

# IMMUNOFERRITIN DETERMINATION OF THE DISTRIBUTION OF $(\text{Na}^+ + \text{K}^+)$ ATPase OVER THE PLASMA MEMBRANES OF RENAL CONVOLUTED TUBULES

## I. Distal Segment

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### ABSTRACT

The distribution of  $(\text{Na}^+ + \text{K}^+)$  ATPase over the plasma membranes of distal convoluted tubules from canine kidney has been determined. This enzyme is responsible for the coupled active transport of  $\text{Na}^+$  and  $\text{K}^+$  across animal cell membranes. Ultrathin frozen sections were cut from fragments of renal cortex and specifically stained with antibodies, which recognize antigenic sites on the enzyme, and ferritin-conjugated goat antirabbit  $\gamma$ -globulins. It is demonstrated that  $(\text{Na}^+ + \text{K}^+)$  ATPase is distributed uniformly and at high concentration over the plasma membranes which form the intercellular spaces of this epithelium. The enzyme is located on the luminal surface of the tubules as well but at a much lower concentration. These results, in combination with those of previous determinations of the cation fluxes across this epithelium, can be used to formulate a complete description of the cation movements through this tissue.

The tubules of the mammalian kidney are responsible for the reabsorption of all useful metabolites and most of the water from the ultrafiltrate which enters at the glomerular end. All of these fluxes across the tubular epithelium require the expenditure of metabolic energy and are associated with significant fluxes of sodium. Reabsorption processes are inhibited by the application of ouabain (5, 6, 9, 43, 53). It is known that ouabain is a highly specific inhibitor of  $(\text{Na}^+ + \text{K}^+)$  ATPase (10), the enzyme responsible for the coupled, active transport of sodium and potassium in opposite directions across the plasma membranes of animal cells (46). These considerations suggest that this enzyme plays a fundamental role in kidney function.

This communication presents a determination of the distribution of  $(\text{Na}^+ + \text{K}^+)$  ATPase over the

plasma membranes of the cells which form the distal convoluted tubules of canine renal cortex. This has been accomplished through the application of a recently described technique for locating antigens in frozen ultrathin sections by staining them with ferritin-antibody conjugates (40). Ferritin conjugates have frequently been used to locate antigens in suitable biological samples, but, in the past, intracellular antigens have been made accessible to the conjugates only by methods that disrupt cells and tissues. The recently described technique of ultracryotomy now allows ferritin conjugates to be used to determine distributions within cells which are ultrastructurally intact. The experiments described here are the first systematic application of this technique to a particular biological problem.

## MATERIALS AND METHODS

Complement fixation was performed by the method of Levine and Van Vunakis (30) as described in an earlier report from this laboratory (28).

### *Ferritin Conjugates*

Goat antirabbit  $\gamma$ -globulin was purified from serum by affinity chromatography (27). Ferritin and this affinity-purified  $\gamma$ -globulin were covalently cross-linked by reaction with glutaraldehyde (1). The following procedure has consistently produced conjugate with low background in high yield.

Spin out an  $(\text{NH}_4)_2\text{SO}_4$  slurry of the  $\gamma$ -globulin at 30,000 g for 20 min. Resuspend (will not necessarily redissolve) the slurry in an absolute minimum of 0.1 M sodium phosphate buffer, pH 7.4 (Buffer A). Dialyze for 2-3 days against Buffer A in a turgid, ballooned dialysis bag. The buffer must be changed many times to dialyze away all of the  $\text{NH}_4^+$ . Final solution should be 6-8% in protein. Store frozen at  $-70^\circ\text{C}$  indefinitely.

Prepare ferritin directly from *unfrozen*, freshly removed horse spleens by the method of Granick (15). No commercial ferritin is adequate. Recrystallize until the crystals redissolve rapidly and completely, leaving no residue. Dialyze exhaustively to eliminate  $\text{NH}_4^+$  and  $\text{Cd}^{2+}$ , concentrate by ultracentrifugation, and sterilize by Millipore filtration (Millipore Corp., Bedford, Mass.). Store ferritin at 15-20% in protein in 0.05 M sodium phosphate, pH 7.5 at  $4^\circ\text{C}$  in a sterile container. It will last for about 1 yr.

Mix 13 mg of ferritin and 7.5 mg of the  $\gamma$ -globulin together in a minimum volume. Add 10-30  $\mu\text{l}$  of 0.5% glutaraldehyde dissolved in Buffer A with rapid stirring. Stand at room temperature after thoroughly mixing for 1 hr. Dialyze for 4 h against 0.1 M  $(\text{NH}_4)_2\text{CO}_3$  at  $4^\circ\text{C}$ . Layer onto a  $2.3 \times 26\text{-cm}$  column containing 6% agarose equilibrated with 0.15 M NaCl, 20 mM sodium phosphate, 0.1 mM ethylenediaminetetraacetic acid, EDTA, pH 7.4 (phosphate-buffered saline). Run the column at 10 ml/h. Read  $A_{440}$  of the fractions. The elution profile will contain two peaks, the void volume (polymers) and the ferritin monomer peak, well separated. The leading edge of the ferritin monomer peak, which appears as a shoulder, will contain the ferritin- $\gamma$ -globulin conjugate. The glutaraldehyde concentration in the original reaction mixture is critical. Each bottle of this reagent must be independently titrated. The only indication of success is the column elution profile. If too little glutaraldehyde is used, there is no intermolecular cross-linking, no void volume peak, and all the ferritin travels at the monomer position. If too much glutaraldehyde is used, all the material polymerizes, and the void volume peak contains most of the absorbance. Choose six to eight fractions in the region of the leading edge of the monomer peak, well removed from the void volume, and spin out each of them individually at 120,000 g for 45 min. Remove most of the supernate. Notice how long it takes for each pellet to

redissolve. Test each fraction for antibody activity by immunodiffusion (a red precipitin line is formed). Choose those fractions which redissolved rapidly (low background) and gave good titers (high activity).

### *Fixation and Sectioning*

The method described by Tokuyasu (48) was used to obtain ultrathin frozen sections attached to carboned, ionized, Formvar-coated grids (Formvar, Belden Mfg. Co., Chicago, Ill.). Tissue fragments (1-2 mm) were cut from the kidney, immediately following death of the animal, and fixed in either 2% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) for 1 hr or 2% formaldehyde (prepared from Matheson paraformaldehyde Matheson Gas Products, East Rutherford, N. J.) overnight, in each case at  $4^\circ\text{C}$  and buffered with Buffer A. The sections were cut at  $-70^\circ$  to  $-90^\circ\text{C}$  in a cryokit on a Sorvall MT-2 microtome (Dupont Instruments, Sorvall Operations, Newtown, Conn.) from these fragments after they had been infused with 30% (wt/wt) sucrose in either Buffer A or Buffer A, 2% in formaldehyde, respectively. The temperature in the cryokit was varied to obtain the optimum hardness.

### *Staining with Ferritin Conjugates*

The method for ferritin staining was devised by Dr. K. T. Tokuyasu.<sup>1</sup> The grids containing the sections are collected face down on a large puddle of phosphate-buffered saline. They are transferred consecutively between 0.2-0.4-ml droplets of the appropriate reagents which were scattered over a sheet of Parafilm (Para Mfg. Co., Inc., Cranford, N. J.). All the different solutions are prepared in phosphate-buffered saline, 10 mM in glycine. A grid is first transferred to a droplet of 10% bovine serum albumin, BSA, for 5 min and then lifted directly out of this droplet on a thick film of the solution trapped in a wire loop. This is done by bringing a special thin loop #2525 (Ladd Research Industries, Inc., Burlington, Vt.) up from below the floating grid. The loop, with the grid floating now on the thick film, is turned over and 10-20  $\mu\text{l}$  of a 0.2-1.0 mg/ml  $\gamma$ -globulin solution is added from above. The loop is again inverted to allow the  $\gamma$ -globulin solution to float up to the section, and held in a humid chamber for 5 min. The section is then transferred onto the surface of a droplet of phosphate-buffered saline by simply immersing the loop. It is then transferred between several similar droplets in rapid succession and washed for 10 min on a final large puddle by gentle jets of buffer from below. The procedure is then repeated exactly, starting with the 10% BSA, but ferritin-conjugated goat antirabbit  $\gamma$ -globulin (0.2-1.0  $A_{440}$ ) is introduced into the thick film instead of a solution of the primary antibody. The sections are transferred from the final rinse to 2% glutaraldehyde in Buffer A, water, and then 0.5% phosphotungstic acid. They are exposed to the

<sup>1</sup> Tokuyasu, K. Personal communication.

negative stain for 10 s and carefully drained. The degree of staining is critical. The ferritin cannot be seen on overstained specimens, and no fine structure is apparent on understained specimens. Fig. 8 of reference 25 illustrates the desired qualities. Sections were examined on a Philips EM-300 electron microscope.

