IMMUNOFERRITIN DETERMINATION OF THE DISTRIBUTION OF $(Na^+ + K^+)$ ATPase OVER THE PLASMA MEMBRANES OF RENAL CONVOLUTED TUBULES

II. PROXIMAL SEGMENT

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ABSTRACT

The distribution of $(Na^+ + K^+)$ ATPase over the plasma membranes of the proximal convoluted tubule from canine renal cortex has been determined. Ultrathin frozen sections of this tissue were stained with rabbit antibodies to this enzyme and ferritin-conjugated goat antirabbit γ-globulin. It is demonstrated that high concentrations of this enzyme uniformly line the intercellular spaces of this epithelium. The consequences of this observation are discussed in terms of the low resistant tight junctions of these tubules and the isotonic fluid transport which they support. Furthermore, antibodies to $(Na^+ + K^+)$ ATPase recognize an antigen on the luminal surfaces of the tubules within the brush border. It is proposed that the enzyme is present in this region of the plasma membrane as well, although at much lower concentration. To further substantiate this conclusion, a brush border fraction has been purified from rabbit kidney and been shown to contain significant $(Na^+ + K^+)$ ATPase. These results contradict earlier conclusions about the location of $(Na^+ + K^+)$ ATPase in this tissue.

The previous paper in this pair (18) has introduced an approach to the localization of $(Na^+ + K^+)$ ATPase on the plasma membranes of renal tubule cells. The distal nephron was examined, and it was demonstrated that high, uniform concentrations of $(Na^+ + K^+)$ ATPase were found over the entire lateral surfaces of these cells and a low concentration of the enzyme was present on the luminal surfaces. This manuscript will present observations made on proximal convoluted tubules from the same specimens. The proximal tubule also absorbs Na^+, but it differs from the distal tubule in two important respects. The fluid which it generates is invariably isotonic (2), and the tight junctions present on the luminal side of the epithelium are of the low resistance type (9). The distribution of $(Na^+ + K^+)$ ATPase which has been observed in the proximal tubule will be discussed in terms of these unusual features.

MATERIALS AND METHODS

Brush Border Fraction

Brush border fractions were purified from rabbit kidney by the method of George and Kenny (11). The following modifications were introduced. The buffer used for homogenization and preliminary differential centrifugation was 11% sucrose, 20 mM sodium phosphate, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0. Resuspension before zonal centrifugation was made

in 10 mM Tris SO_4, 10 mM $MgSO_4$, pH 7.6, and all sucrose solutions for gradients were made in this buffer. The buffer which was added to the tissue fragments before the use of the tissue press and which was used for the homogenization was brought to 1 mM in iodoacetamide and 1 mM in phenyl methyl sulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.). The same additions were made to the buffer in which the crude membranes were resuspended before zonal centrifugation. The crude brush border fractions were run in a Ti-14 zonal rotor (Beckman Instruments Inc., Palo Alto, Calif.) over a continuous isopycnic sucrose gradient (35%–53%). The gradient was a 400-ml exponential one (1) constructed with a 460-ml mixing chamber containing 35% sucrose into which 65% sucrose allowed to flow to displace the exiting fluid. Sucrose velocity gradients of these membranes were run in 5-ml Beckman polyallomer tubes in a desk top clinical centrifuge (International Equipment Company, Needham Heights, Mass.) at top speed. Linear gradients between 15–30% were constructed from a two-chamber gradient maker.

$(Na^+ + K^+)$ ATPase assays were performed on these fractions in the usual fashion (14) following preincubation in 0.4 M KCl, 0.3 mg/ml deoxycholate, 5 mM Tris SO_4, 5 mM $MgSO_4$, pH 7.6, for 10 min on ice. Activity was determined by difference, using 10^{-5} M strophanthidin as an inhibitor. Trehalase was determined by the method of Dahlqvist (8) and alkaline phosphatase by the method of Bessey et al. (4).

RESULTS

The observations presented here of the distribution of antigenic sites related to $(Na^+ + K^+)$ ATPase over the surface of the plasma membrane of the renal proximal tubule cell are based on electron micrographs obtained from the same grids examined in the first paper of this pair (18). For that reason, no further description of the antibodies employed or other procedures will be presented.

Antiholoenzyme Antibody

A low power micrograph of a cross-section of proximal tubule located in an ultrathin frozen section is presented in Fig. 1. A low power micrograph of an Epon section from the same canine cortex is presented as Fig. 2. The basement membranes are at the lower edges of the micrographs; the lumens, filled with microvilli, at the tops. Numerous intercellular spaces are observed in the center of the cytoplasm (arrows). Tight junctions can be observed along the luminal border of the epithelium.

When sections which have been stained with antiholoenzyme antibody are examined at higher power, it is observed that high concentrations of ferritin lie along the intercellular spaces between the mitochondria (Fig. 3). An intercellular space can be seen entering at bottom center and exiting at the right (arrows) after circling the center of the field. This space is filled with ferritin. Plasma membranes can be seen more clearly in proximal tubule cells and appear as faint white lines. When antiholoenzyme antibody is preincubated with purified $(Na^+ + K^+)$ ATPase, staining is eliminated (Fig. 4). The random clumps of ferritin in these images (circles) may be antigen-antibody microprecipitates which result from the preincubation. The staining extends uniformly along the intercellular spaces and also along the plasma membranes adjacent to the basement membrane (Fig. 5). In this figure, a band of ferritin (arrows) can be seen descending along an intercellular space and then out along the plasma membrane over the bottom surface of a cell.

Staining with antiholoenzyme antibody is also observed on the microvilli at the luminal surfaces of the cells (Fig. 6). This is eliminated when the antibody is preincubated with purified $(Na^+ + K^+)$ ATPase (Fig. 7). The pattern of staining is uniform and clearly parallels the plasma membrane of the villi, indicating that the surface of the cells is the location of the antigen. When a larger field at the top of the cells is examined (Fig. 8), it can be seen that the heavy staining of the intercellular spaces runs up to the tight junctions (arrows), abruptly ceases throughout their length, and less concentrated staining begins just above them on the microvilli in the lumen. Fig. 9 is an enlargement of a field from Fig. 8 containing two tight junctions. The dense packing of the ferritin in the intercellular space immediately adjacent to these structures (arrows) should be compared with the rather sparse staining of the microvilli which fill the lumen.

The region marked by an oval in Fig. 8 is one in which an intercellular space lies almost perpendicular to the plane of the section and ferritin is very tightly packed. The pattern observed here again suggests that the majority of the antiholoenzyme antibody is attached to the external surface of the plasma membrane in sections (18).

