

# Localization of Na<sup>+</sup>,K<sup>+</sup>-ATPase $\alpha$ -Subunit to the Sinusoidal and Lateral but Not Canalicular Membranes of Rat Hepatocytes

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**Abstract.** Controversy has recently developed over the surface distribution of Na<sup>+</sup>,K<sup>+</sup>-ATPase in hepatic parenchymal cells. We have reexamined this issue using several independent techniques. A monoclonal antibody specific for the endodomain of  $\alpha$ -subunit was used to examine Na<sup>+</sup>,K<sup>+</sup>-ATPase distribution at the light and electron microscope levels. When cryostat sections of rat liver were incubated with the monoclonal antibody, followed by either rhodamine or horseradish peroxidase-conjugated goat anti-mouse secondary, fluorescent staining or horseradish peroxidase reaction product was observed at the basolateral surfaces of hepatocytes from the space of Disse to the tight junctions bordering bile canaliculi. No labeling of the canalicular plasma membrane was detected. In contrast, when hepatocytes were dissociated by collagenase digestion, Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit was localized to the entire plasma membrane. Na<sup>+</sup>,K<sup>+</sup>-

ATPase was quantitated in isolated rat liver plasma membrane fractions by Western blots using a polyclonal antibody against Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit. Plasma membranes from the basolateral domain of hepatocytes possessed essentially all of the cell's estimated Na<sup>+</sup>,K<sup>+</sup>-ATPase catalytic activity and contained a 96-kD  $\alpha$ -subunit band. Canalicular plasma membrane fractions, defined by their enrichment in alkaline phosphatase, 5' nucleotidase, gamma-glutamyl transferase, and leucine aminopeptidase had no detectable Na<sup>+</sup>,K<sup>+</sup>-ATPase activity and no  $\alpha$ -subunit band could be detected in Western blots of these fractions. We conclude that Na<sup>+</sup>,K<sup>+</sup>-ATPase is limited to the sinusoidal and lateral domains of hepatocyte plasma membrane in intact liver. This basolateral distribution is consistent with its topology in other ion-transporting epithelia.

THE sodium pump is an integral component of plasma membranes of most animal cells. By coupling the energy of ATP hydrolysis to the transport of sodium and potassium, this enzyme functions as a primary driving force in maintaining the differential ion gradients that are critical to cellular homeostasis. The electrochemical gradient generated by the activity of Na<sup>+</sup>,K<sup>+</sup>-ATPase governs a diverse array of processes, including the transepithelial movement of electrolytes, water, and organic solutes (10, 20). In hepatocytes, the transport of solutes such as bile acids and certain amino acids from blood across basolateral membranes (i.e., sinusoidal and lateral) is coupled to and thus driven by the inwardly directed Na<sup>+</sup> gradient (3, 7). Therefore, the resolution of membrane-specific patterns of Na<sup>+</sup> pump localization and the determination of the relative densities of pumps along distinct regions of plasma membrane have great relevance to current concepts of hepatic ion, H<sub>2</sub>O, and solute transport (3, 7, 9, 13). The localization of Na<sup>+</sup>,K<sup>+</sup>-ATPase on the hepatocyte cell surface has been extensively studied by cytochemical (2, 23), biochemical (4, 40, 30, 5, 28), and immunocytochemical (24, 39) procedures. To date, variant results (further discussed later) have been obtained; some studies indicate that the bulk of

Na<sup>+</sup>,K<sup>+</sup>-ATPase activity is localized to the basolateral surface (2, 23, 30, 5, 28), whereas others suggest that the enzyme is present on both basolateral and bile canalicular domains (24, 39).

In this report we present immunocytochemical and immunobiochemical data indicating that hepatocyte Na<sup>+</sup>,K<sup>+</sup>-ATPase is distributed asymmetrically and is restricted to the basolateral plasma membrane.

## Materials and Methods

### Preparation of Antibodies

The preparation and characterization of anti-Na<sup>+</sup>,K<sup>+</sup>-ATPase monoclonal antibody have been described in detail elsewhere (22). Briefly, mice were immunized with membrane fragments (100  $\mu$ g protein) enriched in Na<sup>+</sup>,K<sup>+</sup>-ATPase by the method of Jorgensen (19). Animals exhibiting high antibody titer (in ELISA assay) were used for fusion. Clones were screened both by ELISA and by a functional assay (22). Clone C62.4 was used in these studies.

Canine kidney Na<sup>+</sup>,K<sup>+</sup>-ATPase was purified, according to the method of Jorgensen (19), for generation of polyclonal antibodies. Sodium pump preparations with enzymatic activities indicative of significant enrichment ( $\sim$ 20 mM P/mg protein per min) were mixed with equal volumes of complete Freund's adjuvant (200  $\mu$ g Na<sup>+</sup>,K<sup>+</sup>-ATPase/150  $\mu$ l) and injected in-

tradermally at multiple sites along the backs and shoulders of 5-lb. female New Zealand white rabbits. 1 mo later the initial injection serum was collected and tested for immunoreactivity to Jorgensen-purified  $\text{Na}^+, \text{K}^+$ -ATPase by ELISA (32) assay. Positively responding rabbits were boosted with antigen mixed with incomplete Freund's adjuvant (200 ng/150  $\mu\text{l}$ ) by injection at multiple intradermal sites. Boosting and bleeding continued at 2-wk intervals. The serum used in the studies described in this paper was collected 2 wk after the third boost injection and was stored frozen at  $-20^\circ\text{C}$ .

Antibodies to gamma-glutamyl transferase were a kind gift of Dr. David J. Castle (Department of Cell Biology, Yale University School of Medicine). Details of procedures used for antigen purification and antibody generation as well as characterization of obtained immune sera have been published (7).

### Preparation of Isolated Hepatocytes

Isolated rat hepatocyte couplets and single cells were prepared by a modification of a collagenase perfusion technique as previously described (6, 15). The amount of collagenase (Sigma Chemical Co., St. Louis, MO) was decreased to 0.05% and the dissociated cells were filtered through gauze and resuspended in Leibovitz-15 tissue culture media (Gibco, Grand Island, NY). This preparation currently results in  $31 \pm 6\%$  of the cells isolated as couplets with initial viabilities of  $>90\%$  (as assessed by trypan blue exclusion).

### Immunocytochemical Localization of $\text{Na}^+, \text{K}^+$ -ATPase $\alpha$ -Subunit in Intact Rat Liver and in Isolated Hepatocytes

Sprague-Dawley male rats were anesthetized with Inactin (10 mg/100 g body wt). A cannula was inserted into the aorta via the left ventricle, the vena cava was cut, and the animal was perfused with mammalian Ringers for  $\sim 1$  min. Subsequently, the animal was perfused for 5 min with fixative consisting of 0.01 M Na metaperiodate, 0.75 M lysine, 2% paraformaldehyde in 0.0375 M  $\text{NaH}_2\text{PO}_4$  buffer (25). The final pH of the fixative was 6.2. This fixative provided adequate structural preservation with excellent retention of antigenicity (22). The liver was removed and pieces (2–4  $\text{mm}^3$ ) were postfixed in the same fixative for 6 h at  $4^\circ\text{C}$ . The tissue was washed five times in 0.1 M  $\text{NaH}_2\text{PO}_4$  buffer, incubated in 10% DMSO for 1 h, and then frozen by plunging into swirling liquid Freon 22 (Presto, New Haven, CT) cooled in liquid nitrogen. Frozen tissue was stored in liquid nitrogen until used.