In every instance in which a comparison between control and experimental conditions is made, both grids were stained with the same concentrations of primary antibody and ferritin-conjugated goat antirabbit  $\gamma$ -globulin.

## RESULTS

### Antibodies

( $\text{Na}^+ + \text{K}^+$ ) ATPase is an enzyme composed of two subunits, the large chain (mol wt = 130,000) and the small chain (mol wt = 40,000) (26). It has been purified for these experiments from canine kidney (24). Antibodies were raised against two well-defined protein preparations (27). Antiholoenzyme antibody was obtained from the serum of a rabbit injected with the purified, native enzyme. This antigen contains both subunits in their native conformations. Any  $\gamma$ -globulins which recognized lipids in the enzyme preparations used for injection were eliminated by adsorption (27). Evidence has already been presented which demonstrates that this adsorbed antiholoenzyme  $\gamma$ -globulin reacts specifically with ( $\text{Na}^+ + \text{K}^+$ ) ATPase and binds to the enzyme on both sides of the plasma membrane (27). The large chain and the small chain can be separated from one another by gel filtration in sodium dodecyl sulfate, and the detergent removed (26). The proteins are then completely water soluble. When antiholoenzyme  $\gamma$ -globulin is preincubated with a solution of the homogeneous, water-soluble large chain a certain fraction of its activity, as judged by complement fixation (see Fig. 2, reference 28), is lost. The results of an experiment in which the amount of large chain in the preincubation was varied are displayed in Fig. 1. It can be seen that, as the concentration of purified large chain in the preincubation increases, the antibody activity progressively decreases until 50–55% remains at an apparent saturation. The same experiment, with the same antibody, antiholoenzyme, was repeated using the purified small chain of the enzyme to reabsorb the antibody (Fig. 1). It can be seen that, at saturation, the purified small chain can adsorb 40–45% of the antibody activity. When antiholoenzyme was preincubated with saturating amounts of both chains,

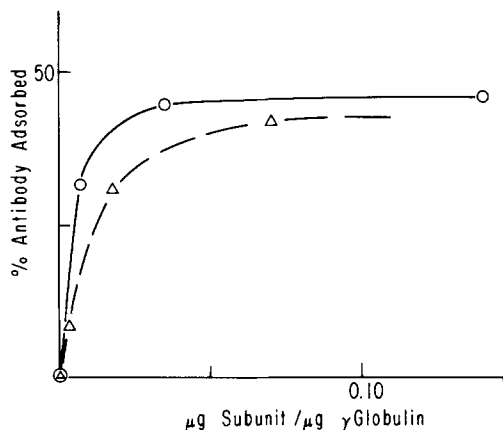


FIGURE 1. Determination of the specificity of the  $\gamma$ -globulins in antiholoenzyme antibody. A solution ( $50 \mu\text{l}$ ) of antiholoenzyme  $\gamma$ -globulin (4.3 mg/ml) was mixed with various amounts of a solution of either the large chain (0.3 mg/ml) (O—O) or the small chain (0.15 mg/ml) ( $\Delta$ — $\Delta$ ) of ( $\text{Na}^+ + \text{K}^+$ ) ATPase to obtain the mass ratios displayed on the abscissa. The mixtures were left at room temperature for 2 h, and the antibody activity remaining was determined by complement fixation against zonal gradient microsomes (27). Antibody was held constant and antigen varied (see Fig. 2, ref. 28). From these data, the percent antibody lost can be calculated. That information is displayed on the ordinate.

greater than 90% of the activity disappeared. These results demonstrate that antiholoenzyme antiserum contains  $\gamma$ -globulins directed against antigenic sites on both the large chain and the small chain of the enzyme. This may be correlated with the ability of this antibody to bind to both sides of the membrane since the small chain is a sialoglycoprotein (26), presumably exposed at the external surface of the cell (39), and the large chain, which spans the membrane (28), contains an antigenic site which is on the cytoplasmic side and which is recognized by anti-large chain antibody (27).

Anti-large chain antibody was obtained from the serum of a rabbit which had been injected with homogeneous, water-soluble large chain. Evidence for the specificity of this antibody has been presented (27, 28). It binds to antigenic sites which are on the large chain of the enzyme and are located on the cytoplasmic side of the plasma membrane (27).

These two antibodies were used as primary ligands to determine specifically the distribution of ( $\text{Na}^+ + \text{K}^+$ ) ATPase over the surface of the plasma membrane of canine distal convoluted

tubule cells. Ferritin-conjugated goat antirabbit  $\gamma$ -globulin was utilized to locate the sites at which the primary antibody was bound.

### *Ferritin Staining*

Ultrathin frozen sections (40, 48) of tissue fragments from canine renal cortex, which had been fixed for 1 h in 2% glutaraldehyde or overnight in 2% formaldehyde, were attached to Formvar-coated grids. The grid and its attached section were floated upsidedown on the surface of a droplet of buffer and were then transferred in succession between droplets of a 10% BSA solution, a solution of antiholoenzyme or anti-large chain  $\gamma$ -globulin, several changes of buffer, 10% BSA, ferritin-conjugated goat antirabbit  $\gamma$ -globulin, several changes of buffer, water, and 0.5% phosphotungstic acid. In this way the fixed sections were first exposed to the primary antibody which was bound by ( $\text{Na}^+ + \text{K}^+$ ) ATPase, and then to ferritin-conjugated goat antirabbit  $\gamma$ -globulin which attached to the primary antibody.

### *Antiholoenzyme*

Fig. 2 is an electron micrograph of an Epon section through a distal convoluted tubule from the cortex of a canine kidney. The basement membrane appears in the lower left, the lumen in the upper right. Intercellular spaces, sandwiched between numerous mitochondria, can be seen throughout the cytoplasm (arrow). Several of these terminate at tight junctions within the field. Fig. 3 is an electron micrograph of an ultrathin frozen section through another distal convoluted tubule from the same kidney cortex. It is presented for comparison. The basement membrane is to the left; lumen, to the lower right. It should be pointed out that, in the present circumstances, membranes appear as white lines (48). The tracework of the intercellular spaces can be observed in several regions (arrow).

When sections such as these, which have been stained with antiholoenzyme, are examined at higher magnification, heavy concentrations of ferritin in ribbon array can be observed in the spaces between the mitochondria in the central regions of the tubule wall (Fig. 4). Although the membranes which form the intercellular spaces cannot be readily distinguished with the negative staining method employed, the patterns which the ferritin assumes are the same as those of these spaces. It can be seen that very little ferritin is located over

the mitochondria themselves. When the antiholoenzyme  $\gamma$ -globulin is preincubated with purified, native ( $\text{Na}^+ + \text{K}^+$ ) ATPase (XAD4 enzyme) (25) at saturating concentrations,<sup>2</sup> most of the ferritin staining is eliminated (Fig. 5). The staining along the intercellular spaces extends uniformly and continuously to the basement membrane (Fig. 6). The arrow indicates a position at which an intercellular space begins. The luminal plasma membrane is also stained by antiholoenzyme antibody (Fig. 7) and this staining is eliminated when the antibody is preincubated with purified ( $\text{Na}^+ + \text{K}^+$ ) ATPase (Fig. 8). These results demonstrate that the antigenic sites, to which antiholoenzyme antibody binds under these conditions, are continuously distributed over the plasma membranes which form the intercellular spaces, and are present at detectable concentrations on the luminal membranes of these cells.

In regions of the section where the plasma membranes which form the intercellular spaces of a convoluted tubule are supported by mitochondria, such that the membranes very likely stand perpendicular to the plane of the grid, it can be seen that the ferritin staining which results from the binding of antiholoenzyme is confined to a very sharp line about 35 nm wide (arrows, Fig. 9). When isolated plasma membranes are liganded with either anti-large chain  $\gamma$ -globulin or antiholoenzyme  $\gamma$ -globulin and ferritin-conjugated goat antirabbit  $\gamma$ -globulin and examined in Epon sections, the outer edge of the ferritin is found to lie 35 nm from the center of the membrane (27). If significant quantities of ferritin were attached to the cytoplasmic surfaces of the two plasma membranes which form the intercellular spaces, the width of the ferritin band in this region should be greater than 80 nm (assuming the thickness of a single plasma membrane to be 10 nm). This lower limit, however, is approached only as the width of the intercellular space goes to zero. This would allow no external attachment of ferritin whatsoever since there would be no room for it. On the other hand, the width of intercellular spaces in glutaraldehyde-fixed, Epon sections of comparable regions is at least 35 nm (Fig. 2). If only this space were filled with ferritin, the band of particles would be about 35 nm wide. Therefore, the most reasonable explanation of the image presented in Fig. 9 is that the majority of the staining with antiholoenzyme antibody is occurring at ( $\text{Na}^+ +$

<sup>2</sup> Determined by complement fixation (30).