The conclusions drawn from these results are that the plasma membranes which form the intercellular spaces contain a uniform high concentration of antigenic sites recognized by antiholoenzyme antibody, and that this dense distribution of antigens is discontinuous with one of a uniform lower concentration over the brush border, and

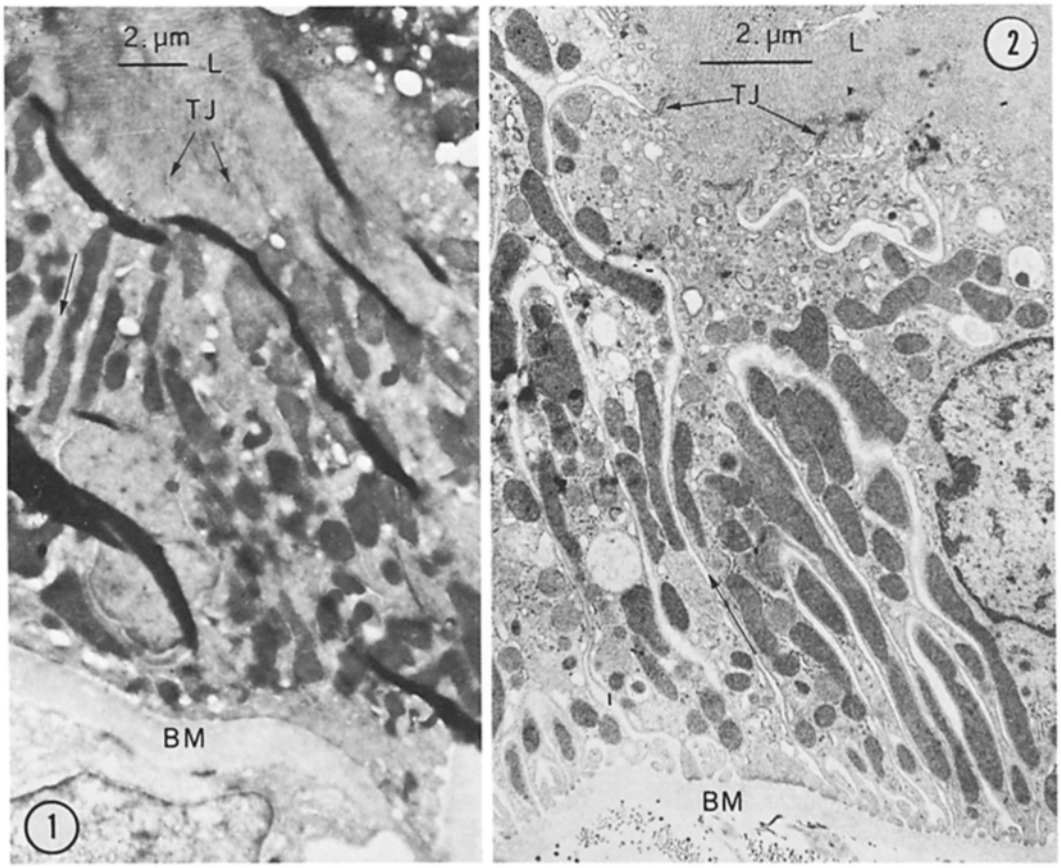


FIGURE 1 Ultrathin frozen section through a proximal convoluted tubule from canine kidney. Basement membrane (*BM*), tight junctions (*TJ*) and lumen (*L*) are clearly seen below and above the central cytoplasm of the tubular epithelium. An intercellular space is indicated by an arrow. × 4,300.

FIGURE 2 Epon section through a proximal tubule from same cortex as that of Fig. 1. The figure is similarly oriented and identically labeled for comparison purposes. × 8,000.

that the discontinuity is formed by the tight junctions.

Anti-Large Chain Antibody

Sections of tissue fixed in 2% formaldehyde overnight were stained with anti-large chain antibody. Fig. 10 is a field in a proximal convoluted tubule and Fig. 11, a distal convoluted tubule in the same grid square. Circles highlight a few of the ferritin molecules on the proximal tubule section (Fig. 10), and arrows point out intercellular spaces. Although the intercellular spaces of the distal tubule are strongly stained, those of the proximal tubule show no significant staining above background.

Brush Border

The antiholoenzyme antibody contains γ-globulins that are directed to the small chain and the large chain of $(Na^+ + K^+)$ ATPase (18). The results of the ferritin staining with antiholoenzyme antibody demonstrate that at least the small chain of the enzyme is present on the brush border of the canine proximal convoluted tubule. It is possible, however, that only the small chain is present, in which case no enzyme activity would be expressed in this region of the cell. In order to provide biochemical evidence that intact, functional $(Na^+ + K^+)$ ATPase is present in this region, brush border was purified from rabbit renal cortex by the procedure of George and Kenny (11). This proce-

