Quantitative Immunoferritin Localization of [Na⁺,K⁺]ATPase on Canine Hepatocyte Cell Surface

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ABSTRACT Distribution of [Na⁺,K⁺]ATPase on the cell surface of canine hepatocytes was investigated quantitatively by incubating prefixed and dissociated liver cells with ferritin antibody conjugates against canine kidney holo[Na⁺,K⁺]ATPase. We found that [Na⁺,K⁺]-ATPase exists bilaterally both on the bile canalicular and sinusoid-lateral surfaces. The particle density on the bile canalicular surface was much higher (approximately 2.5 times) than that on the sinusoid-lateral surface. In the latter region, the enzyme was detected almost equally both on the sinusoidal and lateral surfaces. On all the surfaces, the distribution of the enzyme was homogeneous and no clustering of the enzyme was detected. Total number of the enzyme on the sinusoid-lateral surface was, however, approximately three times higher than that on the bile canalicular region, because the sinusoid-lateral surface represents ~87% of the total cell surface of a hepatocyte. We suggest that the [Na⁺,K⁺]ATPase on the bile canalicular surface is responsible for the bile acid-independent bile flow and the other transport processes on the bile canalicular cell surface, while that on the sinusoid-lateral surface is responsible not only for the active transport of Na⁺ but also for the secondary active transport of various substances in this region.
To 2.5 ml of the Jorgensen’s \(Na^+K^+\)ATPase solution (2 mg/ml), we added trastomy, leupeptin, and pepstatin at a final concentration of 200 \(\mu\)g/ml, and 20 \(\mu\)g/ml, respectively, and 10% C\(_2\)E\(_8\) was added to a final concentration of 1.3. The mixture was incubated at 25°C for 15 min and centrifuged at 100,000 \(g\) for 1 h. The supernatant was dialyzed against 30 mM histidine (pH 7.2) containing 100 mM KCl, 0.04% C\(_2\)E\(_8\), 10 mM choline chloride, and 100 \(\mu\)g/ml phosphatidyl choline (buffer A). The dialyzed solution was mixed continuously at 4°C overnight with WGA-Sepharose 6 MB previously washed extensively with buffer A. The WGA-Sepharose was washed twice with buffer A by low speed centrifugation and packed into a column (0.4 x 8 cm). After washing the column with 10 vol of buffer A, 0.2 M \(\alpha\)-acetylglucosamine in buffer A was used for the elution of holo[Na\(^+\),K\(^+\)]ATPase.

**Purification of Holo[Na\(^+\),K\(^+\)]ATPase:** Holo[Na\(^+\),K\(^+\)]ATPase was purified from the Jorgensen’s ATPase fraction by solubilization with C\(_2\)E\(_8\) (19-21) and subsequent affinity chromatography with WGA-Sepharose 6 MB, which has specific affinity for the \(\beta\) subunit of the ATPase and, therefore, used for the purification of the subunits by Omori et al. (18).

To 2.5 ml of the Jorgensen’s [Na\(^+\),K\(^+\)]ATPase solution (2 mg/ml), we added trastomy, leupeptin, and pepstatin at a final concentration of 200 \(\mu\)g/ml, and 20 \(\mu\)g/ml, respectively, and 10% C\(_2\)E\(_8\) was added to a final concentration of 1.3. The mixture was incubated at 25°C for 15 min and centrifuged at 100,000 \(g\) for 1 h. The supernatant was dialyzed against 30 mM histidine (pH 7.2) containing 100 mM KCl, 0.04% C\(_2\)E\(_8\), 10 mM choline chloride, and 100 \(\mu\)g/ml phosphatidyl choline (buffer A). The dialyzed solution was mixed continuously at 4°C overnight with WGA-Sepharose 6 MB previously washed extensively with buffer A. The WGA-Sepharose was washed twice with buffer A by low speed centrifugation and packed into a column (0.4 x 8 cm). After washing the column with 10 vol of buffer A, 0.2 M \(\alpha\)-acetylglucosamine in buffer A was used for the elution of holo[Na\(^+\),K\(^+\)]ATPase.