Hepatocytes, isolated from rat liver as described above, were maintained on glass coverslips for 1–4 h. The cells were then fixed by immersing the coverslip in the fixative described above and incubating for 6 h. The cells were cryoprotected and frozen by the same techniques used for whole tissue.

For antigen localization at the light level, sections (0.5–1.0- $\mu\text{m}$  thick) were cut with an Ultracut equipped with an FC-4 cryounit (Reichert Scientific Instruments, Buffalo, NY). Sections were incubated in 50 mM ammonium chloride for 15 min to quench free aldehyde groups. The sections were incubated for 1 h in a solution containing PBS, 1% BSA, and a primary antibody (either sera or ascites fluid) at 1:100 dilution. We used either monoclonal antibodies to  $\alpha$ -subunit of  $\text{Na}^+, \text{K}^+$ -ATPase or, for a double-label experiment, a mixture of monoclonal antibodies to  $\alpha$ -subunit of  $\text{Na}^+, \text{K}^+$ -ATPase and polyclonal antibodies to gamma-glutamyl transferase. The sections were washed in PBS and incubated with either rhodamine-labeled goat anti-mouse IgG (Cappel Laboratories, Malvern, PA) to detect adsorbed monoclonal antibodies, or, for a double-label experiment, with a mixture of rhodamine-labeled goat-anti-mouse IgG and fluorescein-labeled goat anti-rabbit IgG (Cappel Laboratories). In the latter case, both  $\alpha$ -subunit of  $\text{Na}^+, \text{K}^+$ -ATPase and gamma-glutamyl transferase will be detected on the same section. After washing, sections were examined with a Zeiss incident light fluorescence microscope.

The method for immunocytochemical labeling at the ultrastructural level was essentially that described by Brown and Farquhar (8) and recently applied to the ultrastructural localization of  $\text{Na}^+, \text{K}^+$ -ATPase in the kidney (22). 16- $\mu\text{m}$  thick cryostat sections, or pieces of coverslip with attached hepatocytes, were incubated overnight at  $4^\circ\text{C}$  in antibody against the  $\alpha$ -subunit of the sodium pump. The tissue was washed five times with PBS followed by a 2-h incubation in sheep anti-mouse (Fab) conjugated to horseradish peroxidase (HRP).<sup>1</sup> After washing, the tissue was fixed for 1 h in 1.5%

glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) containing 5% sucrose. After three washes in 0.1 M Na cacodylate buffer with 7.5% sucrose and three washes in 50 mM Tris-HCl (pH 7.4) with 7.5% sucrose, peroxidase reaction product was developed in a solution of 0.2% diaminobenzidine in the Tris-sucrose buffer to which  $\text{H}_2\text{O}_2$  had been added to yield a final concentration of 0.01%. The reaction was stopped after 10–20 min by washing in cold Tris-sucrose buffer. Osmium fixation was carried out using the reduced osmium method of Karnovsky (21). The tissue was dehydrated and embedded in Epon 812, and unstained thin sections were examined with a Zeiss 10B electron microscope.

### Isolation and Characterization of Canalicular and Basolateral Liver Plasma Membrane Fractions

The procedure for isolation of canalicular and basolateral liver plasma membrane (cLPM and bLPM, respectively) subfractions has been previously described in detail (28). Briefly, livers from 160–180-g male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, MA) were homogenized and the homogenate was spun down (7000 g for 10 min) to give a nuclear pellet fraction. A mixed LPM subfraction was isolated from the nuclear pellet by rate zonal flotation. This material was tightly homogenized and the vesiculated LPM elements were separated on a three-step sucrose gradient (31, 34, and 38% wt/wt). The membranes collected at each interphase (cLPM at 31/34 and bLPM at 34/38) were then spun down (105,000 g for 60 min) and resuspended in 0.25 M sucrose, 0.2 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgSO}_4$ , 10 mM Hepes-Tris, pH 7.5. The degree of purification of cLPM and bLPM was analyzed extensively by measuring intracellular and plasma membrane marker enzyme activities as in reference (28). These studies have indicated minimal contamination of both LPM fractions by intracellular organelles and virtually complete separation of bLPM from cLPM as indicated by the absence of glucagon-stimulatable adenylate cyclase or secretory component in cLPM (28). bLPM subfraction was contaminated with cLPM elements by  $\sim 10\%$ .

### Western Blots

Madin-Darby canine kidney (MDCK) cells were grown and lysed, and cellular membranes were prepared as described in reference 22. MDCK membranes and LPM subfractions were processed for SDS PAGE and subjected to electrophoresis as described previously (38). Upon completion of electrophoresis, separated proteins were transferred to nitrocellulose (NC) filters for 4 h at room temperature and constant current (150 mA). The filters were immediately quenched in 12.5 mg/ml hemoglobin (2 h at room temperature) and then incubated overnight at room temperature with either nonimmune serum or a polyclonal anti- $\text{Na}^+, \text{K}^+$ -ATPase  $\alpha$ -subunit serum (1:100 dilutions in 12.5 mg/ml hemoglobin). The filters were washed ( $2 \times 10$  min) with PBS, then with PBS containing 0.05% Nonidet P-40 ( $2 \times 10$  min), and finally rinsed ( $2 \times 5$  min) with PBS. Adsorbed IgGs were detected by incubating the filters in  $^{125}\text{I}$ -labeled protein A ( $\sim 20 \times 10^6$  cpm in 200 ml of 12.5 mg/ml hemoglobin) for 4 h at room temperature, followed by washing (as above), drying, and autoradiography.

### Inhibition of ATPase Activity in Isolated Membrane Subfraction

Rat liver basolateral membranes were isolated as described (28). Membrane samples (25  $\mu\text{l}$ ) containing 50–70  $\mu\text{g}$  protein were added to 25  $\mu\text{l}$  bovine serum albumin fraction V (Sigma Chemical Co.) and 2.5  $\mu\text{l}$  1% SDS (14). All membrane fractions were solubilized in 0.05% SDS (Bio-rad Laboratories, Richmond, CA) and incubated at room temperature for 10 min before enzyme analysis, since preliminary studies determined that concentrations of 0.06% SDS and above decreased enzyme activity. 10  $\mu\text{l}$  of either deionized water, preimmune rat serum, control rat ascites, polyclonal or monoclonal (C62.4) antibodies were added to the membranes.  $\text{Na}^+, \text{K}^+$ -ATPase and  $\text{Mg}^{2+}$ -ATPase activities were assayed after a 30-min room temperature incubation by a standard spectrophotometric assay (34).

## Results

### Characterization of Polyclonal and Monoclonal Antibodies against $\text{Na}^+, \text{K}^+$ -ATPase

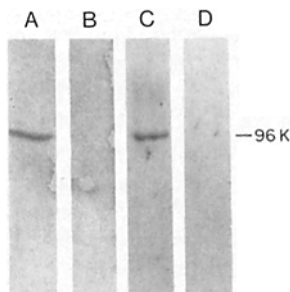
The monoclonal antibody C62.4 has been characterized previously (22). This antibody inhibits  $\text{Na}^+$ -dependent but not

1. *Abbreviations used in this paper:* bLPM, basolateral liver plasma membrane; cLPM, canalicular liver plasma membrane; HRP, horseradish peroxidase; NC, nitrocellulose.