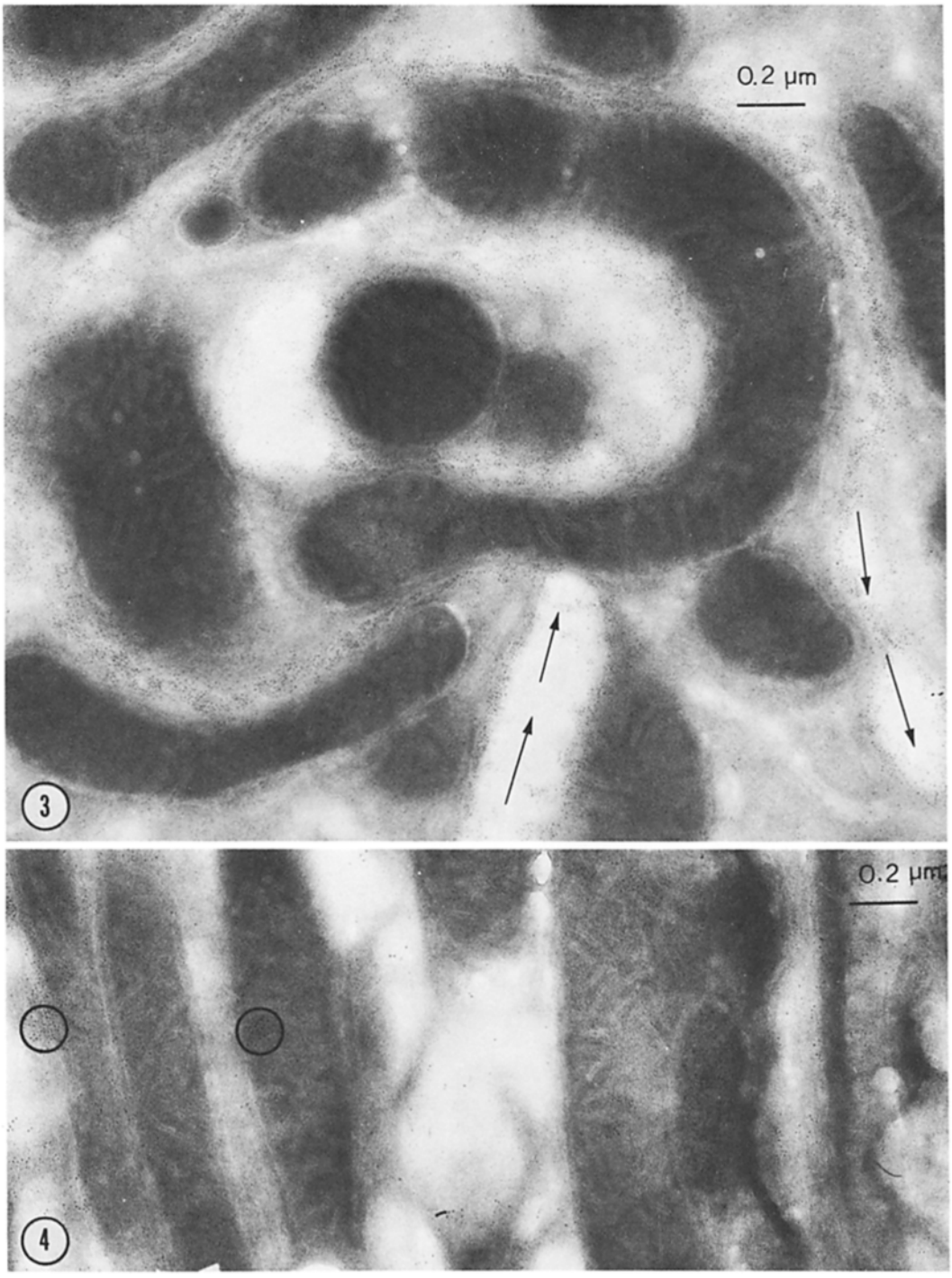
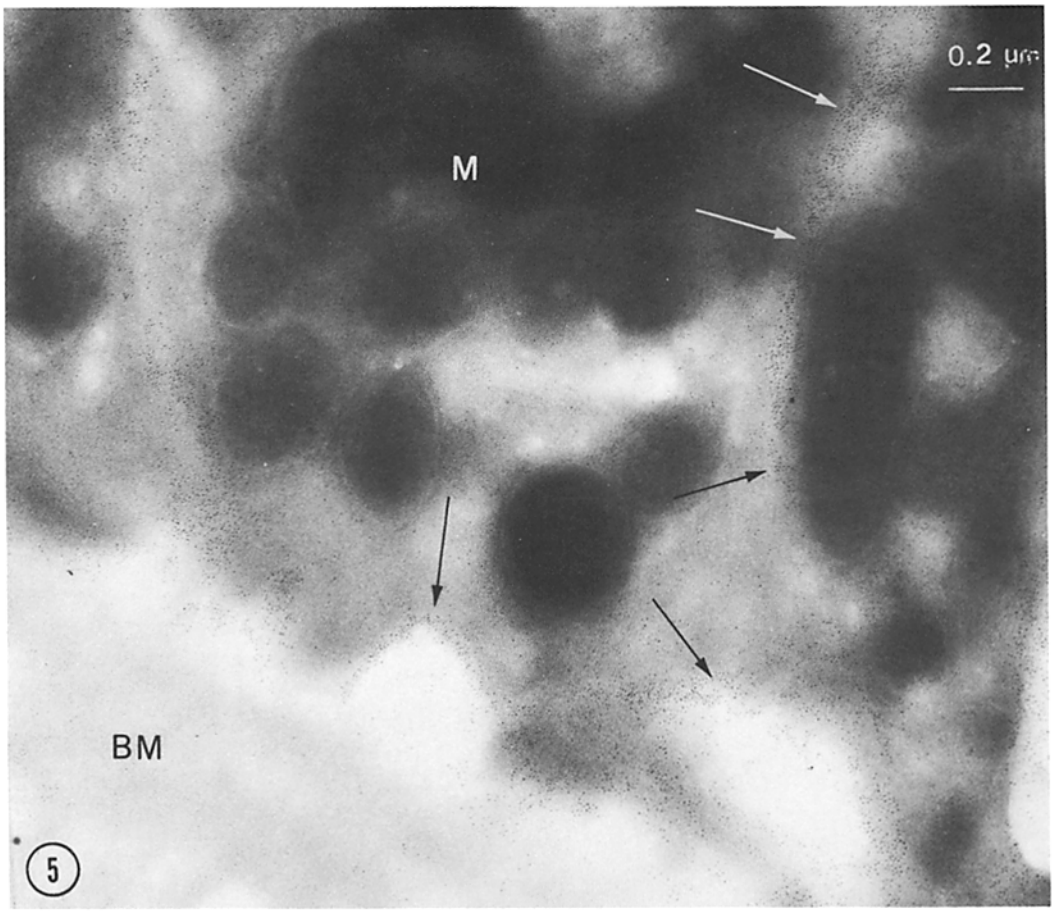


FIGURE 3 Field from the central, cytoplasmic region of proximal tubular epithelium in an ultrathin frozen section which has been stained with antiholoenzyme γ-globulin. Arrows point out the track of an intercellular space. × 50,000.

FIGURE 4 Similar field from a section which has been stained with the same concentrations of γ-globulin and ferritin-conjugated goat antirabbit γ-globulin as in Fig. 3. Antiholoenzyme γ-globulin, however, was preincubated with purified ($Na^+ + K^+$) ATPase. Circles surround random clumps of ferritin. × 50,000.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M
BM
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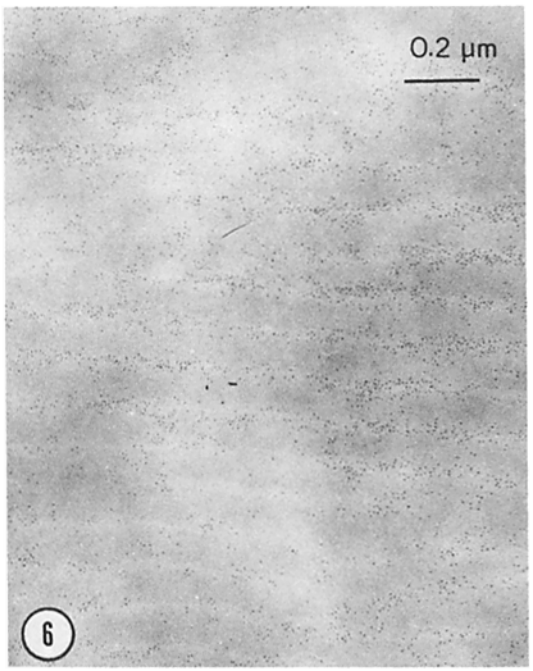
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dure could not be adapted to the canine tissue. The only change introduced was to use a continuous isopycnic gradient during the final step. In preliminary experiments, $(Na^+ + K^+)$ ATPase activity was found in brush border fractions as long as they were assayed within a day following sacrifice of the animal. If these fractions were assayed after two days, no activity remained (Table I). In order to prevent this instability of the enzyme, a series of steps was instituted to prevent proteolytic activity, which is assumed to be mainly due to the release of cathepsins (12). During the purification procedure, buffers were used which had pH of 8.0, lacked Cl^-, and contained 10^{-3} M EDTA. At several steps during the purification, including the initial homogenization, the preparation was made 1.0 mM in iodoacetamide and 1.0 mM in phenyl methyl sulfonyl fluoride. In this way, it was possible to obtain a brush border preparation in which $(Na^+ + K^+)$ ATPase activity was completely stable (Table I).

The distribution of brush border fragments on sucrose gradients can be determined by measuring trehalase and alkaline phosphatase (11). It can be seen that these activities migrate coincidentally when the partially purified cell fragments are sorted either on the basis of density (Fig. 12) by isopycnic centrifugation, or by sedimentation coefficient (Fig. 13) in a velocity gradient.