**Purification of the \(\alpha\) and \(\beta\) Subunits:** The \(\alpha\)- and \(\beta\)-subunits of Freund’s complete adjuvant were injected into rabbits subcutaneously (100-200 \(\mu\)g protein/animal) or popliteal lymph nodes according to Goudie et al. (22) (50-100 \(\mu\)g protein/animal). 1 wk before bleeding, the rabbits were intravenously immunized with the corresponding proteins (100-200 \(\mu\)g protein/animal). The IgG fractions of these antisera were prepared by repeated precipitation with ammonium sulfate, followed by DEAE cellulose column chromatography, and concentrated to the original serum volume.

**Specific Inhibition of the Kidney and Liver [Na\(^+\),K\(^+\)]ATPase Activities by the Antibodies:** The immunological specificity of the antibodies was tested by specific inhibition of the [Na\(^+\),K\(^+\)]ATPase activities of kidney [Na\(^+\),K\(^+\)]ATPase fraction of Jorgensen and of the liver plasma membrane fraction of Neville (23).

In the former case, 0-200 \(\mu\)l of the nonimmunized rabbit serum or antisera, which were previously dialyzed extensively against 0.15 M imidazole/HCl buffer, pH 7.4, and 20 \(\mu\)l of the kidney [Na\(^+\),K\(^+\)]ATPase fraction (0.2 mg/ml), was added to 109 concentrated buffer B (3 mM MgCl\(_2\), 130 mM NaCl, 20 mM KCl, 30 mM histidine, pH 7.5, with or without 1 mM ouabain) at a final volume of 0.9 ml. The solution was preincubated at 4°C for 12 h. Then, after preheating at 37°C for 2 min, 100 \(\mu\)l of 30 mM ATP was added and the incubation continued for 5 min. The reaction was stopped by adding 100 \(\mu\)l of 50% trichloroacetic acid and cooling to 0°C. The amount of inorganic phosphate liberated was measured by the method of Parvin and Roberts (24).

In the latter case, canine liver plasma membrane fraction was prepared according to Neville (23), and the inhibition of the [Na\(^+\),K\(^+\)]ATPase activity by the antibodies was measured. Nonimmunized rabbit IgG (control IgG) or specific IgG (0-400 \(\mu\)g), which were previously dialyzed against 0.15 M Tris-HCl buffer, pH 7.4, and the plasma membrane fraction (50 \(\mu\)g) was added to the 10:9 concentrated buffer B at a final volume of 0.9 ml. This solution was preincubated at 4°C for 12 h. Then, after heating at 37°C for 2 min, 100 \(\mu\)l of 30 mM ATP was added and the incubation continued for 20 min at 37°C. The reaction was stopped and the amount of liberated inorganic phosphate was measured as before.

**Characterization of the Antibodies by Immunoblotting:** The immunological specificity of the antibodies was also tested by immunoblotting of [Na\(^+\),K\(^+\)]ATPase from canine liver plasma membranes according to Burnett (25). Briefly, plasma membrane fractions were prepared from canine liver by the method of Neville (23), and the membrane proteins were separated by SDS PAGE and transferred electrophoretically to a nitrocellulose sheet. The sheet was incubated with IgG specific for holo[Na\(^+\),K\(^+\)]ATPase and subsequently with \(^{32}\)P-labeled protein A and visualized by autoradiography.

As a control, the Jorgensen’s [Na\(^+\),K\(^+\)]ATPase fraction was analysed simultaneously.

**Preparation of Ferritin Antibody Conjugates:** Ferritin was purified from horse spleen according to the procedures of Granick (26), with a slight modification. Ferritin and the antibodies were coupled together by using glutaraldehyde as a coupling agent (27), and 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 (13). The conjugates were concentrated to -2 mg of ferritin and 0.5 mg IgG/ml. The control conjugates were prepared in the same way by coupling ferritin with nonimmunized rabbit IgG (control IgG).

**Dissociation of Prefixed Liver Cells:** Prefixed liver cells were dissociated from canine liver according to the improved procedures of Matsuura et al. (14). By this procedure we could prepare the dissociated hepatocytes and endothelial cells preserving well their original polygonal shape (14).