K<sup>+</sup>-dependent Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. Immunoprecipitation of MDCK cells, biosynthetically labeled with [<sup>35</sup>S]methionine, demonstrated that the antibody recognizes a 96-kD protein. Furthermore, this antibody precipitated a 96-kD protein labeled in vitro with [<sup>3</sup>H]NAB ouabain. Immunocytochemical localization revealed that the antigenic site recognized by this antibody is on the cytoplasmic domain of basolateral plasma membranes of renal tubular epithelium.

Presence of polyclonal antibodies in sera of immunized rabbits was tested by Western blots. As shown in Fig. 5, the antibodies reacted with  $\alpha$ -subunit on NC transfers of purified canine Na<sup>+</sup>,K<sup>+</sup>-ATPase. To test whether antibodies to contaminating (non-Na<sup>+</sup>,K<sup>+</sup>-ATPase) antigens were perhaps also generated, we used the immune sera in Western blots of membranes from MDCK cells and from rat liver. As shown in Fig. 1, only a single polypeptide with a molecular mass of 96 kD was detected in both MDCK and rat liver membrane fractions. No reactive band was seen when non-immune serum was used. This polyclonal antibody also immunoprecipitated  $\alpha$ -subunit labeled with a photoaffinity derivative of ouabain, a highly specific inhibitor of sodium pump (Smith, Z., M. J. Caplan, and J. Jamieson, manuscript submitted for publication), thus indicating that it recognizes the Na<sup>+</sup>,K<sup>+</sup>-ATPase. Because no immunoreactivity to the  $\beta$ -subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase was observed with either monoclonal or polyclonal antibody, we conclude that both were monospecific for the  $\alpha$ -subunit of the enzyme.

Both antibodies were tested for their ability to inhibit enzymatic activity of two hepatic ATPases. As shown in Table I, the polyclonal serum inhibited Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by ~50%, while having no effect on Mg<sup>2+</sup>-ATPase. The monoclonal C62.4 antibody inhibited Na<sup>+</sup>,K<sup>+</sup>-ATPase activity of hepatocyte plasma membrane even further (19.7% of normal activity), and also had no effect on Mg<sup>2+</sup>-ATPase. Both antibodies showed inhibition only when intact right-side out LPM vesicles were permeabilized with SDS. (The slight decline in ATPase activity in the permeabilized membrane is caused by SDS inactivation). Since the vesicles retain their right-side out orientation during preparation, the results indicate that the antibodies recognize a cytoplasmic domain of the  $\alpha$ -subunit, not accessible to the antibodies in the nonpermeabilized vesicles. The same level of Na<sup>+</sup>,K<sup>+</sup>-ATPase inhibition was found when C62.4 was incubated with dog kidney membranes (22).



**Figure 1.** Western blot analysis of polyclonal antibodies to the  $\alpha$ -subunit of canine Na<sup>+</sup>,K<sup>+</sup>-ATPase. Total cell membranes and a mixed LPM fraction were prepared from MDCK cells and from rat liver, respectively. Membrane proteins were separated by SDS PAGE electrophoresis and then transferred to NC filters. The filters were incubated with either anti-Na<sup>+</sup>,K<sup>+</sup>-ATPase or nonimmune

sera, followed by <sup>125</sup>I-protein A. Shown are autoradiograms of filters. (Lane A) Western blot of MDCK cell membrane proteins with immune sera; (lane B) western blot of MDCK cell membrane proteins with nonimmune sera; (lane C) western blot of liver membrane proteins with immune sera; (lane D) western blot of liver membrane proteins with nonimmune sera.

**Table I.** Inhibition of Enzymatic Activities of Na<sup>+</sup>, K<sup>+</sup>-ATPase and Mg<sup>2+</sup>-ATPase by Polyclonal and Monoclonal Antibodies

	Nonimmune serum	Polyclonal antibody	Normal ascites	Monoclonal C62.4
<b>Na<sup>+</sup>,K<sup>+</sup>-ATPase</b>				
Nonpermeabilized vesicles	12.25	10.96	12.41	8.22
SDS-permeabilized vesicles	9.51	4.76	10.24	2.02
<b>Mg<sup>2+</sup>-ATPase</b>				
Nonpermeabilized vesicles	24.50	23.05	23.05	23.05
SDS-permeabilized vesicles	25.22	25.22	23.20	23.20

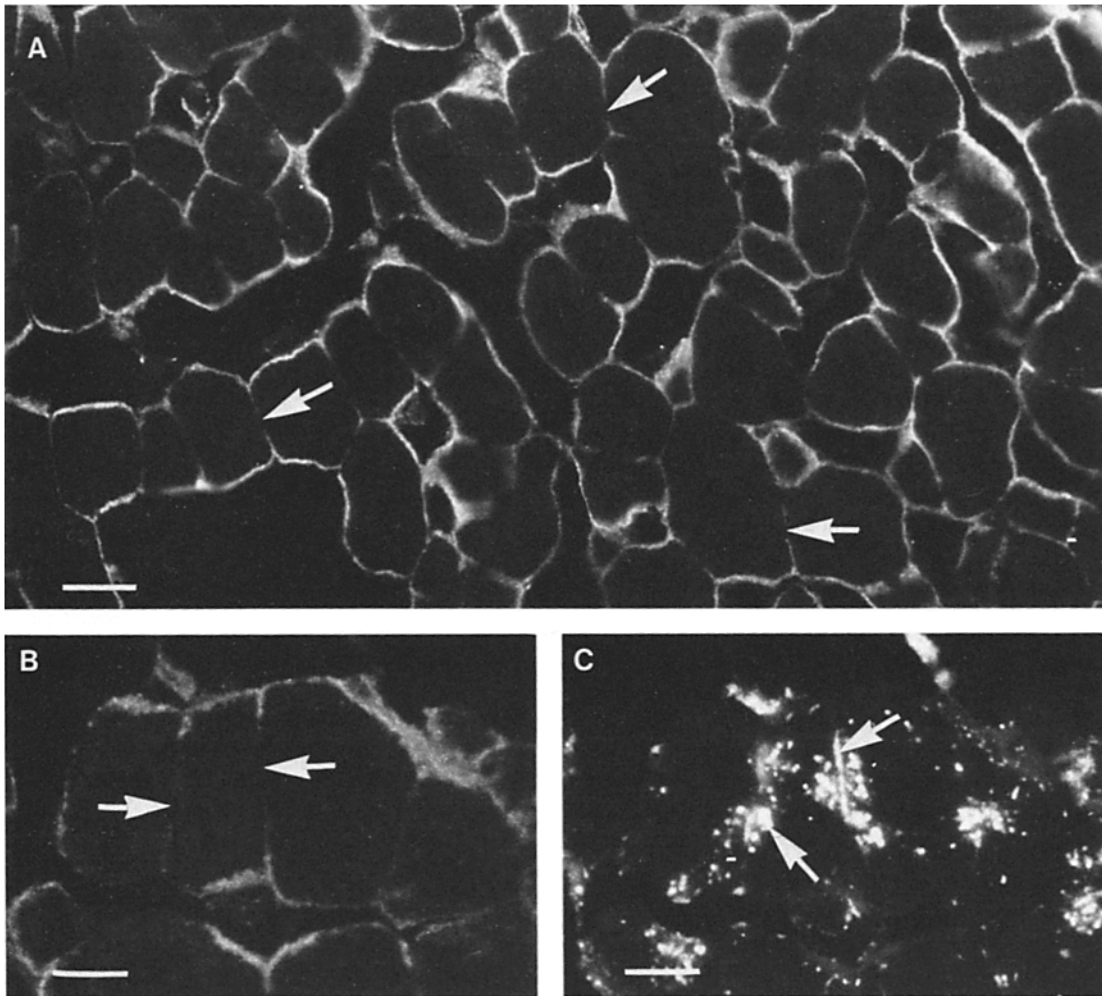
Isolated LPM vesicles were incubated with control or immune antibodies as described in Materials and Methods. Na<sup>+</sup>,K<sup>+</sup>-ATPase and Mg<sup>2+</sup>-ATPase activities were then assayed and are represented as  $\mu$ mol P released per mg protein per min. Polyclonal antibodies inhibited Na<sup>+</sup>,K<sup>+</sup>-ATPase activity by 50.1%, while monoclonal antibodies inhibited by 80%. In both cases activity of Mg<sup>2+</sup>-ATPase was unaffected. The data represent averages of three determinations.