When brush border fragments were partially purified from rabbit kidney and run on an isopycnic zonal gradient, centrifuged to equilibrium, those fractions containing the brush border fragments, as judged by marker enzyme activities, also contained significant $(Na^+ + K^+)$ ATPase (Fig. 12). When the fractions indicated by the bar in Fig. 12 were pooled, concentrated, and run on a sedimentation velocity gradient, at least 60% of the $(Na^+ + K^+)$ ATPase activity cosedimented with brush border fragments (Fig. 13) whose position was determined by marker enzymes. The remaining 40% was concentrated at the top of the gradient, presumably associated with much smaller fragments. These results demonstrate that a fraction of the $(Na^+ + K^+)$ ATPase present in a homogenate of rabbit renal cortex has the same density and sedimentation coefficient as brush border fragments and is therefore presumably located upon those fragments.

When fractions 25–28 from the zonal gradient (Fig. 12) were pooled, concentrated, and preincubated with 0.3% deoxycholate in 0.4 M KCl in order to improve substrate access to $(Na^+ + K^+)$ ATPase, it was found that the specific activity of the material was 20 μmol P_i/mg h. When the pure enzyme is incubated with the same concentrations of iodoacetamide and phenyl methyl sulfonyl fluoride used during the purification of brush border fragments, it lost 50% of its activity. Since the brush border fragments were exposed twice to the inhibitors, a minimum estimate of the initial specific activity of the brush border in the proximal tubule is 40 μmol P_i/mg h. Under similar assay conditions, the specific activity of the pure enzyme is 650 nm P_i/mg h (16).

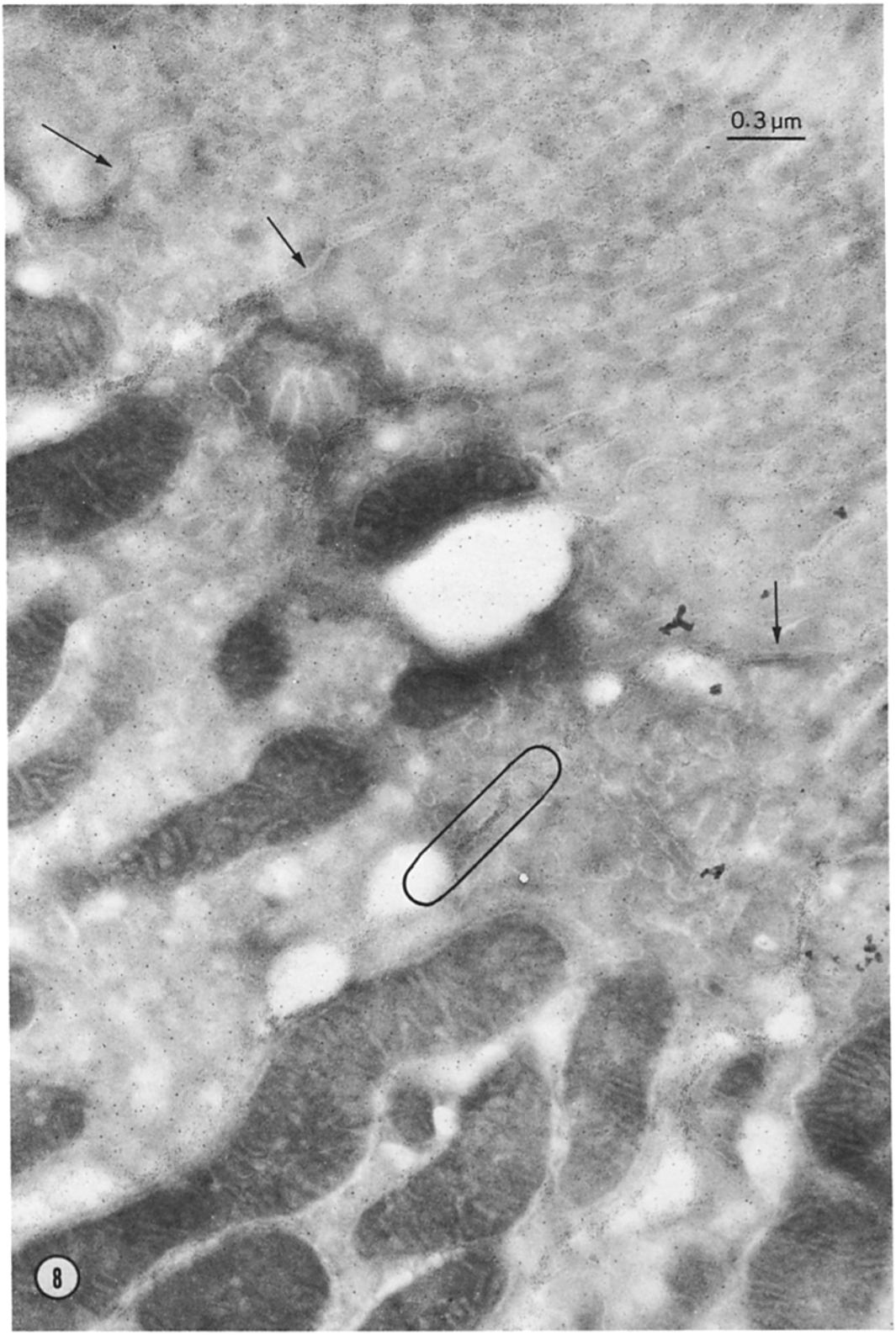
DISCUSSION

The proximal convoluted tubule reabsorbs 70% of the Na^+ and water from the urine in an isotonic fashion (2) as well as 80% of the K^+ (20). The Na^+ and water fluxes are completely eliminated by the addition of ouabain (5, 26). The counterions in this process are HCO_3^- and Cl^-. In addition, sugars and amino acids are reabsorbed in a Na^+-dependent fashion (13). The situation in the proximal tubule is similar to that in the distal tubule except for the fact that the tight junctions of these cells have significant permeability to ions, e.g. they are of the low resistance

FIGURE 5 Field from the basal region of a proximal tubule in an ultrathin frozen section stained with antiholoenzyme γ-globulin. The basement membrane (*BM*) and mitochondria (*M*) are identified. Arrows indicate a band of ferritin descending along an intercellular space and then over the basal plasma membrane of a tubule cell. × 50,000.

FIGURE 6 Field from the lumen of a proximal tubule in an ultrathin frozen section which has been stained with antiholoenzyme γ-globulin. × 50,000.