**Labeling of Isolated Liver Cells with Antibody Conjugates and Electron Microscopy:** Prefixed canine liver cells were incubated for 2-3 h at 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 (28), and the corresponding concentration of control conjugates were used for the control experiments.

The other procedures for the quantitative ferritin immunoelectron microscopy were carried out according to the previous paper from this laboratory (13, 14, 28). As a control, hepatocytes were incubated with the control conjugates.

**Other Methods:** Protein was measured by the method of Lowry et al. (29) using bovine serum albumin as the standard. SDS PAGE was run according to Laemmli (30) using 8% polyacrylamide gels.
The properties of the antibodies against the α- and β-subunits have been described in detail elsewhere (18). It is evident, however, that small amounts of the impurities are effectively eliminated by the affinity chromatography with WGA-Sepharose 6 MB (Fig. 1, lane 5), and the WGA-Sepharose bound fraction is now exclusively composed of the α- and β-subunits (Fig. 1, lane 4). We have previously reported that the α-subunit, when denatured with SDS, does not bind to the WGA-Sepharose (18).

Table I shows yield in the amount of protein and the specific activity of [Na⁺,K⁺]ATPase in the course of the purification procedures of holo[Na⁺,K⁺]ATPase. Starting from the [Na⁺,K⁺]ATPase fraction of Jorgensen, the protein yield and the enzyme specific activity of the final preparation (WGA-Sepharose bound fraction) was ~10% and 50%, respectively. The specific activity of the WGA-Sepharose bound fraction was even lower that of the original Jorgensen's fraction. Since [Na⁺,K⁺]ATPase is originally a membrane-bound enzyme, it is suggested that the enzyme, although purified extensively, was partially inactivated by the detergent treatment as reported by Brotherus et al. (31).

Some Properties of the Antibodies against Canine [Na⁺,K⁺]ATPase

The properties of the antibodies against the α- and β-subunits have been described in detail elsewhere (18). These antibodies did not inhibit [Na⁺,K⁺]ATPase activity under various conditions in agreement with the report by McDonaugh et al. (32). Immunoelectron microscopic observation indicated that the ferritin-antibody conjugates against these α- and β-subunits do not bind to the cell surface of hepatocytes.

On the contrary, the antibody against the holoenzymes markedly inhibited not only canine kidney [Na⁺,K⁺]ATPase activity but also liver ATPase activity as shown in Figs. 2 and 3, respectively, and we could show characteristic labeling of canine hepatocyte cell surface as described in the following. In the subsequent immunoelectron microscopic analyses, the antibody against holo[Na⁺,K⁺]ATPase was exclusively used.

The monospecificity of the antibody against holo[Na⁺,K⁺]ATPase was tested by the “Western blotting” of the canine kidney [Na⁺,K⁺]ATPase fraction and the canine liver plasma membrane fraction. As shown in Fig. 4, the antibody bound preferentially to the α subunit of [Na⁺,K⁺]ATPase from both kidney and liver, and no other membrane proteins were labeled. By longer exposure, however, their β-subunits were also visualized. When the control IgG from non-immunized rabbit sera was used, none of the membrane proteins were labeled.

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein yield, ( n = 5 )</th>
<th>Specific activity, ( n = 3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jorgensen's [Na⁺,K⁺]ATPase</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C₂₂E₆ solubilized</td>
<td>58.3 ± 5.1</td>
<td>44.8 ± 6.8</td>
</tr>
<tr>
<td>C₂₂E₆ insoluble</td>
<td>32.1 ± 2.1</td>
<td>0</td>
</tr>
<tr>
<td>WGA-Sepharose bound</td>
<td>9.7 ± 2.9</td>
<td>48.7 ± 6.3</td>
</tr>
<tr>
<td>WGA-Sepharose flow-through</td>
<td>14.7 ± 6.4</td>
<td>0</td>
</tr>
</tbody>
</table>