### Immunolocalization of $\alpha$ -Subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase in Intact Rat Liver

**Immunofluorescent Localization.** When the monoclonal anti-Na<sup>+</sup>,K<sup>+</sup>-ATPase antibodies were used to label rat liver cryosections, sinusoidal and lateral plasma membranes of hepatocytes were uniformly labeled (Fig. 2 A). Bile canaliculi (arrows) were consistently negative, thus suggesting that Na<sup>+</sup>,K<sup>+</sup>-ATPase is distributed asymmetrically, with a high concentration of  $\alpha$ -subunit per surface area on sinusoidal and lateral membranes and no detectable pumps on the biliary domain of the plasmalemma.

To determine whether the lack of bile canalicular staining represents bonafide distribution of the enzyme, or perhaps is caused by inaccessibility of the biliary region of the hepatocyte to antibodies, we performed a double-label experiment in which we localized  $\alpha$ -subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase and gamma-glutamyl transferase (a known bile canalicular marker) on the same section. Liver sections were incubated with a mixture of mouse anti-Na<sup>+</sup>,K<sup>+</sup>-ATPase antibodies and rabbit anti-gamma-glutamyl transferase antibodies, followed by a mixture of rhodamine-conjugated goat anti-mouse antibodies and fluorescein-conjugated goat anti-rabbit antibodies. As shown in Fig. 2 B, when a rhodamine-detecting filter was used, fluorescence was observed along sinusoidal and lateral membranes up to the tight junctions delineating bile canaliculi (arrows). When a fluorescein-detecting filter was used (Fig. 2 C), a distinct pattern was observed. Sinusoidal and lateral membranes were not stained while bile canaliculi (arrows) showed strong fluorescent signals. In addition, a number of intracellular organelles was stained. This distribution of label is compatible with the previously observed (7) apical distribution of gamma-glutamyl transferase in liver and in other epithelia. These data indicate that the bile canalicular domain of hepatocytes is readily accessible for immunolabeling and suggest that Na<sup>+</sup>,K<sup>+</sup>-ATPase  $\alpha$ -subunit is not present in detectable amounts in that membrane region.

**Ultrastructural Localization.** Cryostat sections were incubated with monoclonal antibody C62.4 followed by goat



**Figure 2.** Immunofluorescent localization of  $\text{Na}^+, \text{K}^+$ -ATPase in rat liver. In *A* sections were incubated with anti- $\text{Na}^+, \text{K}^+$ -ATPase monoclonal antibodies. In *B* and *C*, sections were incubated with a mixture of anti- $\text{Na}^+, \text{K}^+$ -ATPase monoclonal antibodies (labeling visualized in *B*) and anti- $\gamma$ -glutamyl transferase antibodies (labeling visualized in *C*). *A* clearly indicates that staining is restricted to the sinusoidal and lateral domains of the hepatocyte and is absent from bile canaliculi (arrows). *B* shows sinusoidal and lateral staining of hepatocytes and absence of staining from bile canaliculi (arrows). *C* shows staining of bile canaliculi (arrows) and neighboring intracellular organelles. Bars, (*A*) 12.5  $\mu\text{m}$ ; (*B* and *C*) 6.3  $\mu\text{m}$ .

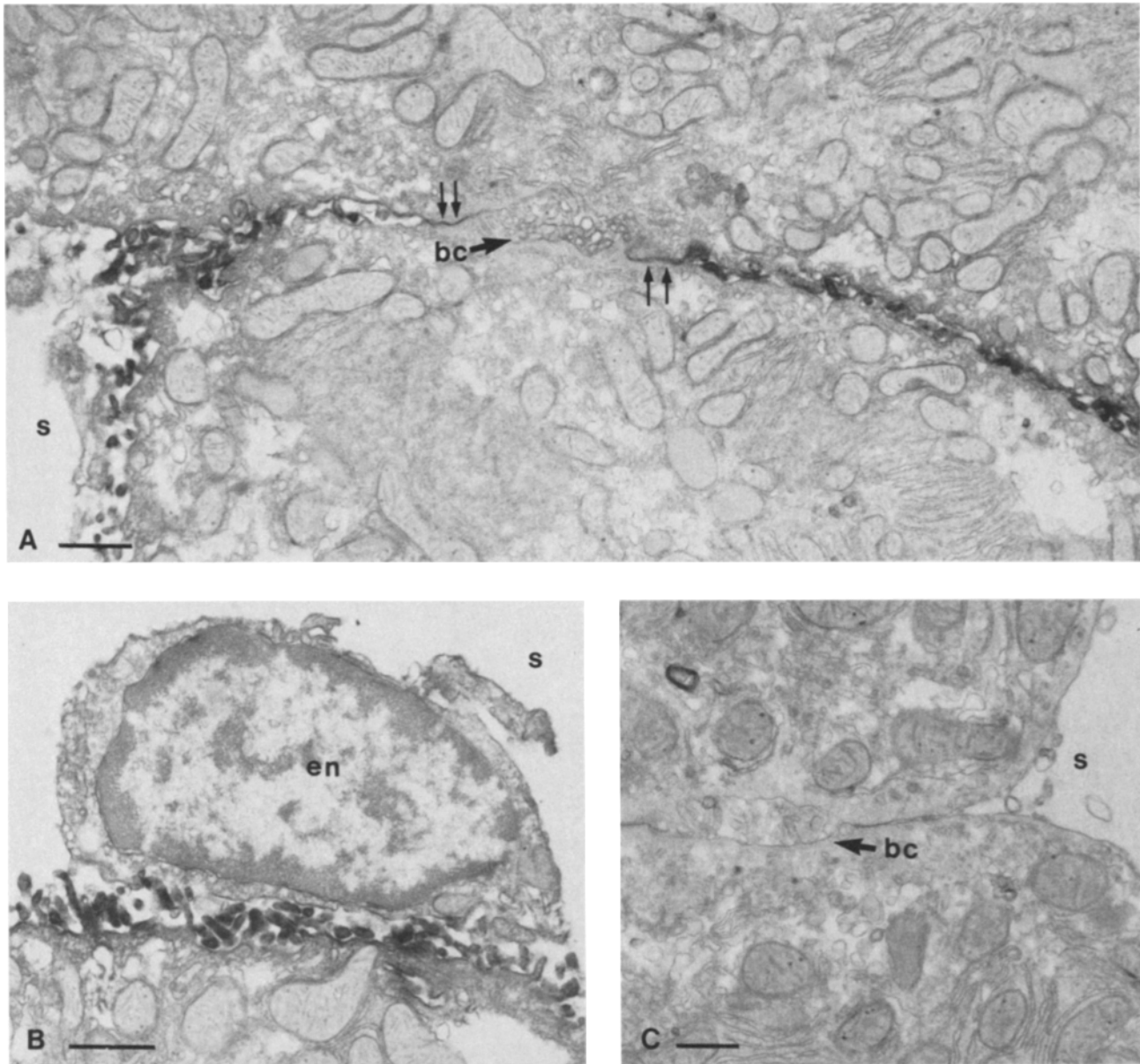
anti-mouse F(ab) conjugated to HRP. As seen in Fig. 3 *A*, HRP reaction product was restricted to the cytoplasmic domain of basolateral plasma membranes of hepatocytes. On the sinusoidal surface (Fig. 3 *B*), the distribution of reaction product appeared to be uniform over the entire plasmalemma, a finding in accord with the distribution of  $\text{Na}^+, \text{K}^+$ -ATPase at the basolateral invaginations of the kidney epithelium (22).<sup>2</sup> A similar lack of clustering of  $\text{Na}^+, \text{K}^+$ -ATPase has been previously reported in dog hepatic tissue by Takemura et al. (39). Lateral plasma membranes (Fig. 3 *A*, double arrows) were uniformly labeled with HRP reaction product up to tight junctions defining the bile canaliculus. It is important to note that the bile canalicular membrane with its microvilli was consistently unlabeled with reaction product. While we realize that the immunoperoxidase method is