FIGURE 7 Similar field from a section which has been stained with the same concentrations of γ-globulin and ferritin conjugate as in Fig. 6. Antiholoenzyme γ-globulin, however, was preincubated with purified $(Na^+ + K^+)$ ATPase. × 50,000.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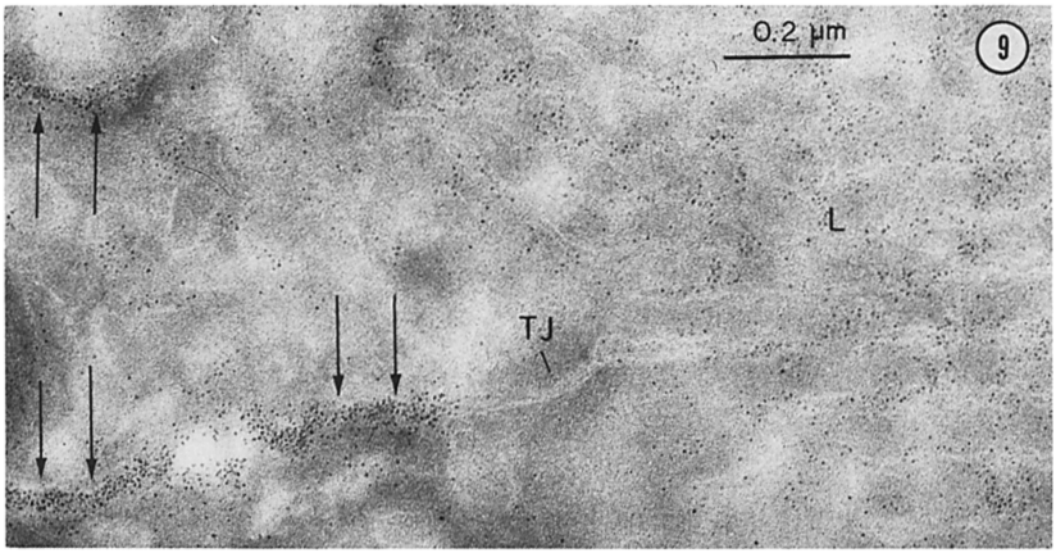

FIGURE 9 Region from the field displayed in Fig. 8 at higher magnification. Arrows indicate the mucosal ends of two intercellular spaces. Lumen (*L*) and tight junction (*TJ*) are identified. × 80,000.

type (26). It has already been noted (18) that the morphology observed in cross-sections of the proximal tubule is the result of the complex expansion of a rather simple system of intercellular spaces represented diagrammatically in Fig. 14.

Antigenic sites in the proximal convoluted tubule which are recognized by the antiholoenzyme antibody can be readily located (Fig. 3) but sites accessible to (18) and recognized by the anti-large chain antibody are present at concentration below the sensitivity of the staining method (Fig. 10). Since these two antibody solutions, when present at saturating concentration, elicit approximately the same density of staining on purified plasma membranes (17), the sites to which anti-large chain γ-globulins are directed are very likely sterically hindered in the section as discussed previously (18). It is known that the concentration of (Na^+ + K^+) ATPase in the proximal convoluted tubule is five times less than that in the distal tubule (27). This is consistent with the difference in anti-large chain antibody staining displayed in Figs. 10 and 11. Nevertheless, since all previous results agree that there is a significant (Na^+ + K^+) ATPase concentration in the basal portions of the renal proximal tubule (28), it is concluded that the staining observed with antiholoenzyme antibody in this region reflects the presence of the enzyme and, further, that the enzyme is distributed continuously along the intercellular spaces.

The following description of sodium fluxes in the proximal convoluted tubule is essentially an abbreviated version of an elegant quantitative treatment presented by Spring (33) for *Necturus* proximal tubule. (Na^+ + K^+) ATPase, located along the borders of the intercellular spaces, is responsible for a large net flux of Na^+ into those spaces such that, in the absence of compensating water flux, large concentrations of NaCl and $NaHCO_3$ would be created within them. The net flux of Na^+ across the epithelium results from the balance of four fluxes (Fig. 14):

$$J_{Na_{l,s}} = J_{Na_{l,c}} - J_{Na_{ls,l}} = J_{Na_{ls,s}} - J_{Na_{s,c}}$$

where $J_{Na_{l,s}}$ = net flux of Na^+ from lumen to serum; $J_{Na_{l,c}}$ = net flux of Na^+ from lumen into cell; $J_{Na_{ls,l}}$ = net flux of Na^+ from intercellular spaces into lumen; $J_{Na_{ls,s}}$ = net flux of Na^+ from intercellular spaces into serum; and $J_{Na_{s,c}}$ = net

FIGURE 8 Field from the luminal side of proximal tubular epithelium in an ultrathin frozen section stained with antiholoenzyme γ-globulin. Tight junctions are indicated by arrows. The oval highlights a segment of intercellular space oriented normal to the plane of the section. × 40,000.

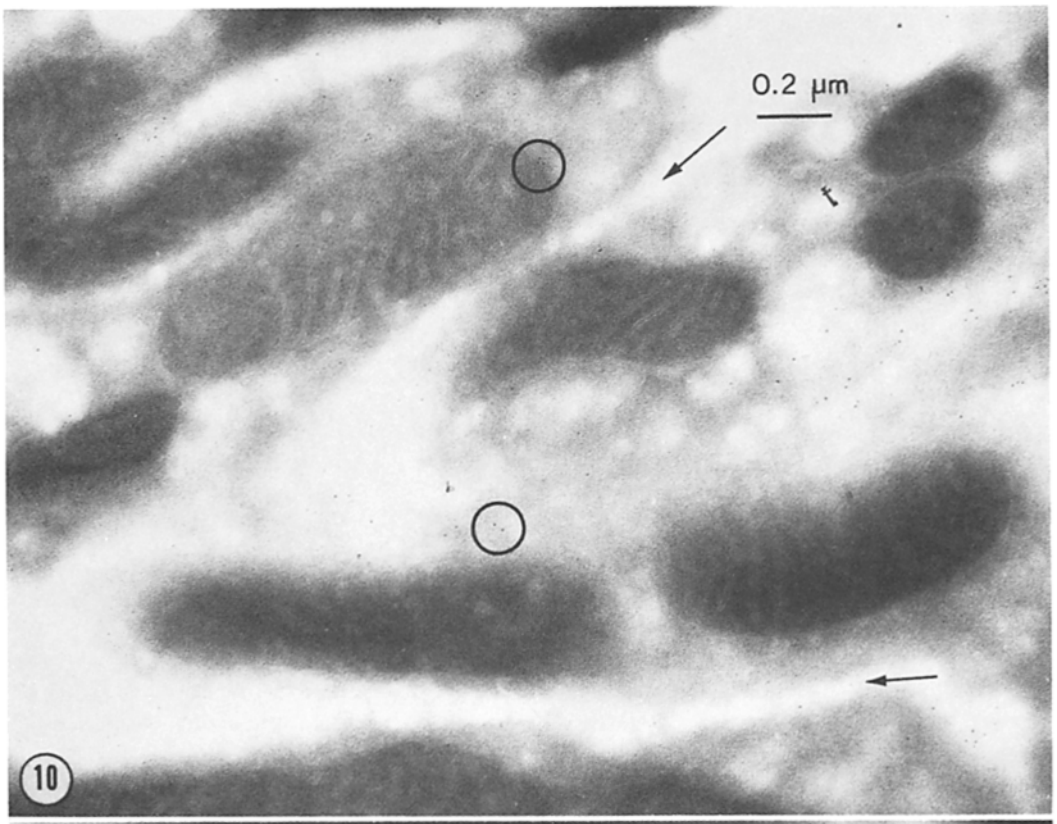

FIGURE 10 Field from the central cytoplasmic region of proximal tubular epithelium in an ultrathin frozen section stained with antilarge chain γ-globulin. Arrows indicate intercellular spaces; circles, random background ferritin. × 50,000.