FIGURE 2 Inhibition of canine kidney [Na⁺,K⁺]ATPase activity by the addition of antisera against canine kidney holo[Na⁺,K⁺]ATPase. The Jorgensen's [Na⁺,K⁺]ATPase fraction prepared from canine kidney (16, 17) was preincubated with various amounts of non-immunized rabbit serum (○) or antisemur (●) at 37°C for 20 min. After addition of ATP, the solution was further incubated for 5 min. The reaction was stopped by the addition of trichloroacetic acid and cooling. The amount of inorganic phosphate liberated was measured by Parvin and Roberts (28). [Na⁺,K⁺]ATPase activities were measured as a difference in the amount of inorganic phosphate liberated in the presence and absence of 1 mM ouabain. The ATPase activity in the absence of the antisera was presented as 100% of the orginate.

FIGURE 3 Inhibition of canine liver [Na⁺,K⁺]ATPase activity by the addition of antibodies against canine kidney holo[Na⁺,K⁺]ATPase. The canine liver plasma membrane fraction was prepared according to Neville (23). These membranes were preincubated either with control IgG prepared from non-immunized rabbit sera or specific IgG prepared from immunized sera for 12 h at 4°C. ATP was added and the solutions were further incubated at 37°C for 20 min. The reaction was stopped and the amount of liberated inorganic phosphate was determined. [Na⁺,K⁺]ATPase activities were measured as described in the legend for Fig. 2, and Mg²⁺-ATPase activities were measured in the presence of 1 mM ouabain. These two ATPase activities in the absence of IgG were presented as 100% of the orginate. [Na⁺,K⁺]ATPase, + specific IgG (●); + control IgG (○). Mg²⁺-ATPase, + specific IgG (●); + control IgG (○).
cytes incubated with control conjugates. When washed well, hardly any ferritin particles were found attached to the hepatocytes cell surface, including the bile canalicular surface.

Sinusoidal and Lateral Surfaces of Hepatocytes

As shown in Figs. 8 and 9, sinusoidal surface of hepatocytes was labeled with ferritin antibody conjugates rather uniformly. Both microvillar and intermicrovillar regions were labeled and no clustering of ferritin particles on the sinusoidal surface were observed. It is apparent, however, that the degree of labeling on the sinusoidal surface was much less than that on the bile canalicular surface.

Figs. 10 and 11 show that the lateral surface of hepatocytes is also labeled rather uniformly with ferritin particles. No marked clustering of ferritin particles was observed.

Endothelial Cell Surface

Fig. 12 shows an endothelial cell. It is apparent that the surface of the endothelial cells is labeled evenly with ferritin particles. Again, no clustering of ferritin particles was observable.

Particle Density on the Various Regions of Canine Hepatocyte Cell Surface

The particle density or the number of ferritin particles per \( \mu \text{m} \) of various regions of canine hepatocyte cell surface was calculated and shown in Table II. The total number of ferritin particles counted was about 11,000 and the total length of cell surface surveyed was \( \sim 800 \mu \text{m} \).

The particle density of the bile canalicular surface was about 2.5 times higher than that of the sinusoidal surface, and no marked difference in the particle density was noticed between the lateral and sinusoidal surfaces. Table II also shows the particle density at various surfaces of the control experiments. At the bile canalicular surface the particle density of the control specimens was \( \sim 1\% \) of the experimental particle density. These control values were subtracted from the experimental values shown in Table II.

In the previous paper the average area per cell of sinusoidal, lateral, and bile canalicular surfaces of hepatocyte were determined by morphometry as 1,756, 785, and 407 \( \mu \text{m}^2 \), respectively (17). Assuming that the average thickness of the thin sections is 70 nm, we can calculate the approximate total number of ferritin particles on the various cell surfaces per cell as shown in Table III. Although the particle density of the bile canalicular surface is \( \sim 2.5 \) times higher than that of the sinusoid-lateral surfaces, the total number of \([\text{Na}^+,\text{K}^+]\)-ATPase on the sinusoid-lateral surface is about three times higher than that on the bile canalicular surface.