2. Flat regions of plasmalemma directly opposed to the basement membrane were devoid of reaction product, suggesting a microdomain sorting of  $\text{Na}^+, \text{K}^+$ -ATPase in the kidney (22).

not very quantitative and has a finite level of detection, the lack of reaction product clearly indicates that  $\text{Na}^+, \text{K}^+$ -ATPase concentration in apical domain is significantly below that in basolateral membrane. Reaction product was not observed in other intracellular organelles in our sections. This may be the result of low levels of intracellular antigen (below detection level of the antibody HRP method) or a change in the conformation of the  $\alpha$ -subunit in some intracellular membranes. (The C62.4 antibody reacted with the membranes of the medial Golgi cisternae in cells of the thick ascending limb of rat kidney medulla [22].) The specificity of the immunoreaction was established by the lack of immunolabeling when sections were incubated with control monoclonal antibody (Fig. 3 *C*).

#### ***Immunolocalization of $\text{Na}^+, \text{K}^+$ -ATPase in Isolated Rat Hepatocytes and in Hepatocyte Couplets***

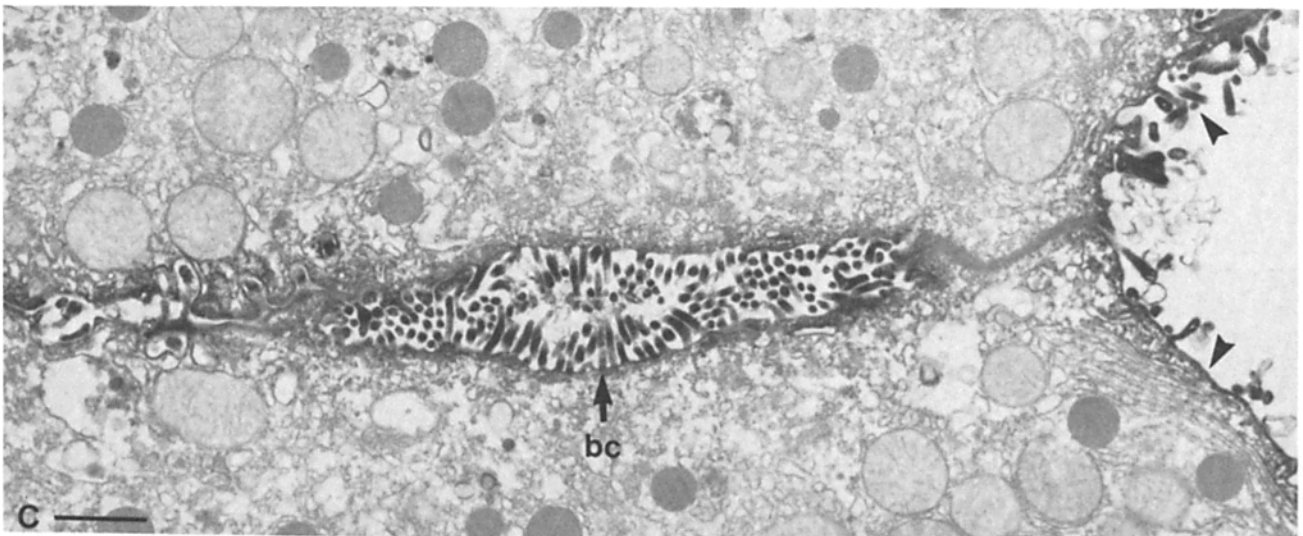
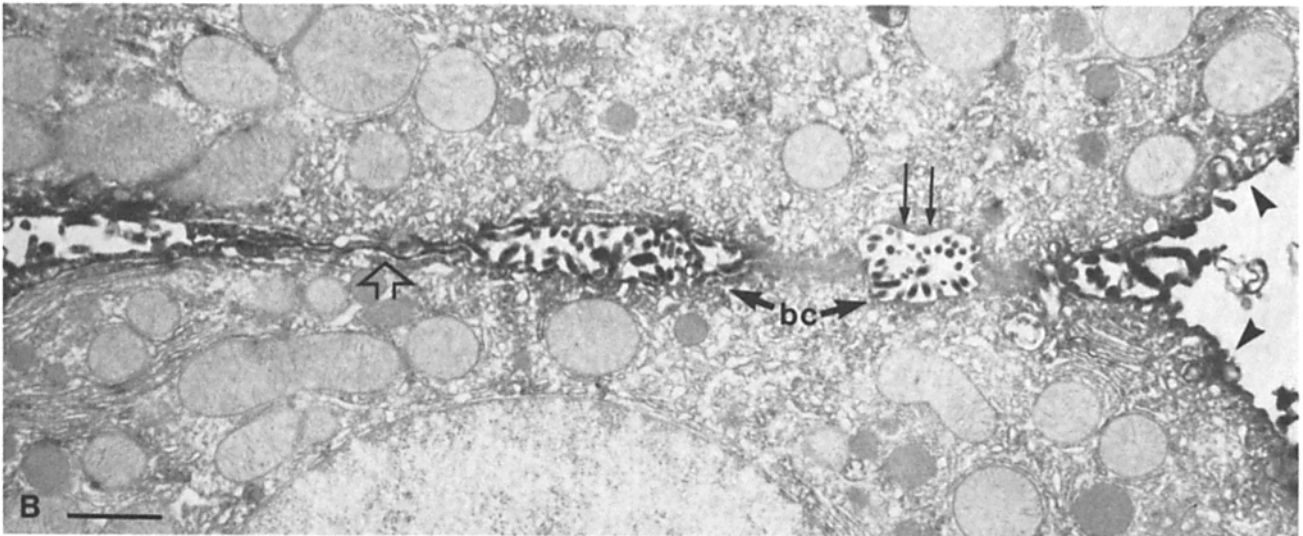
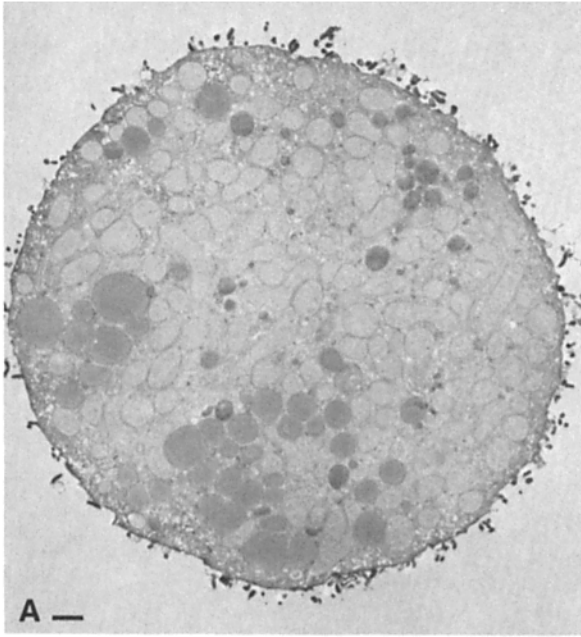
Rat hepatocytes and hepatocyte couplets were isolated after collagenase disruption, fixed, and processed for immuno-