FIGURE 11 Field from the central cytoplasmic region of distal tubular epithelium, in the same section as Fig. 10, stained with anti-large chain γ-globulin. × 50,000.

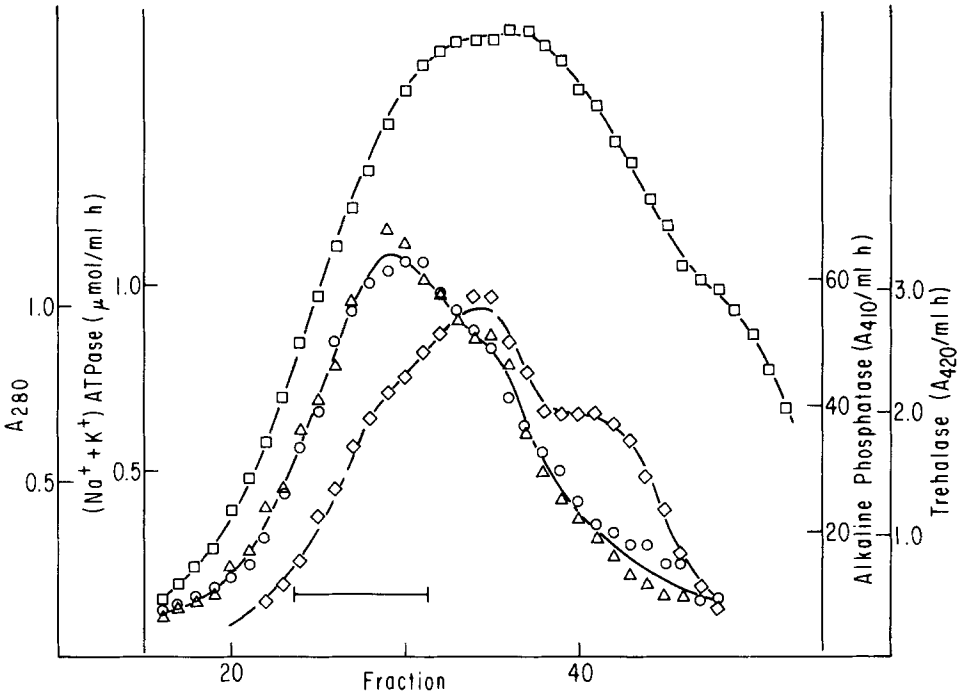

FIGURE 12 Distribution of enzymatic activity over an isopycnic density gradient. Crude brush border fraction was purified by a modification of the method of George and Kenny (11). This material was layered onto a sucrose gradient in a 14-Ti rotor and spun at 37,500 rpm for 1 h. The gradient was then pumped out of the rotor and fractions assayed for A_{280} (□——□) (mainly light scattering), trehalase (○——○), alkaline phosphatase (△——△) and (Na^+ + K^+) ATPase (◇——◇). Sucrose concentrations (wt/wt) of selected fractions were: 20, 37.4%; 30, 40.0%; and 40, 41.9%. The bar indicates the location of fractions pooled for sedimentation velocity analysis.

flux of Na^+ from serum into cell. The fluxes are written in their most probable physiological direction. The active flux of Na^+ into the intercellular spaces catalyzed by (Na^+ + K^+) ATPase does not

TABLE I

Specific Activity of Brush Border Fractions as a Function of Storage Time

Days after preparation	1	2	5
(Na^+ + K^+) ATPase ($\mu mol/P_i$)/(mg/h)			
Normal conditions	20	3	
Cathepsins inhibited	30		20

Brush border fractions were prepared by isopycnic centrifugation. A region similar to that indicated by the bar in Fig. 12 was pooled, diluted, and concentrated by centrifugation. The brush border fragments obtained were stored in 11% sucrose, 10 mM $MgSO_4$, 10 mM Tris SO_4, pH 7.6 at 4°C. Samples were taken for assay at noted times. Brush border fragments prepared under normal conditions (11) are compared with those prepared under conditions which minimize cathepsin activity.

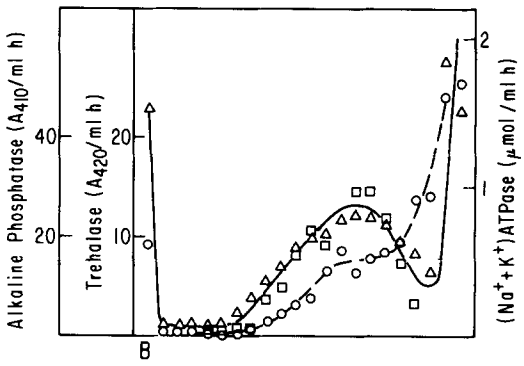

FIGURE 13 Sedimentation velocity analysis of brush border fragments. The fractions indicated in Fig. 12 were pooled, diluted, and concentrated by centrifugation. A sample of this material was layered onto a 15-30% linear sucrose gradient which was spun at full speed at 4°C in a clinical centrifuge. The tube was punctured, and fractions were collected. They were assayed for trehalase (△——△), alkaline phosphatase (□——□), and (Na^+ + K^+) ATPase (○——○). The letter B in the figure marks the bottom of the gradient.

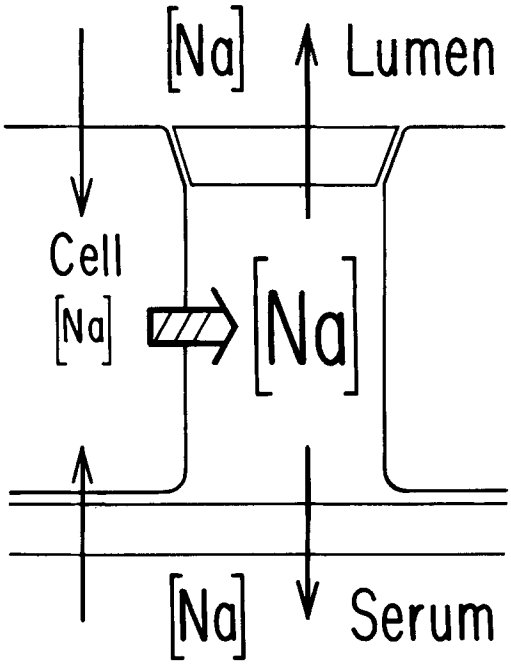

FIGURE 14 Diagrammatic portrayal of the Na^+ fluxes in a transporting epithelium. The fluxes described in the text are indicated by arrows. The hatched arrow denotes active Na^+ flux. Relative sodium concentrations are denoted (not quantitatively) by the size of the lettering.

appear in the equation because it occurs entirely within the epithelium. Each one of these fluxes is the result of an electrochemical gradient and a conductance. The conductances are those of the luminal cell membrane, the spaces themselves and the tight junction, the spaces themselves and the basement membrane, and the serosal cell membrane. Each of these conductances can be developmentally controlled by the organism. In the case of the proximal convoluted tubule, the conductance of the luminal plasma membrane to Na^+ is greater than that of the serosal, and the conductance of the spaces and the basement membrane is presumably greater than that of the spaces and tight junction (33). As a result, the net flux of Na^+ is from the lumen to the serum. Nevertheless, it should be noted that the net flux of Na^+ is always the balance of several opposing fluxes which are occurring at all times.