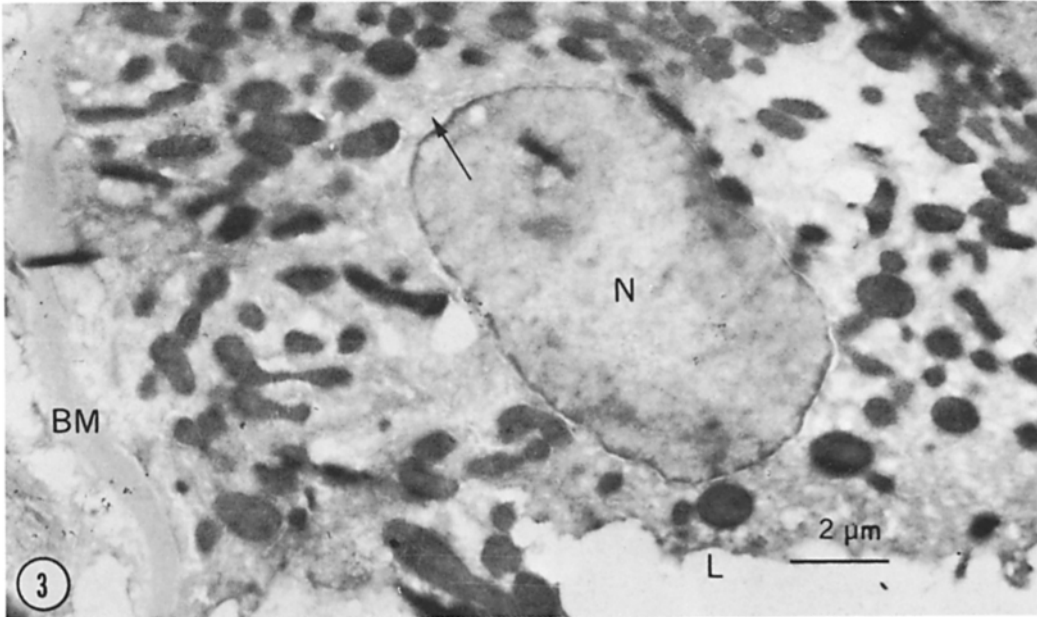
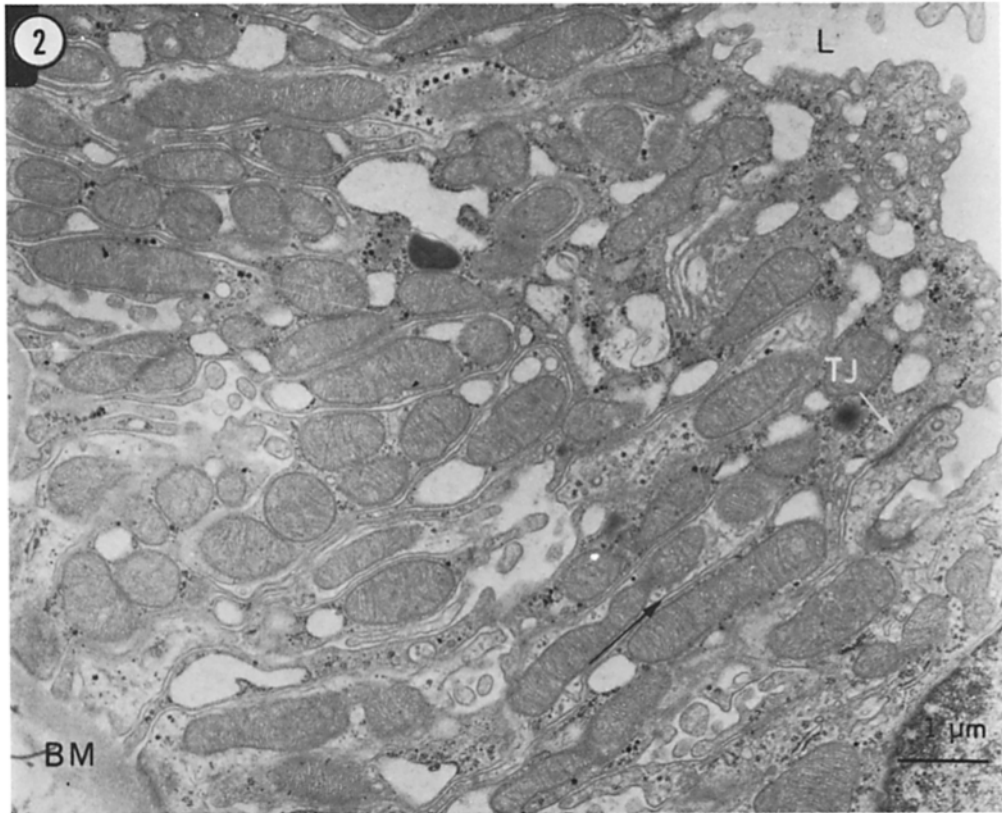


FIGURE 2. Epon section through a distal convoluted tubule from canine kidney. Basement membrane (*BM*), tight junction (*TJ*), and lumen (*L*) are identified. Arrow indicates an intercellular space.  $\times 12,000$ .

FIGURE 3. Ultrathin frozen section through a distal convoluted tubule from the same cortex as that of Fig. 2. Basement membrane (*BM*), nucleus (*N*), and lumen (*L*) are identified. Arrow indicates region in which intercellular spaces can be seen.  $\times 6,400$ .

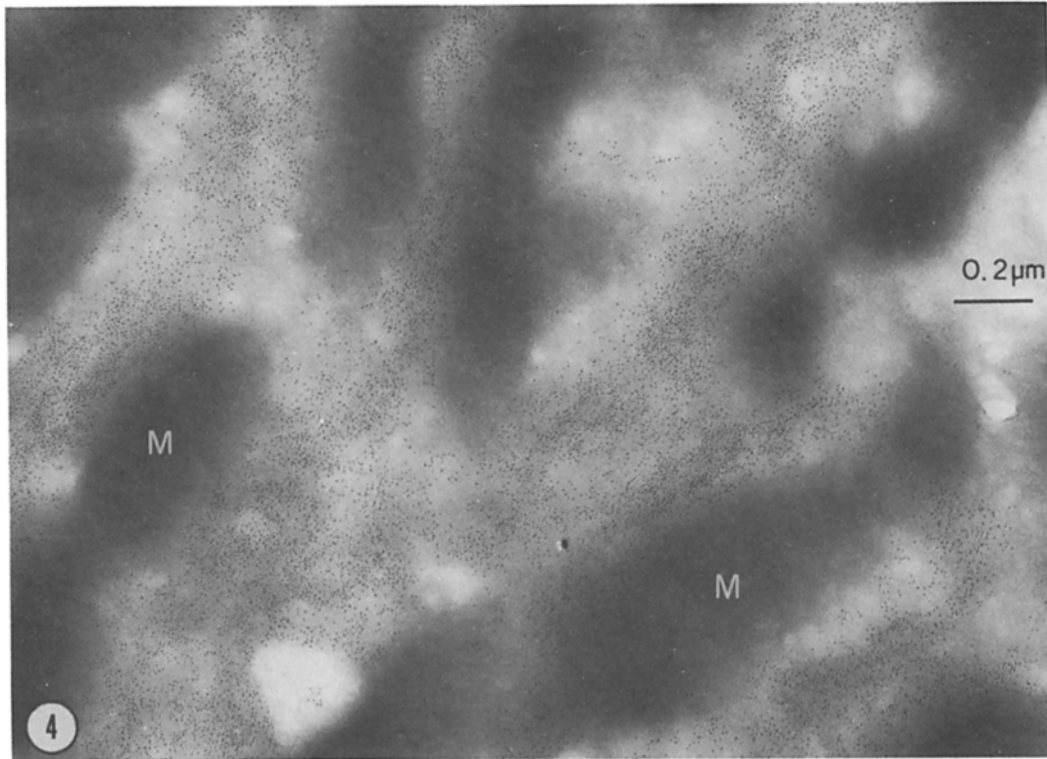


FIGURE 4. Field from the central, cytoplasmic region of distal tubular epithelium in an ultrathin frozen section stained with antiholoenzyme  $\gamma$ -globulin. Mitochondria (*M*) are identified.  $\times 50,000$ .