**Figure 3.** Ultrastructural localization of  $\text{Na}^+, \text{K}^+$ -ATPase  $\alpha$ -subunit in intact rat liver. **A** demonstrates sinusoidal (*s*) and lateral distribution of HRP reaction product. Note that the reaction product is confined to the cytoplasmic domain of the membrane. Bile canalicular (*bc*) membranes are unlabeled. **B** demonstrates the almost uniform deposition of reaction product on sinusoidal plasma membrane adjacent to unlabeled endothelial (*en*) cell. **C** shows lack of immunolabeling when control nonimmune antibody was used. Bars, (**A** and **B**) 1  $\mu\text{M}$ ; (**C**) 0.5  $\mu\text{M}$ .

localization using the monoclonal C62.4 antibody. When isolated hepatocytes were used for  $\text{Na}^+, \text{K}^+$ -ATPase localization, HRP reaction product was seen over their entire plasmalemma (Fig. 4 *A*). We therefore conclude that in isolated hepatocytes, where the polarity of the plasma membrane domains has been destroyed, relocation of plasma membrane proteins occurs, leading to the observed, uniform membrane distribution of  $\alpha$ -subunit of  $\text{Na}^+, \text{K}^+$ -ATPase. When hepatocyte couplets with morphologically distinct sinusoidal, lateral, and bile canalicular domains were used for  $\text{Na}^+, \text{K}^+$ -ATPase localizations, HRP reaction product was seen along their entire plasma membrane surface (Fig. 4, *B* and *C*). Cellular plasma membrane domains analogous to the sinu-

oidal and lateral domains in intact liver were strongly labeled. However, bile canalicular region in hepatic couplets, unlike that in intact liver, contained HRP reaction product. As seen in Fig. 4, *B* and *C*, most canaliculi contained HRP reaction product over their entire membrane surface, while some (Fig. 4 *B*, double arrows) showed restriction of labeling to certain biliary domains. Whether the partially labeled canaliculi represent those least perturbed by the collagenase treatment or those which have partially regained their polarity by removal of membrane components (as exemplified by  $\text{Na}^+, \text{K}^+$ -ATPase) from the bile canalicular region is not currently known. Irrespective of the above, it is clear that the disruption of tight junctions during collagenase perfusion



may result in relocation of membrane proteins from the lateral to the canalicular domain.

### Immunoblots of Isolated cLPM and bLPM Fractions

Highly purified bLPM and cLPM fractions were isolated from the same rat liver homogenate by rate zonal and discontinuous sucrose density centrifugation. Analysis of isolated fractions for marker enzyme activities resulted in data analogous to those reported previously by Meier et al. (28). Actual values fell within the range of values published in that report. Based on the yields and relative enrichment of various enzymes, we conclude that the bLPM was enriched in Na<sup>+</sup>,K<sup>+</sup>-ATPase and glucagon-stimulatable adenylate cyclase, while cLPM was enriched in leucyl-naphthyl-amidase, gamma-glutamyl transferase, and alkaline phosphodiesterase I. Proteins of both subfractions were separated by SDS PAGE and then transferred to nitrocellulose filters. Polyclonal anti-Na<sup>+</sup>,K<sup>+</sup>-ATPase antibodies were used to probe the presence and relative quantities of the corresponding antigen. The amounts of ATPase present in fractions were correlated with signals obtained with known amounts of purified dog kidney ATPase. As shown in Fig. 5, the enzyme was detected in bLPM but was absent from pure cLPM fraction. Since 300 μg protein of each fraction was loaded per well and since cLPM contains twice the total phospholipid/mg protein as bLPM (28), 300 μg of cLPM represents twice the membrane area of 300 μg bLPM. Therefore, the lack of detectable α-subunit of Na<sup>+</sup>,K<sup>+</sup>-ATPase in the cLPM sample is not a result of differences in protein loading on the gels but rather indicates that the antigen is absent from canalicular domain (or present at minor concentrations below the level of detection with this technique). These results are in direct contrast to those obtained by Takemura et al. (39) in dog hepatocytes, in which the density of Na<sup>+</sup>,K<sup>+</sup>-ATPase on cLPM is more than twice that on bLPM. If that were the case, we would expect the signal in the cLPM lane to be four times that in the bLPM lane.