The only ions present at significant concentrations in the intercellular spaces are Na^+, Cl^-, and HCO_3^-, since K^+ is being continuously taken up into the cells. As a result, it is NaCl and $NaHCO_3$ which exit from the spaces in both directions. These considerations can explain several unusual physiological observations. When intestinal epithelium is incubated with choleragen, the unidirectional flux of Na^+ from lumen to serum is decreased while the unidirectional flux of Na^+ from serum to lumen remains the same (22). The result is to reverse the net flux and create a net secretion of $NaHCO_3$ and NaCl from the serum to the lumen, which is completely inhibited by ouabain (23). It can be seen that the effect of choleragen can easily be achieved in a transporting epithelium by simply eliminating $J_{Nal,c}$ The consequence is net secretion of $NaHCO_3$ and NaCl from the serum to the lumen through the tight junction. This secretion of Na^+ is occurring even under normal circumstances and may be satisfying the requirement for luminal Na^+ in the uptake of sugars and amino acids (30). In a similar fashion, it can be seen that by a simple alteration of the various conductances for Na^+ and water the epithelium can be made to secrete a NaCl solution of high concentration from the serum to the lumen, through the tight junction. The salt gland of certain marine birds is an epithelium which is able to generate such a secretion (29).

The above discussion has assumed that the only significant active Na^+ flux is that into the intercellular spaces catalyzed by ($Na^+ + K^+$) ATPase. Although it will be argued later that ($Na^+ + K^+$) ATPase is present on the luminal surface of these cells, it is apparently present in these regions at a lower concentration (Figs. 8 and 9) and at a much greater distance from mitochondrial sources of MgATP (Fig. 2) compared to the enzyme at the intercellular spaces. ($Na^+ + K^+$) ATPase is also located in the regions of the plasma membrane adjacent to the basement membrane of the tubule (Fig. 5). The surface area of this portion of the cell, however, is small compared with that of the intercellular spaces, and it is separated from mitochondrial sources of MgATP by the basal labyrinth (Fig. 2).

The fluid which emerges from the intercellular spaces into the serum in the proximal convoluted tubule is invariably isotonic (2). Presumably the active flux of Na^+ (and the accompanying Cl^- and HCO_3^-) into these spaces creates a standing osmotic gradient which promotes a coordinate water flux, as described by Diamond and Bossert in their mathematical model (10), such that an isotonic NaCl solution emerges into the serum.

One of the predictions which this description makes, with regard to isotonically transporting epithelia such as the proximal tubule, is that the sites of active solute transport will be confined to regions of the intercellular spaces near the tight junctions and distant from the openings at the basement membrane. This arrangement allows fluid within the spaces to reach osmotic equilibrium with the interior of the cell before it emerges into the plasma. The observations presented here demonstrate that the ferritin staining which results from the attachment of γ-globulins which recognize ($Na^+ + K^+$) ATPase is distributed densely and uniformly over the plasma membranes which form the intercellular spaces of the proximal tubule (Fig. 5). It has already been pointed out that when antigens are present in regions on these sections at concentrations in excess of a certain saturation level, discrimination between differences in actual concentration no longer occurs (18). Nevertheless, because no obvious nonuniformity of staining is observed in these regions, it is assumed that ($Na^+ + K^+$) ATPase itself is uniformly distributed over the plasma membranes which form the intercellular spaces of the proximal convoluted tubule. This would be consistent with present views that membranes are fluid structures in which membrane-bound proteins diffuse rapidly over the surface in the absence of constraints (32). Furthermore it has been demonstrated that ($Na^+ + K^+$) ATPase is uniformly distributed over the plasma membranes which form the intercellular spaces of the distal tubule (18). If this is also the case in the proximal tubule, it is difficult to explain the invariable isotonicity of the emerging fluid by utilizing the parameters chosen by Diamond and Bossert (10). The mathematical model which they describe predicts that the fluid secreted by an epithelium becomes progressively more hypertonic as the distribution of solute flux is spread over the intercellular space. Several possible resolutions of this difficulty should be considered.

(*a*) Since all intercellular space is continuous with a tight junction, there may be significant water flux from the lumen into the intercellular space and Na^+ backflux from the intercellular space into the lumen across these structures such that the solution within the spaces is brought to isotonic equilibrium more rapidly. Those epithelia which transport fluid isotonically usually have low resistant tight junctions (9).

(*b*) It is possible that the water permeability of the plasma membranes which bound the intercellular spaces is much greater than was originally thought. Wright et al. (36) have recently demonstrated that previous measurements of water permeability across the gall bladder were actually lower limits of the true value due to the presence of sweeping away effects which occur in the unstirred layers adjacent to the epithelium. Furthermore, the value for water permeability across an entire epithelium, even corrected for unstirred layers, can be used to calculate only a lower limit to the permeabilities of the various membranes which compose the epithelium. If the plasma membranes which form the intercellular spaces are more permeable to water than the luminal membranes, only the latter contribute significantly to measurements of the overall permeability.

(*c*) The distribution of mitochondria in the cytoplasm may significantly affect the flux densities at the plasma membrane, causing far greater fluxes to occur in the central regions of the cell. In the proximal tubule between those regions of the intercellular spaces, which are in direct contact with the mitochondria, and the basement membrane, there is an extensive labyrinth which is simply a final anastamosis of the intercellular space (3). The result of this device is to increase the amount of cellular surface area between the mitochondria and the basement membrane. If, in the steady state, significant Na^+ flux occurs only adjacent to mitochondria, and their supply of MgATP,[1] this morphological structure allows the intercellular space to have a significant surface area, beyond the sites of cation transport, over which osmotic equilibrium can be reached. It has

[1] Rat kidney mitochondria are capable of producing, at 37°C and at saturation of MgADP, 200 nmol of ATP mg^{-1} min^{-1} (19, 31). Mitochondria are 50% by weight protein, proximal tubule epithelium is 20% by volume mitochondria (Fig. 2), and rat proximal tubule is a cylinder 50 μm in diameter (28) with a wall 15 μm thick (3). ($Na^+ + K^+$) ATPase will transport 3 Na^+ per MgATP produced within the mitochondrion (18). From these measurements it can be calculated that the rat proximal tubule can transport 0.8-nl isotonic solution mm^{-1} min^{-1}. It has been observed that a rabbit proximal tubule can transport 1–2 nl mm^{-1} min^{-1} under optimum condition (5). These calculations suggest that the production of MgATP by the mitochondria could be the rate limiting step in fluid transport and therefore it would not be unreasonable to postulate a significant gradient in (MgATP) as a function of the distance from these organelles.

been noted by Tormey and Diamond that in the gall bladder there is a definite gradient of mitochondria, the more luminal regions of the cells containing the higher concentration (34).