FIGURE 5. Similar field from a section which has been stained with the same concentrations of  $\gamma$ -globulin and ferritin conjugate as in Fig. 4. Antiholoenzyme  $\gamma$ -globulin, however, was preincubated with purified ( $\text{Na}^+ + \text{K}^+$ ) ATPase.  $\times 50,000$ .

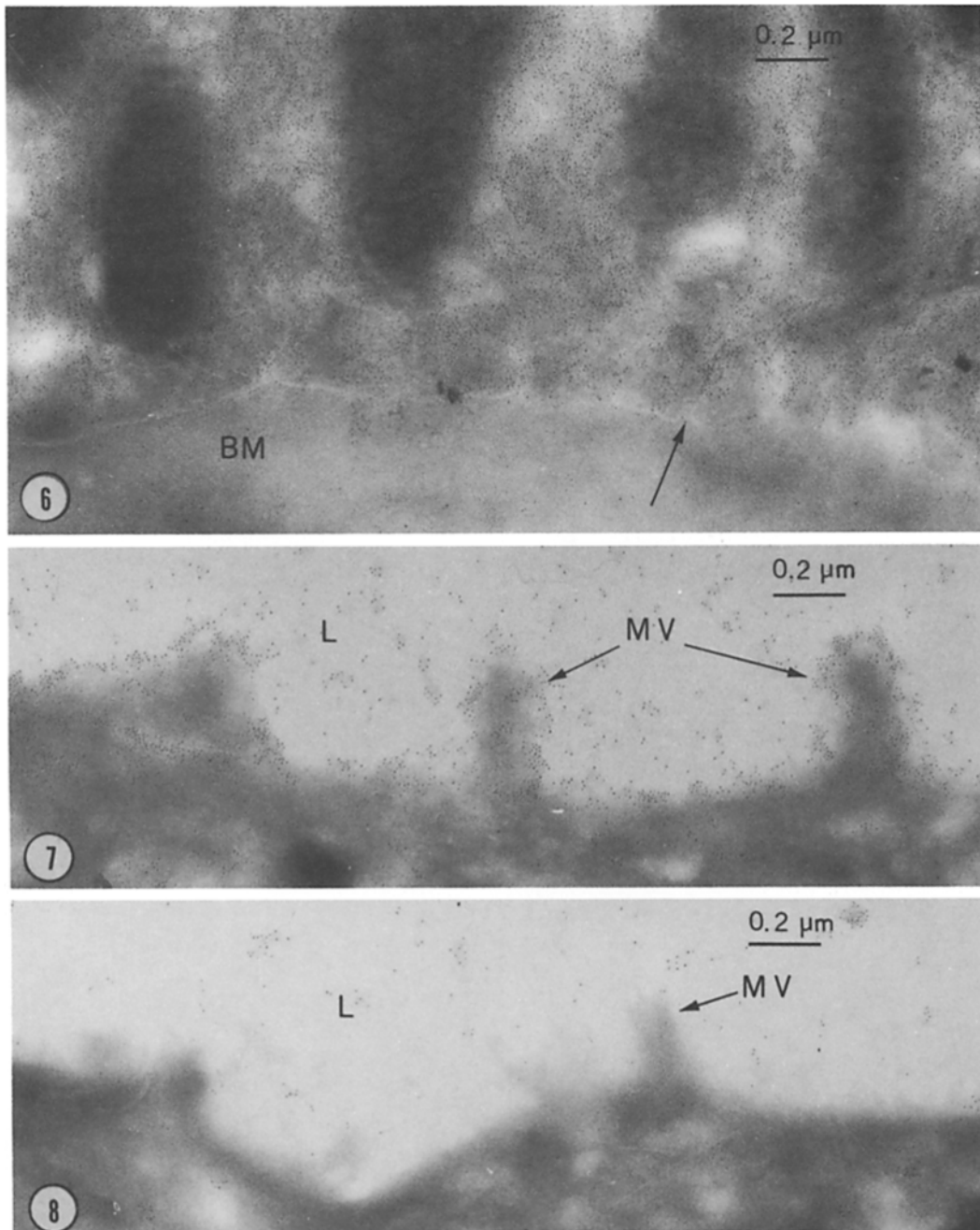


FIGURE 6. Field from the basal region of a distal tubule in an ultrathin frozen section stained with antiholoenzyme  $\gamma$ -globulin. The basement membrane (*BM*) is identified. The arrow marks the basal end of an intercellular space.  $\times 50,000$ .

FIGURE 7. Field from the luminal surface of distal tubular epithelium in an ultrathin frozen section stained with antiholoenzyme  $\gamma$ -globulin. Lumen (*L*) and microvilli (*MV*) are identified.  $\times 50,000$ .

FIGURE 8. Similar field from a section which has been stained with the same concentration of  $\gamma$ -globulin and ferritin conjugate as in Fig. 7. Antiholoenzyme  $\gamma$ -globulin, however, was preincubated with purified ( $\text{Na}^+ + \text{K}^+$ ) ATPase. The lumen (*L*) and a microvillus (*MV*) are identified.  $\times 50,000$ .

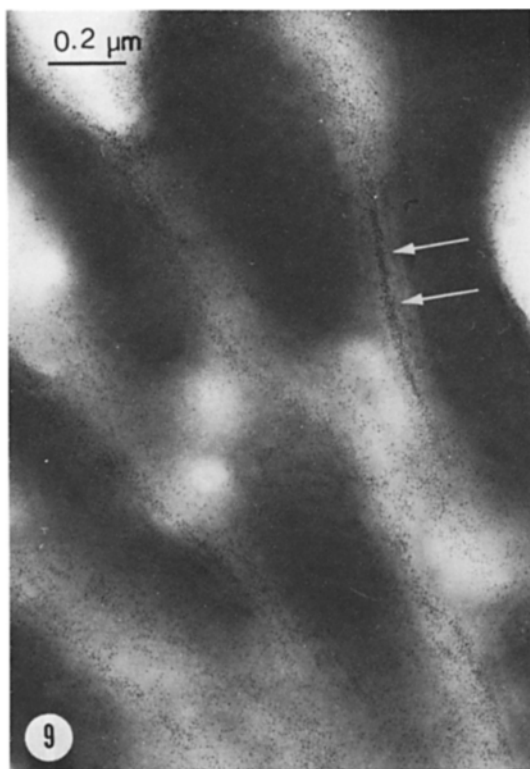


FIGURE 9. Field from the central cytoplasmic region of tubular epithelium in an ultrathin frozen section stained with antiholoenzyme  $\gamma$ -globulin. Arrows indicate a segment of intercellular space oriented normal to the plane of the section.  $\times 50,000$ .

$K^+$ ) ATPase antigenic sites which face out from the exterior surfaces of the membranes.

#### *Anti-Large Chain Antibody*

The antigenic sites on native ( $Na^+ + K^+$ ) ATPase which react with anti-large chain  $\gamma$ -globulin are sensitive to fixation with glutaraldehyde (Fig. 10). When the purified enzyme is treated with 2% glutaraldehyde for 1 h at  $0^\circ C$  about 70% of its antigenicity is lost. These sites are, however, stable to fixation in 2% formaldehyde at  $4^\circ C$  overnight (Fig. 11). In fact, fixation appears to increase their antigenicity slightly.

Canine renal cortex was fixed in 2% formaldehyde overnight, infused with a 30% (wt/wt) sucrose solution 2% in formaldehyde, frozen, and sectioned. When sections from this tissue are stained with anti-large chain  $\gamma$ -globulin, ferritin is found between the mitochondria of the distal tubule epithelium at the positions of the intercellu-

lar spaces (Fig. 12). Little ferritin is found over mitochondria. When the antibody is preincubated with purified, water-soluble large chain, at saturation (see footnote 2), most of the staining is lost (Fig. 13). The circles highlight some of the scattered ferritin particles in this image. It is again observed that ferritin extends along the intercellular spaces all the way to the base of the cells (Fig. 14). A faint white line, marking the lower edges of the cells, can be observed (arrows). The luminal sides of the cells, however, do not stain under these circumstances (Fig. 15). Arrows in this figure indicate the outline of the luminal surface, and a circle points out that only background levels of ferritin can be seen in this region.

These results demonstrate that the antigenic sites recognized by anti-large chain antibody are distributed uniformly over the plasma membranes which form the intercellular spaces but are not detectable on the luminal surface of the cells which constitute the distal tubule.