DISCUSSION

The cytochemical localization of \([\text{Na}^+,\text{K}^+]\)ATPase in hepatocyte has been studied first as Mg-activated ATPase by using a lead phosphate capture method as described by Wachstein and Meisel (34). It had been uncritically assumed that any cytochemically demonstrable plasma membrane Mg-ATPase is likely to be \([\text{Na}^+,\text{K}^+]\)ATPase (35). This ATPase reaction product was demonstrated exclusively at the bile canalicular membranes of hepatocytes (34-37). The validity of this method, however, has been criticized, because Pb\(^{2+}\) inhibits \([\text{Na}^+,\text{K}^+]\)ATPase strongly, Pb\(^{2+}\) can cause nonenzymatic hy-

![Figure 4](image-url)
FIGURES 5-7  Bile canalicular region of canine hepatocyte incubated with ferritin antibody conjugates against canine kidney holo[Na⁺,K⁺]ATPase (Figs. 5 and 6) or with control conjugates (Fig. 7). The bile canalicular region was easily identified by the presence of a pair of junctional complex regions (arrows). The microvillar and intermicrovillar regions of the bile canalicular surface are heavily labeled. Note that the canine bile canalicular microvilli are not vesiculated. × 64,500.
Figs. 8 and 9. Sinusoidal region of canine hepatocyte incubated with ferritin antibody conjugates against canine kidney holo[Na⁺,K⁺]ATPase. The microvillar and intermicrovillar regions of the sinusoidal surface are evenly labeled by the antibody conjugates. Note that the degree of labeling on the sinusoidal surface is much less than that on the bile canalicular surface.

...drolysis of ATP, and Pb²⁺ and ATP can form insoluble, electron-dense complexes in the absence of hydrolysis of ATP.

Then Ernst devised a strontium-phosphate capature technique and used it to identify the site of a ouabain-sensitive, K-dependent phosphatase in salt gland (39, 40). This enzyme was assumed to be identical with [Na⁺,K⁺]ATPase. This technique has been applied for cytochemical localization of [Na⁺,K⁺]ATPase in rat hepatocyte by Blitzer and Boyer (8) and Latham and Kashgarian (9). They found that the enzyme is localized exclusively to the sinusoidal and lateral portion of the rat hepatocyte plasma membrane and is not detectable on the bile canaliculi. Thus the localization of the enzyme activity in hepatocytes is similar to that reported for other transporting epithelia as reviewed by DiBona and Mills (2). This conclusion has been generally accepted and the mechanisms of hepatocyte bile formation is now discussed based on this conclusion (6, 7).

Biochemical investigation of the localization of [Na⁺,K⁺]-ATPase in rat liver plasma membrane has been reported by Boyer and Reno (10) and Toda et al. (11). Both of them independently arrived at a same conclusion that the ATPase is present in fractions of rat liver plasma membranes that are enriched in bile canaliculi. Similar results have been reported by Reichen and Paumgartner (41), who pointed out the relationship between bile flow and [Na⁺,K⁺]ATPase activity in liver plasma membrane enriched in bile canaliculi. Poupon and Evans (12), however, have presented evidence for the localization at the lateral region.

Since [Na⁺,K⁺]ATPase was localized histochemically on the sinusoidal and lateral surface in the rat hepatocytes by Blitzer and Boyer (8), the biochemical data by Boyer and Reno (10) was interpreted as contamination of the bile canalicular membrane fraction by sinusoidal and lateral membrane fragments (8).

The localization of [Na⁺,K⁺]ATPase on the hepatocyte cell surface is thus quite confusing and is not firmly established. We have, therefore, localized [Na⁺,K⁺]ATPase on the hepatocyte by applying quantitative ferritin immunoelectron microscopy to the isolated hepatocytes prefixed by perfusion with dilute glutaraldehyde. This technique has been successfully applied for the quantitative distribution analyses of rat liver plasma membrane proteins such as asialoglycoprotein receptor (13) and 5'-nucleotidase (14), and the polarized distribution of such membrane proteins on hepatocyte cell surface has been clearly demonstrated.

