hepatocytes. We believe that such liberation of ectoenzymes into bile should be a physiological process, although it is markedly elevated in the case of cholestasis.

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