#### DISCUSSION

Although ferritin-conjugated  $\gamma$ -globulins were introduced more than a decade ago (45), the applica-

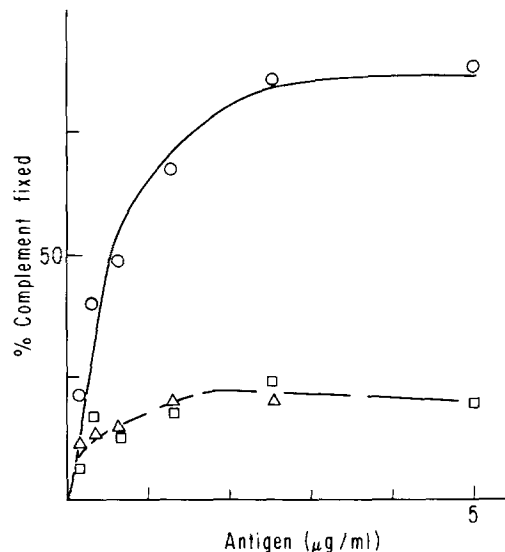


FIGURE 10. Sensitivity of the antigenicity of ( $Na^+ + K^+$ ) ATPase to glutaraldehyde. XAD4 enzyme (25) ( $33 \mu g/ml$ ) in Buffer A was brought to 0% (O—O), 0.5% ( $\Delta$ — $\Delta$ ), or 2.0% ( $\square$ — $\square$ ) in glutaraldehyde. These mixtures stood for 1 h at  $0^\circ C$  and were then dialyzed against complement fixation buffer. They were assayed as antigens, following dialysis, against anti-large chain  $\gamma$ -globulin ( $20 \mu g/ml$ ).



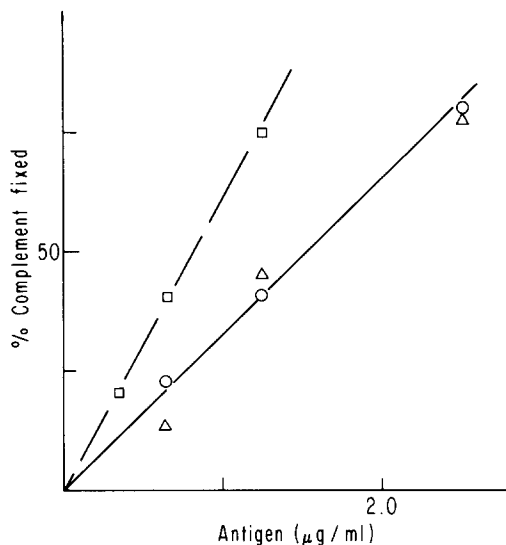


FIGURE 11. Sensitivity of the antigenicity of ( $\text{Na}^+ + \text{K}^+$ ) ATPase to formaldehyde. Salt detergent-extracted microsomes (24) (1.5 mg/ml) in Buffer A were brought to 2% in formaldehyde and incubated at  $0^\circ\text{C}$  for 5 h ( $\Delta$ — $\Delta$ ) or overnight ( $\square$ — $\square$ ) while a control was kept ( $\text{O}$ — $\text{O}$ ) which never saw the reagent. These samples were then dialyzed into complement fixation buffer and assayed as antigens in complement fixation against antiholoenzyme antibody ( $3 \mu\text{g/ml}$ ).

tion of these reagents to the precise determination of antigen distributions inside cells and within animal tissues has been delayed by the problem of assuring access of the conjugate to the antigenic sites under conditions which retain both the ultrastructure of the cell and the capacity of the antigen to bind its specific antibody (35). The introduction of a technique which allows ultrathin sections to be cut from frozen, fixed tissue fragments (48) has greatly improved the prospects of overcoming these difficulties. These sections can be specifically stained with ferritin-conjugated antibodies (40). This report and the one which accompanies it (29) represent the first systematic application of this technique to a particular problem, a determination of the distribution of ( $\text{Na}^+ + \text{K}^+$ ) ATPase over the plasma membranes of the proximal and distal tubular epithelium from the canine renal cortex. Certain considerations of the present limitations of the method itself must be discussed, however, before conclusions which have been reached are examined.

Although the procedure for preparing the sections allows the tissue to be stained in a hydrated

state, prior fixation is required (48). The fixative which is currently most widely employed is glutaraldehyde. Unfortunately, a number of protein antigens, including the large chain of ( $\text{Na}^+ + \text{K}^+$ ) ATPase, are destroyed by this reagent (Fig. 10). Overnight fixation with formaldehyde<sup>3</sup> has been used to prepare the tissue stained with anti-large chain  $\gamma$ -globulin. This fixation procedure retains intracellular ultrastructure and appears to preserve the antigenicity of proteins better than glutaraldehyde (Figs. 10 and 11).

A problem related to that of fixation is steric hindrance to the molecular contact of the antibodies and the antigens. It must be recalled that the cytoplasm of most cells is 30% in protein; furthermore, there is evidence that plasma membranes may have associated networks of contractile proteins on the cytoplasmic surface and a glycocalyx, on the external surface. Aldehyde reagents presumably preserve the structure of tissues by covalently cross-linking all of these proteins, including the membrane-bound polypeptides, into a continuous network. In well-fixed preparations there may be significant steric hindrance to the bimolecular association of a particular antigen, bound within the section, and the antibody which recognizes it. This problem appears to have been encountered in the present experiments. It is known that antiholoenzyme  $\gamma$ -globulin, when it is allowed access to both sides of purified plasma membrane fragments from canine renal medulla, binds to both sides of these membranes (27). Since all of the enzyme molecules in these fragments are oriented in the same direction across the membrane, this result demonstrates that the enzyme itself has unique antigenic sites exclusively located on each side of the membrane. Nevertheless, when tubular epithelium is stained with this antibody, the majority of the ferritin which attaches appears to be bound only to the external surface of the plasma membranes (Fig. 9). This does not result from a difference in sensitivity to glutaraldehyde of the two sides of the membrane since fixation of the purified enzyme with glutaraldehyde does not reduce its overall antigenicity for this antibody.<sup>4</sup> Furthermore, when purified plasma membrane fragments are stained with either antiholoenzyme or anti-large chain  $\gamma$ -globulins, similar densities of ferritin are observed (27). When thin sections which have been

<sup>3</sup> Tokuyasu, K. Personal communication.

<sup>4</sup> Kyte, J. Unpublished observation.