The relative intensity of the Na<sup>+</sup>,K<sup>+</sup>-ATPase α-subunit band in bLPM is approximately ten times that obtained with 70 pg purified dog kidney Na<sup>+</sup>,K<sup>+</sup>-ATPase. (70 pg of Na<sup>+</sup>,K<sup>+</sup>-ATPase prepared by the Jorgenson method [19] was loaded on the gel. This preparation contains 35 pg of the Na<sup>+</sup> pump and 23 pg of the α-subunit.) We can therefore assume that the loaded sample, i.e., 300 μg of bLPM, contains 230 pg of α-subunit. Since 200 μg of bLPM is recovered per gram liver weight (28), we can estimate that 1 g liver contains ~154 pg of α-subunit in recovered bLPM. Since the isolated bLPM subfraction contains only 5% of total cellular Na<sup>+</sup>,K<sup>+</sup>-ATPase (28), it follows that there are 3.1 ng of α-subunit per gram liver. (We are defining the minimal level of α-subunit since only active ATPase has been assayed in our measurements. α-subunit not associated into a functional entity, if such exists, would not be included in these calculations.) Compositionally, Na<sup>+</sup>,K<sup>+</sup>-ATPase is a heterodimer made up of a β-subunit and a catalytic (ATP and ouabain binding) α-subunit. Our antibody recognizes only

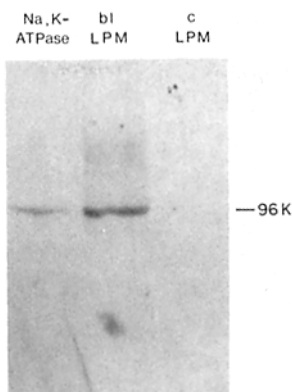


Figure 5. Western blot quantitation of Na<sup>+</sup>,K<sup>+</sup>-ATPase in bLPM and cLPM. 300 μg of each fraction protein and 70 pg of purified dog kidney Na<sup>+</sup>,K<sup>+</sup>-ATPase were separated by SDS PAGE and transferred to NC filters. Filters were incubated with polyclonal anti-Na<sup>+</sup>,K<sup>+</sup>-ATPase sera, followed by <sup>125</sup>I-protein A. An autoradiograph of the filter is shown.

the 96-kD α-subunit. From the above data we can calculate that  $1.93 \times 10^{13}$  molecules of Na<sup>+</sup>,K<sup>+</sup>-ATPase α-subunit are present per gram liver. Based on the value of  $1 \times 10^6$  hepatocytes in 1 mg of whole homogenate (36) and 164.7 mg whole homogenate per gram liver (28) we can conclude that there are  $164.7 \times 10^6$  hepatocytes per gram liver, and, hence, 115,675 α-subunits per hepatocyte. This value is in good agreement with Na<sup>+</sup>,K<sup>+</sup>-ATPase concentrations obtained by Schenk et al. (36) in rat liver (238,000 sites per cell) and within the range ( $1 \times 10^5$ – $1 \times 10^6$  sites per cell) obtained in other cell systems (e.g., muscle [1], or HeLa cells [29]). We therefore conclude that the vast majority of cellular Na<sup>+</sup>,K<sup>+</sup>-ATPase α-subunit is located on the basolateral PM domain.

### Discussion

In this report we present immunocytochemical and immunocytochemical evidence that Na<sup>+</sup>,K<sup>+</sup>-ATPase in rat hepatocytes is localized to the sinusoid-lateral domain of the plasma membrane and is restricted from the bile canalicular surface. Immunolocalization of the antigen in intact tissue at both the light and electron microscope levels indicates that Na<sup>+</sup>,K<sup>+</sup>-ATPase is limited to the sinusoidal and lateral domains of hepatocytes, providing the polarity of the cell membrane is maintained. When the tight junctions defining membrane domains are disrupted (as shown in isolated hepatocytes or hepatocyte couplets), previously restricted proteins relocate, thus leading to uniform distribution on the plasmalemma. Disruption of the junctional complexes in hepatocyte couplets has been previously demonstrated by penetration of extracellularly added Ruthenium red into the canalicular lumen (6).

Since detection of antigens in fixed frozen tissue is governed by (a) antigen stability and conformation and (b) antigen accessibility, lack of immunolabeling must always be interpreted with caution. To address this concern we tested whether, using our methodology, we could detect an antigen known to be localized to the bile canalicular membrane. Our

Figure 4. Ultrastructural localization of Na<sup>+</sup>,K<sup>+</sup>-ATPase α-subunit in isolated hepatocytes and in hepatocyte couplets. A shows HRP reaction product distributed evenly over the plasmalemma of a dissociated hepatocyte. B and C demonstrate HRP reaction product present on sinusoidal (arrowheads), lateral (empty arrow), and canalicular (bc) membranes of hepatocyte couplets. Some canaliculi (double arrows) show incomplete staining. Others exhibit more uniform labeling. Bars, 1 μM.

results, using antibodies to gamma-glutamyl transferase, clearly show that we can detect a bile canalicular protein and hence that our conclusions about  $\text{Na}^+, \text{K}^+$ -ATPase localization are correct. To further strengthen our argument we tested  $\text{Na}^+, \text{K}^+$ -ATPase presence in isolated fractions using Western blots, a technique in which the membrane proteins are SDS-treated, resulting in equivalent accessibility of all antigens to the antibody. After transfer to NC filters,  $\text{Na}^+, \text{K}^+$ -ATPase  $\alpha$ -subunit was detected only in bLPM but was absent from cLPM, thus confirming the results of the immunolocalization studies.

These data, though consistent with  $\text{Na}^+, \text{K}^+$ -ATPase topology in most other ion-transporting epithelia, are at variance with recent immunocytochemical results by Leffert et al. (24) and Takemura et al. (39). To reconcile these differences a brief review of the pertinent findings is necessary.

The electron microscopic cytochemical localization of  $\text{Na}^+, \text{K}^+$ -ATPase was originally reported by Blitzer and Boyer (2) and Latham and Kashgarian (23). Using the Ernst nitrophenyl phosphatase technique (12) both groups independently reported basolateral distribution of this enzyme. Reaction product deposition was  $\text{K}^+$  and  $\text{Mg}^{+2}$  dependent and was inhibited by ouabain, a specific inhibitor of  $\text{Na}^+, \text{K}^+$ -ATPase. Furthermore, reaction precipitate was present on the cytosolic side of the plasmalemma, consistent with the topology of  $\text{Na}^+, \text{K}^+$ -ATPase-mediated phosphate release.

Subcellular fractionation studies (including this report) further supported the basolateral distribution of hepatic  $\text{Na}^+, \text{K}^+$ -ATPase (30, 5, 28). Isolated basolateral membrane fractions containing glucagon-stimulated adenyl cyclase and secretory component were greatly enriched in  $\text{Na}^+, \text{K}^+$ -ATPase, while canalicular membrane fractions lacked both enzymes and were devoid of secretory component (28). The validity of these findings was supported by nearly complete recovery of  $\text{Na}^+, \text{K}^+$ -ATPase activity in these studies.