(*d*) In an early model for active water transport, proposed by Curran (6), it was demonstrated that a system composed of two membranes in series, the first permiselective and the second porous, could couple fluid transport from one reservoir to another to an active flux of solute into the space between them (7). The basolateral plasma membranes correspond to the permiselective barrier and the basement membrane to the porous one. In a mathematical examination of this model, Patlak et al. (21) has demonstrated that this system would generally transport hypertonically but, under certain circumstances (if σ of the permiselective membrane $\cong 1$), flows that are almost isotonic can be generated. Even in the regions where ($Na^+ + K^+$) ATPase lies directly opposite the basement membrane (Fig. 5), isotonic fluxes may still be generated if this two-membrane model is applicable to these circumstances.

It can be seen in Figs. 8 and 9 that the tight junction forms a boundary between two regions of the plasma membrane of the proximal tubule cell which are stained with a different intensity by antiholoenzyme antibody. A more striking difference in staining intensity between luminal and basolateral surfaces was observed when sections of the distal tubule were exposed to anti-large chain antibody (18). Antiholoenzyme antibody reacts mainly with antigens on the external surface of the plasma membrane in thin sections (Fig. 8) (18). It has been demonstrated that the glycocalyx on the brush border of the renal proximal tubule is more dense than that on the plasma membranes which form the intercellular spaces (25). A difference, on the two sides of the cell, in steric hindrance to the binding of the antibody to the external surface of the membrane may, therefore, contribute to the differences in density of staining observed. Nevertheless, there appears to be a lower concentration of antigens recognized by antiholoenzyme antibody on the luminal surface than the basolateral surface, and this difference sharply coincides with the discontinuity formed by the tight junction (Fig. 9). If ($Na^+ + K^+$) ATPase is free to diffuse translationally over the plasma membranes of the proximal tubule cells, the polarity of the enzyme distribution must be maintained by a barrier to diffusion which is presented by the tight junction. A similar conclusion has been reached by Revel who has shown that discontinuities in the distribution of surface carbohydrate and intramembrane particles occur at tight junctions (25).

The staining observed at the luminal surface of the proximal tubule cells within the brush border when antiholoenzyme antibody is used as a primary ligand (Fig. 6) indicates that at least the small chain of the ($Na^+ + K^+$) ATPase is present in this region (18). Although the proximal tubule is responsible for 80% of the K^+ uptake from the urine (20), this observation cannot be used to clarify the question of whether ($Na^+ + K^+$) ATPase is located on the luminal surface of the cell because the situation is complicated by the low resistance of the proximal tubule tight junction. The net flux of K^+ which occurs across the proximal tubule must occur either through the tight junction or across the luminal plasma membrane. Since the electrical potential across the epithelium is negligible there would have to be a significant K^+ concentration gradient between the lumen and the intercellular space, to drive K^+ flux across the tight junction. In this case, the concentration of K^+ in the intercellular space must be much less than 4 mM (20). This is not impossible since ($Na^+ + K^+$) ATPase is actively removing K^+ from the space. If K^+ concentrations are of this order, however, the efficiency of the enzyme may be lowered since it would be operating below saturation ($K_{m_{K^+}} = 0.7$ mM @ 100 mM Na^+) (15). On the other hand, if K^+ is absorbed across the luminal plasma membrane, ($Na^+ + K^+$) ATPase must be located within it. It can be seen that there is no definite physiological requirement for the presence of ($Na^+ + K^+$) ATPase at the luminal plasma membrane in this tubule.

In general, brush border fractions which have been purified from the kidney have contained significant ($Na^+ + K^+$) ATPase activity (11). The major evidence against the presence of significant concentrations of ($Na^+ + K^+$) ATPase in the brush border is based on the microdissection study (28). These experiments, however, suffer from two difficulties. It has been shown that ($Na^+ + K^+$) ATPase activity in brush border fractions is sensitive to proteolytic inactivation (Table I). Furthermore, since brush border is a complicated membrane system, and substrates must approach ($Na^+ + K^+$) ATPase from opposite sides of the membrane, access of substrates to the enzyme may be inhibited if these preparations contain resealed membranes. In order to overcome these difficulties, purification of brush border was carried out

under conditions which inactivate proteolytic enzymes (Table I). The assay was performed on preparations incubated with deoxycholate to open up the membranes to substrate. With these precautions, it is demonstrated that a significant fraction of the ($Na^+ + K^+$) ATPase activity migrates with the same density and sedimentation coefficient as brush border membranes and is presumably present in them (Figs. 12 and 13). A criticism of this conclusion is that basolateral plasma membranes of high specific activity may still contaminate these fractions due to their similar physical properties or to their direct attachment to the brush border fragments. A way to resolve this difficulty would be to demonstrate immunochemically that the majority of the ($Na^+ + K^+$) ATPase molecules in these fractions was attached to brush border regions. This could be accomplished by staining these membrane fragments with antibodies against the enzyme and ferritin conjugate. Unfortunately, the procedure of George and Kenny yielded no brush border fraction (determined enzymatically and visually) when it was applied to canine renal cortex.

From the observed specific activities it can be calculated that 5–10% of the protein in brush border fractions is ($Na^+ + K^+$) ATPase. If the distal tubule intercellular spaces are lined with pure ($Na^+ + K^+$) ATPase and the proximal tubule spaces are lined by membranes which have no more than a fivefold lower concentration, the brush border of the proximal tubule should contain more than a four times lower ($Na^+ + K^+$) ATPase concentration than the intercellular spaces. These rough estimates are consistent with the pattern observed in Fig. 8.

It is not an unusual result to find ($Na^+ + K^+$) ATPase on the mucosal surface of a transporting epithelium since it has been shown both autoradiographically and physiologically that high concentrations of the enzyme are located along the mucosal surface of the choroid plexus (24, 35). Furthermore, in epithelia such as the insect malpighian tubule, mitochondria are found within villi (3), presumably feeding ($Na^+ + K^+$) ATPase located there. If ($Na^+ + K^+$) ATPase is located in the brush border of renal proximal tubules, it may be involved in K^+ reabsorption (20). It also may guarantee that the concentration gradient of Na^+ across the luminal plasma membrane remains large enough to insure net uptake of sugars and amino acids (30).

These results, obtained with antibodies to ($Na^+ + K^+$) ATPase, approach the problem of renal absorption from a different perspective than physiological measurements. From knowledge of the distribution of an enzyme which catalyzes a well-defined chemical reaction, certain chemical consequences can be predicted. Models of renal absorption must be consistent with these chemical consequences.

I would like to thank Dr. S. J. Singer for encouragement and candid advice. Dr. K. Tokuyasu was a patient teacher who is responsible for the art of this presentation. I would also like to thank Dr. Richard Painter and Thomas Cope. Discussions with Dr. John Tormey assisted greatly in the preparation of this manuscript.

This research was supported by Grants GM 15971 (S. J. Singer) and HL 17879-01 (J. Kyte) from U. S. Public Health Service and was initiated while I was a postdoctoral fellow of the Damon Runyon Fund.

Received for publication 20 June 1975, and in revised form 8 September 1975.

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