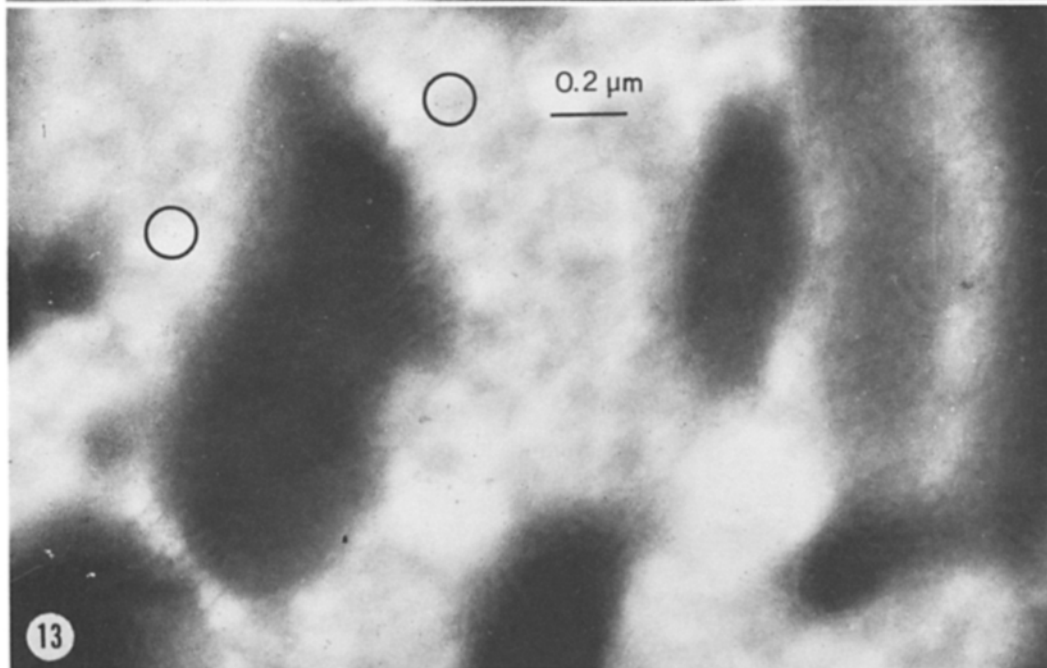
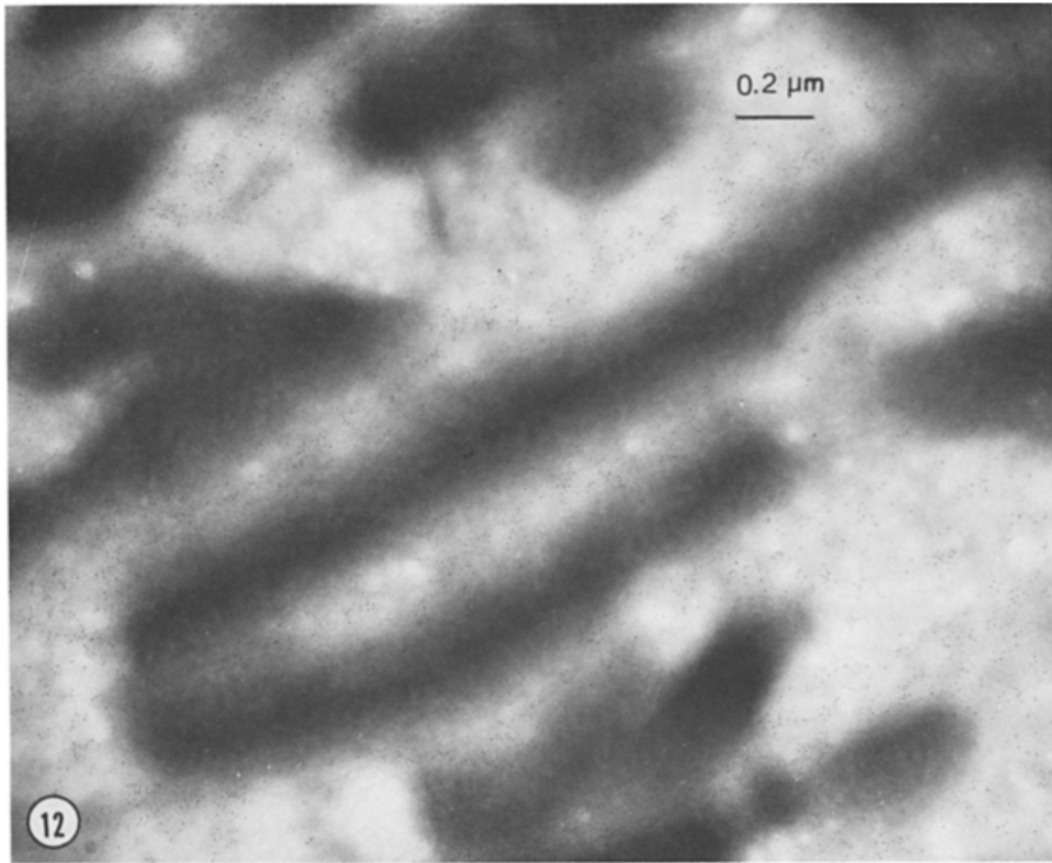


FIGURE 12. Field from the central, cytoplasmic region of distal tubular epithelium in an ultrathin frozen section stained with anti-large chain  $\gamma$ -globulin.  $\times 50,000$ .

FIGURE 13. Similar field from a section which has been stained with the same concentrations of  $\gamma$ -globulin and ferritin conjugate as in Fig. 12. Anti-large chain  $\gamma$ -globulin, however, was preincubated with purified large chain. Circles highlight occasional ferritin.  $\times 50,000$ .

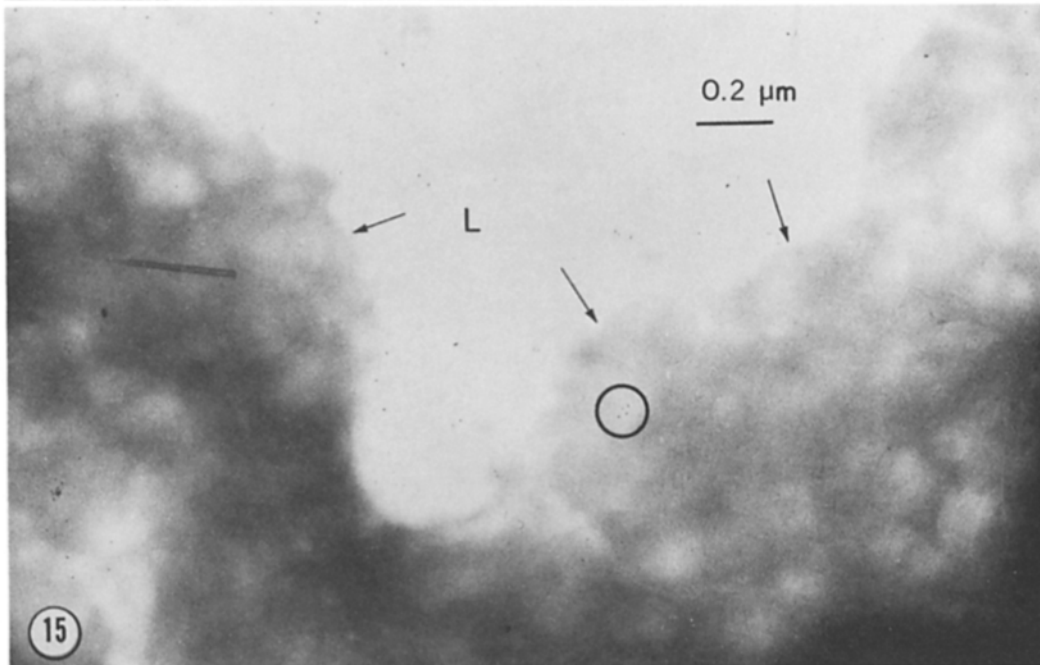
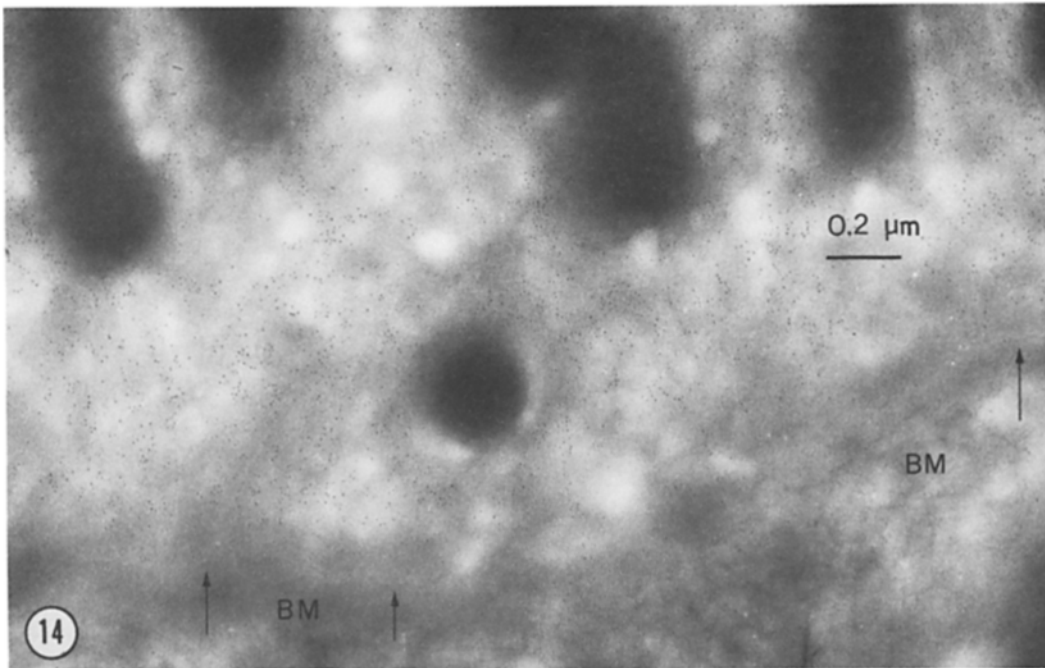


FIGURE 14. Field from the basal region of a distal tubule in an ultrathin frozen section stained with anti-large chain  $\gamma$ -globulin. The basement membrane (*BM*) is identified. Arrows indicate basal boundary of the cells.  $\times 50,000$ .