The results of our present experiment can be summarized as follows: (a) The antigenic sites of [Na⁺,K⁺]ATPase are detected bilaterally, that is, both on the bile canalicular and sinusoidal-lateral surfaces. (b) The average particle density of the enzyme on the bile canalicular region was approximately 2.5 times higher than on the sinusoidal-lateral region. In the latter region, the enzyme is detected almost equally both on the sinusoidal and lateral regions. (c) In each region, the...
FIGURES 10 and 11  Lateral region of canine hepatocyte incubated with ferritin antibody conjugates against canine kidney holo[Na+,K+]ATPase. The lateral surface is evenly labeled by ferritin particles. × 64,500.

Figure 12  Endothelial cells incubated with ferritin antibody conjugates against canine kidney holo[Na+,K+]ATPase. The surface of endothelial cells is also evenly stained with ferritin particles. × 64,500.

<table>
<thead>
<tr>
<th>Cells and cell surfaces</th>
<th>Antibody conjugates</th>
<th>Control conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocyte</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile canalicular</td>
<td>33.9 ± 7.5</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Sinusoidal</td>
<td>13.6 ± 1.5</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Lateral</td>
<td>15.2 ± 1.6</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Endothelial cell (n = 1)</td>
<td>15.5</td>
<td>2.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell surface</th>
<th>No. of ferritin particles/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile canalicular</td>
<td>$1.97 \times 10^5$ (27.8%)</td>
</tr>
<tr>
<td>Sinusoidal</td>
<td>$3.41 \times 10^5$ (48.2%)</td>
</tr>
<tr>
<td>Lateral</td>
<td>$1.70 \times 10^5$ (24.0%)</td>
</tr>
<tr>
<td>Total</td>
<td>$7.08 \times 10^5$ (100%)</td>
</tr>
</tbody>
</table>

The distribution of [Na⁺,K⁺]ATPase was homogeneous, no clustering of the enzyme being detected. The total number of enzyme sites on the sinusoid-lateral region is, however, approximately three times higher than that on the bile canalicular region, because the canalicular surface represents only 13% of the hepatocyte surface membrane (13, 42).

The most interesting finding in our experiment is that the distribution of [Na⁺,K⁺]ATPase is not unilateral but bilateral, existing both the bile canalicular and sinusoid-lateral regions. This distribution of the enzyme on the bile canalicular surface is in contradiction to the enzyme cytochemical localization results on [Na⁺,K⁺]ATPase.

The present immunoferritin localization technique is based on the high specificity of immunological reaction and is sensitive and quantitative. The use of isolated and prefixed hepatocytes have made it possible to determine with high accuracy the surface density of [Na⁺,K⁺]ATPase on the various domains of the hepatocytes. The disadvantage of immunocytochemical techniques is, however, that it does not necessarily establish the localization of active enzyme since only enzyme protein antigenicity is assayed by immunoferritin staining (38).

Similar bilateral localization of [Na⁺,K⁺]ATPase on the cell surface of the renal convoluted tubules has been reported by Kyte (43, 44) by applying the immunoferritin antibody conjugates to the ultrathin frozen sections. According to his interpretation, the density of the enzyme at the luminal surface of the tubules was much lower. It may be argued that the active enzyme does not exist on the luminal surface of...
Published October 1, 1984

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Received for publication 17 February 1984, and in revised form 6 July 1984.

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The most probable interpretation is that the [Na+,K+]ATPase on the bile canalicular plasma membrane is intimately correlated with the bile acid-independent bile flow. If this is the case, [Na+,K+]ATPase on the bile canalicular surface is oriented in the right direction to transport sodium into the canalicular lumen as schematically illustrated in Fig. 13.

Then what are the functions of [Na+,K+]ATPase on the sinusoid-lateral surface of hepatocyte? It may be concerned primarily with the active transport of sodium into the sinusoid-lateral spaces and hence indirectly with the secondary active transport of various substances coupled with the active transport of sodium (symport and antiport). It is possible that the bile flow is indirectly controlled by the [Na+,K+]ATPase on the sinusoid-lateral surface of the hepatocytes.

It is interesting to note here that recently an immunofluorescence study appeared suggesting that most hepatocyte Na+ pumps exist in canalicular membranes (45).

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