In a recent report Takemura et al. (39) used ferritin immunolabeling of prefixed isolated canine hepatocytes to demonstrate  $\text{Na}^+, \text{K}^+$ -ATPase antigenic sites. Both sinusoidal and canalicular domains were labeled with the canalicular domain containing two and a half times the  $\text{Na}^+, \text{K}^+$ -ATPase concentration of the basolateral domain. However, caution must be exercised in interpreting their results. As shown by our experiments with isolated rat hepatocytes or hepatocyte couplets, perfusion conditions that result in the dissociation of hepatocytes lead to disruption of their tight junctions. Under these conditions  $\text{Na}^+, \text{K}^+$ -ATPase may be found on all plasma membrane surfaces. Thus, our findings suggest that the reported localization of  $\text{Na}^+, \text{K}^+$ -ATPase to canalicular domain may be artifactual. Nevertheless, the finding that the bile canalicular plasma membrane contains two and a half times the concentration of  $\text{Na}^+, \text{K}^+$ -ATPase in basolateral plasma membrane can not be explained by distribution alone. Based on simple diffusion of proteins in the plane of the membrane, an equal concentration of  $\text{Na}^+, \text{K}^+$ -ATPase in both domains would be expected. We therefore propose that either their polyclonal antibodies produced against holo  $\text{Na}^+, \text{K}^+$ -ATPase recognize proteins such as  $\text{Ca}^{+2}$  or  $\text{Mg}^{+2}$  ATPase that may share certain antigenic determinants with  $\text{Na}^+, \text{K}^+$ -ATPase or that the original antigen preparation contained a highly antigenic contaminating protein. By immunoblotting, their antibody recognized a band of  $\sim 100$  kD and it could be proposed that multiple proteins of similar

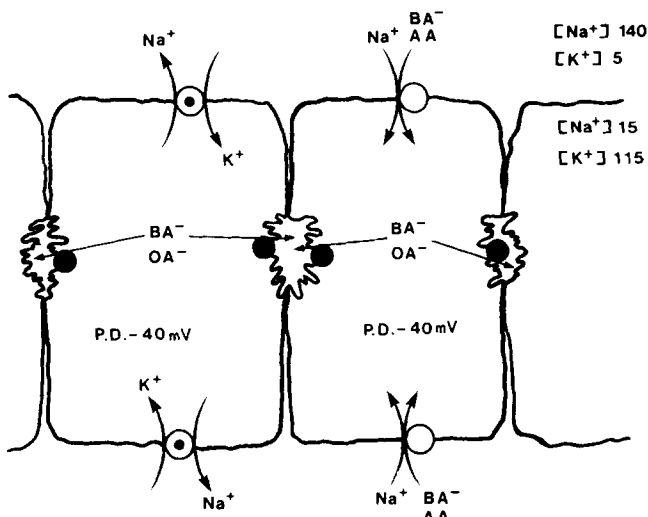
molecular mass might be recognized. Since other ATPases, e.g.,  $\text{H}^+, \text{K}^+$ -ATPase (31) and  $\text{Ca}^{+2}$ -ATPase (41), have catalytic subunits of  $\sim 100$  kD and sequence data reveal extensive homologies with  $\text{Na}^+, \text{K}^+$ -ATPase (37, 26), it is possible that ATPases other than  $\text{Na}^+, \text{K}^+$ -ATPase have been localized at the canalicular domain of the hepatocyte at the same time that  $\text{Na}^+, \text{K}^+$ -ATPase was detected at the basolateral surface. Alternatively, a nonrelated protein of  $\sim 100$  kD could have been localized at the canalicular domain. This latter suggestion is supported by the results of Kashgarian et al. (22) in which another monoclonal antibody developed to enriched  $\text{Na}^+, \text{K}^+$ -ATPase and immunoprecipitating a 96-kD band from biosynthetically labeled rat kidney membranes localized exclusively to brush borders of rat kidney cells, indicating that the original antigen preparation contained a non- $\text{Na}^+, \text{K}^+$ -ATPase component.

Recently, Leffert et al. used monoclonal antibodies to the rat renal  $\text{Na}^+, \text{K}^+$ -ATPase (35) to localize the enzyme in fixed rat liver tissue by immunofluorescence (24). The published photographs and text indicate that labeling occurred on the basolateral membrane as well as on the canalicular domain. The author's conclusion that "fluorescence intensities of punctate regions" (assumed to represent bile canaliculi) "exceed those generated by polygonal and hexagonal array" (assumed to represent basolateral surfaces) is proposed as evidence that the density of pumps is higher on the canalicular membrane. Irrespective of the methodological problems inherent in quantitation of proteins by immunofluorescence, this finding is qualitatively similar to that of Takemura et al. (39). If we assume that lateral diffusion of basolateral proteins into canalicular domain did not occur, then the question is raised as to whether the monoclonal antibodies of Leffert and co-workers may recognize an epitope on the  $\text{Na}^+, \text{K}^+$ -ATPase that is also shared by other canalicular ATPase(s).

In conclusion, a substantial body of published evidence reviewed in this discussion and presented in this report supports a basolateral localization for the hepatic enzyme  $\text{Na}^+, \text{K}^+$ -ATPase.

Localization of this ion pump on sinusoid lateral cell membranes has a number of important implications for mechanisms of hepatic transport of ions and solutes, and supports a model of biliary secretion shown in Fig. 6. Bile acids, a major determinant of the osmotic driving force for bile secretion, are initially removed from blood and transported against electrical and chemical gradients into the hepatocyte before excretion into bile. It is well established (27, 33, 18, 11) that the uptake of organic anions across sinusoidal and lateral plasma membranes is a carrier-mediated process, and, for the bile acid taurocholate, is driven by the inward directed  $\text{Na}^+$  gradient. This gradient is in turn generated and maintained by the basolateral pump  $\text{Na}^+, \text{K}^+$ -ATPase. In contrast, the excretion of taurocholate and possibly other anions from the cells into bile canaliculi is mediated in part by an  $\text{Na}^+$ -independent anion carrier that appears to be driven by the intracellular negative membrane potential (27, 17). Our results that localize  $\text{Na}^+, \text{K}^+$ -ATPase to only the sinusoidal-lateral domain of the hepatocyte are consistent with these biochemical and physiologic results. Furthermore, both the morphological and biochemical findings are supported by electrophysiologic data. In this system the electrogenic  $\text{Na}^+, \text{K}^+$ -ATPase can be activated when  $\text{K}^+$  is first omitted (pump inhibition) and then reintroduced into the





**Figure 6.** Model for hepatic solute transport. In this model, the sodium gradient and the negative electric potential (both maintained by  $\text{Na}^+, \text{K}^+$ -ATPase activity) are the driving forces for transmembrane solute transport (4).  $\text{OA}^-$ , organic anions;  $\text{BA}^-$ , bile acids;  $\text{AA}$ , amino acids; P.D., electric membrane potential.

perfusion media (pump activation). Intracellular and intracanalicular potentials reveal hyperpolarization during  $\text{K}^+$  readmission (16). If  $\text{Na}^+$  were pumped into the canalicular lumen an initial depolarization of the luminal potential should be observed. In contrast, the potential difference in the lumen mirrored the intracellular hyperpolarization as predicted by basolateral localization of  $\text{Na}^+, \text{K}^+$ -ATPase.<sup>3</sup>

Altogether these findings strongly support sinusoid-lateral plasma membrane localization of  $\text{Na}^+, \text{K}^+$ -ATPase and are in agreement with the model for ion and solute transport illustrated in Fig. 6.

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3. The data are suggestive of removal of the majority of  $\text{Na}^+, \text{K}^+$ -ATPase from the biliary space of the couplet 4-8 h after isolation, i.e., at a time when couplets are used for electrochemical studies. This finding is in agreement with the kinetics of  $\text{Mg}^{2+}$ -ATPase relocation into the biliary space (4-5 h after isolation) in the same system (15). We would therefore propose that the partially labeled bile canaliculus seen in Fig. 4 B represents one from which  $\text{Na}^+, \text{K}^+$ -ATPase has been largely removed.

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