FIGURE 15. Field of the luminal surface (arrows) of distal tubular epithelium in an ultrathin frozen section stained with anti-large chain  $\gamma$ -globulin. The lumen (*L*) is identified. A circle highlights a cluster of ferritin.  $\times 50,000$ .

stained with these two antibodies are compared, however, those stained with anti-large chain  $\gamma$ -globulin display much lower densities of ferritin staining (Figs. 4 and 12). In fact,  $(\text{Na}^+ + \text{K}^+)$  ATPase is not present at sufficient concentration in the proximal convoluted tubule to elicit staining with this antibody (29). This may be related to the fact that the anti-large chain antibody recognizes antigenic sites which reside exclusively on the cytoplasmic surface of the plasma membrane (27). These comparisons between the staining of ultrathin sections and that of isolated membrane fragments in which  $\gamma$ -globulins have free access to sites suggest that, in the fixed section, there is considerable steric hindrance to the binding of antibodies to antigens at the cytoplasmic surface of the plasma membrane. A similar conclusion has recently been reached in experiments with ultrathin sections of human erythrocytes in which antibodies to spectrin (38) were employed to label this membrane-associated protein located on the cytoplasmic surface (37). No staining could be observed in cells well fixed with formaldehyde. In cells which were poorly fixed, however, so that most of the hemoglobin dissolved away when the frozen section was thawed, strong, specific staining occurred (see footnote 3). These results suggest that the problem of steric hindrance may ultimately be solved by appropriate manipulation of fixation conditions, and experiments of this kind are being carried out in our laboratory. In the experiments reported in this paper, however, this problem was recognized but was not circumvented.

In order to draw conclusions about the relative distribution of  $(\text{Na}^+ + \text{K}^+)$  ATPase over the plasma membranes of tubule cells, comparisons must be made between the densities of different ferritin staining patterns. Even if these comparisons are made within the same cell, they may be complicated by the problems of steric hindrance. There is still another difficulty which arises when quantitative comparisons are attempted. Consider the effect of varying the actual concentration of  $(\text{Na}^+ + \text{K}^+)$  ATPase upon the density of the ferritin labeling expected. As a result of the significant molecular dimensions of the antibody and the ferritin-antibody conjugates and the limitations of steric hindrance, there should be a concentration,  $C_s$ , of  $(\text{Na}^+ + \text{K}^+)$  ATPase above which no further increase in the density of ferritin staining will be observed. It follows that whenever concentrations of  $(\text{Na}^+ + \text{K}^+)$  ATPase in the membrane are greater than  $C_s$ , no discrimination

between regions of different enzyme concentration will be observed when an excess of the reagents is used for staining.

It is possible that where dense ribbons of ferritin are observed in sections of distal tubule stained with antiholoenzyme antibodies (Fig. 4), the concentrations of enzyme in these membranes is greater than  $C_s$ . When anti-large chain  $\gamma$ -globulin is used as primary antibody, a widely spaced ferritin distribution is observed in the same regions. With such sparse staining, differences in density of the ferritin very likely reflect actual differences in the concentration of the enzyme. In other words, the antiholoenzyme antibodies provide a sensitive reagent for the detection of low concentrations of  $(\text{Na}^+ + \text{K}^+)$  ATPase (Fig. 7) because they react with sites on  $(\text{Na}^+ + \text{K}^+)$  ATPase on the external surface of the plasma membrane, but they saturate the sites and are therefore likely to be quantitatively insensitive to differences in enzyme concentration above  $C_s$ . On the other hand, anti-large chain  $\gamma$ -globulins require that high concentrations of  $(\text{Na}^+ + \text{K}^+)$  ATPase be present in order to promote specific staining of the membrane at levels above background, but they allow the observer to discriminate significant differences in the actual concentration of  $(\text{Na}^+ + \text{K}^+)$  ATPase (Figs. 12 and 15).

In order to control for nonspecific adsorption of antibody and obtain some indication of background levels of ferritin adhesion, each  $\gamma$ -globulin was mixed with its antigen before application to the section. Although some ferritin still attaches to the section (Figs. 5, 8, and 13), it is in much lower concentration than in the experimental cases. Furthermore, although significant amounts of ferritin remain in the controls, it is always randomly scattered. On the experimental sections, however, it is distributed in distinct patterns (Figs. 4 and 12). These two points of discrimination, decrease in intensity and loss of pattern, strongly suggest that the staining observed on the experimental grids is due to the presence of antigens associated with  $(\text{Na}^+ + \text{K}^+)$  ATPase. It should be pointed out that although those  $\gamma$ -globulins directed against the enzyme are complexed by their respective antigens during the control preincubation, the majority of the  $\gamma$ -globulins remains. If the staining observed on the experimental grids were due to nonspecific attachment, all  $\gamma$ -globulins should participate and no change would occur upon elimination of only the anti-ATPase antibodies.

The plasma membranes which form the basolat-

eral boundaries of the cells of the distal convoluted tubule and define the intercellular spaces stain uniformly with both antiholoenzyme and anti-large chain antibodies, although much denser with the former (Figs. 4 and 6) than the latter (Figs. 12 and 14). From the discussion presented above, it can be concluded that the concentration of ( $\text{Na}^+ + \text{K}^+$ ) ATPase is high and uniform over these surfaces.

These results are in agreement with recent observations of the distribution of the nitrophenyl phosphatase activity catalyzed by ( $\text{Na}^+ + \text{K}^+$ ) ATPase over the renal cortex (12, 13). In these cytochemical studies, the reaction product of the enzyme is indirectly visualized in plastic-embedded material. It was found that the basolateral plasma membrane of the distal tubule cell was the only location in the renal cortex where ( $\text{Na}^+ + \text{K}^+$ ) ATPase could be detected by this procedure. In this instance, the results obtained with the immunoferritin technique and those obtained with the cytochemical technique reinforce each other. Each method suffers from drawbacks which are overcome by the other. The immunoferritin technique cannot be used to determine whether the antigens which bind the  $\gamma$ -globulin are associated with active enzyme molecules. The cytochemical technique, on the other hand, suffers from the problem of diffusion of the reaction product before precipitation. This difficulty was demonstrated by Marchesi and Palade (34) in their cytochemical study of ( $\text{Na}^+ + \text{K}^+$ ) ATPase in the red cell. Although about 50% of the phosphate deposition under their experimental conditions was the result of ( $\text{Na}^+ + \text{K}^+$ ) ATPase activity, the precipitates were found to be uniformly distributed over the inside surface of glutaraldehyde-fixed ghosts, rather than at the approximately 100 ( $\text{Na}^+ + \text{K}^+$ ) ATPase sites per cell (18). Furthermore, the cytochemical technique requires that a threshold level of ( $\text{Na}^+ + \text{K}^+$ ) ATPase be present before sufficiently high local concentrations of  $\text{P}_i$  are generated to cause precipitation in the vicinity of the enzyme. On the luminal surfaces of the distal convoluted tubule (Fig. 7) and on the plasma membranes of the proximal convoluted tubule (29), significant, but much lower, concentrations of the antigens recognized by the antiholoenzyme  $\gamma$ -globulin could be demonstrated by the immunoferritin technique. No ouabain-sensitive nitrophenyl phosphatase reaction product appears in these locations (12) in cytochemical studies. If the antigens stained are associated with active enzyme

molecules, these results suggest that the immunoferritin procedure has greater sensitivity than the nitrophenyl phosphatase procedure under these circumstances.

The luminal regions of the plasma membranes of the distal tubule epithelial cells contain at least one of the antigens recognized by antiholoenzyme  $\gamma$ -globulin (Fig. 7). Since the small chain of ( $\text{Na}^+ + \text{K}^+$ ) ATPase is a cell surface sialoglycoprotein and must have an externally exposed surface (26, 39) and since antiholoenzyme  $\gamma$ -globulin directs most of the staining to the external surface of the plasma membrane (Fig. 9), the most likely antigen which is recognized on the luminal surface is the small chain of the enzyme. If this is the case, at least the small chain of ( $\text{Na}^+ + \text{K}^+$ ) ATPase is located on the luminal surfaces of the distal tubule cells. Since anti-large chain  $\gamma$ -globulin stains the basolateral surfaces of these cells and not the luminal surfaces (Figs. 12 and 15), it follows that the concentration of ( $\text{Na}^+ + \text{K}^+$ ) ATPase is much smaller on the luminal surfaces, provided the steric hindrance to the cytoplasmic surfaces of the enzyme is equivalent in each of these two regions of the plasma membrane within the same cell. These conclusions, in combination with certain observations which have been made of the physiology of the distal convoluted tubule, allow a more significant analysis to be made of the cation fluxes catalyzed by ( $\text{Na}^+ + \text{K}^+$ ) ATPase in this epithelium than has heretofore been possible. The description given by Giebisch (16) forms the basis for this discussion.

The urine arrives at the distal convoluted tubule with low concentrations of  $\text{Na}^+$  and  $\text{K}^+$ , the major counterion  $\text{Cl}^-$  (5). During its passage through this segment, there is continued net  $\text{Na}^+$  reabsorption from the lumen to the serum. The tubule either absorbs or secretes  $\text{K}^+$ , depending on the dietary state of the animal (33). The major counterions in these net fluxes are  $\text{Cl}^-$  and  $\text{HCO}_3^-$ . All of these net ionic fluxes are eliminated by the addition of ouabain (5, 9, 53). These physiological effects must be explained in terms of the morphology of the distal tubule cells and the complement of membrane-bound enzymes which they have at their disposal.

In order to simplify the situation somewhat, a point should be made with regard to the morphology of the intercellular spaces. When a tubule is sectioned for electron microscope examination, only a two-dimensional image is obtained. Upon cursory examination of a cross-section of either the

distal (Fig. 2) or the proximal tubule, it would be supposed that the basolateral plasma membranes of the cells are structured to produce numerous infoldings. Tormey (49), however, has examined the three-dimensional morphology of the ciliary epithelium, a tissue which displays two-dimensional images very similar to those of the distal and proximal convoluted tubules, and has shown that the patterns actually observed are the result of lateral interdigitations of cytoplasmic processes of adjacent cells. Bulger (4) has reached similar conclusions from an examination of renal convoluted tubules. Photographs of specimens observed by scanning electron microscopy also support this description (36). It follows that the large majority, if not all, of the narrow, membrane-limited spaces observed within the walls of these epithelia are really intercellular spaces, spaces between separate cells of the tissue. All of these spaces are, therefore, continuous with the tight junctions. There are no dead-end infoldings of the basal plasma membrane. As a result, the rather complicated pattern of intercellular spaces within these tubules can be schematically represented by the simple diagram shown in Fig. 16. The system is simply one of an intercellular space bounded above by a tight junction and below by the basement membrane and bounded on each side by the plasma membrane of a different tubule cell. This is the morphological apparatus available. These conclusions are equally valid for both the distal and the proximal (29) tubule.

A basic conclusion which has been drawn from the observations made in the present manuscript is

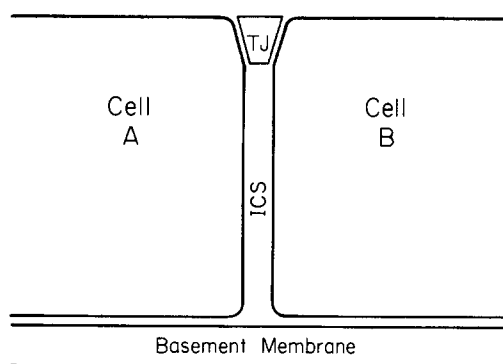
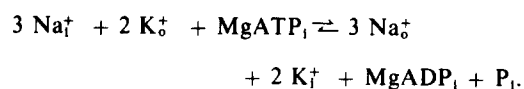


FIGURE 16. Diagrammatic representation of the morphology of cortical tubular epithelium. The intercellular space (ICS) is formed from the plasma membranes of two adjacent cells, the tight junction (TJ) and the basement membrane.

that the intercellular spaces within the distal convoluted tubules are bounded by plasma membrane uniformly paved with  $(\text{Na}^+ + \text{K}^+)$  ATPase. These enzyme molecules are closely associated with the most potent energy source the cell can provide, mitochondria.  $(\text{Na}^+ + \text{K}^+)$  ATPase is the enzyme which catalyzes the following chemical reaction (44):



The subscripts *i* and *o* indicate substrates on the inside or the outside of the cell. The enzyme is oriented in the plasma membrane so that each molecule has a unique cytoplasmic surface and a unique extracellular surface and every enzyme molecule has the same orientation at all times (28). As a result, the enzyme only catalyzes active transport of  $\text{Na}^+$  out of cells and  $\text{K}^+$  into cells (50, 51), never the reverse. All active fluxes, into any cell or across any tissue, which are inhibited by ouabain result from the turnover of this enzyme (10). Whenever the situation has been closely scrutinized, it has always been shown that this enzyme catalyzes the coupled transport of both  $\text{Na}^+$  and  $\text{K}^+$  (42).<sup>5</sup> Although this has not been directly demonstrated in the kidney, the similarity of the structure of the enzyme and its *in vitro* reaction among many species and tissues (2, 3) as well as the universal observation that the stoichiometry of the chemical reactions catalyzed by enzymes never changes, argues that  $(\text{Na}^+ + \text{K}^+)$  ATPase catalyzes the same reaction in the kidney as in other organs. This conclusion is supported by recent immunological experiments. The measurements which were made of the enzyme's stoichiometry utilized human erythrocytes (44), but it has been observed that there is significant immunological cross-reaction between the porcine renal enzyme and the human erythrocyte enzyme (22). Although it had been proposed that certain net  $\text{Na}^+$  fluxes could occur which were not catalyzed by this enzyme, in particular those fluxes inhibited by ethacrynic acid and furosemide (19), it has subsequently been shown that these are due to

<sup>5</sup> Glynn and Karlish (14) have recently described a ouabain-sensitive  $\text{Na}^+$  efflux which is not coupled to  $\text{K}^+$  or  $\text{Na}^+$  influx. This particular flux, however, is completely eliminated at a 5-mM external  $\text{Na}^+$  concentration and, for this reason, would not operate in the renal epithelium.

$\text{Na}^+:\text{Na}^+$  exchange diffusion (31) which does not result in the net flux of  $\text{Na}^+$  (11).

The distribution of  $(\text{Na}^+ + \text{K}^+)$  ATPase which has been observed in the present experiments should generate a significant flux of  $\text{Na}^+$  out of the cell into the intercellular spaces through the membranes lining these spaces. Approximately equivalent amounts of  $\text{K}^+$  must be transported from the intercellular space into the cell by the enzyme at the same time. The basal membrane has been shown to be selectively  $\text{K}^+$  permeable (17, 47). In the steady state, the active flux of  $\text{K}^+$  into the cell catalyzed by the enzyme must be coupled to a passive leak from the cell into the intercellular space. Although it had been proposed that the transport of  $\text{Na}^+$  out of these cells does not require the presence of significant concentrations of  $\text{K}^+$  in the intercellular space (52), when this particular problem was examined more closely it was found that the presence of  $\text{K}^+$  at the serosal surface of the tubule was a requirement for  $\text{Na}^+$  and water flux (41). Electrical measurements are also consistent with the operation of coupled active transport of  $\text{Na}^+$  and  $\text{K}^+$  at this surface (47).

It is the active flux of  $\text{Na}^+$  out of the basolateral side of the cells, catalyzed by  $(\text{Na}^+ + \text{K}^+)$  ATPase, which drives the net reabsorption of  $\text{Na}^+$  across the distal tubule.  $\text{Na}^+$  flows into the cell at the luminal surface down a concentration gradient and is then actively transported out of the cell by this enzyme at the basolateral surface (16). It is the polarity of enzyme distribution (Figs. 12 and 15), the polarity of the  $\text{Na}^+$  permeabilities (17, 47), and the enormous increase in the surface area of plasma membrane of the basolateral surface (Fig. 2) which determine the direction of this net reabsorption. This picture is essentially the one initially proposed by Koefoed-Johnson and Ussing (23).

The observations which were made of the luminal plasma membranes may now be discussed in more detail. These surfaces of the cells are formed from a plasma membrane within which at least the small chain of  $(\text{Na}^+ + \text{K}^+)$  ATPase is located (Fig. 7). It has been shown that  $(\text{Na}^+ + \text{K}^+)$  ATPase is a specific complex of the two polypeptide chains. It is possible covalently to cross-link these two proteins to form an  $\alpha\beta$ -dimer under conditions where no other cross-linked products are observed (26). The enzyme, when purified from several species, always contains both the small and the large chains (20, 21). When the protein is dissolved in Triton X-100, such that it is in a homogeneous

dispersion, both the small and the large chains remain associated (7). Nevertheless, the large chain is more intimately associated with the catalytic function of the enzyme (28) and the possibility exists that the small chain may be present in regions of the plasma membrane which lack the large chain. Lymphocytes, erythrocytes, and endothelial cells in the renal cortex, however, lack detectable quantities of the antigens which are recognized by antiholoenzyme. This observation suggests that the antigens are not indiscriminately distributed. Furthermore, when the complement fixation reactions of crude microsomes from renal medulla and of purified membranes are compared, they coincide when the results are plotted as a function of activity present (27). If significant quantities of antigens were present which were not associated with functional  $(\text{Na}^+ + \text{K}^+)$  ATPase molecules, the crude microsomes should have shown much more extensive complement fixation.

The distal tubule epithelium is able either to secrete or to absorb  $\text{K}^+$  depending upon the dietary state of the animal (33). The net flux of  $\text{K}^+$  across the epithelium is the result of two opposing fluxes across the luminal plasma membrane, the passive diffusion of  $\text{K}^+$  out of the cells into the lumen down a concentration gradient (secretion) and an active uptake from the lumen (absorption). de Mello-Aires et al. (8) have outlined the several pieces of evidence that demonstrate that there is an active, ouabain-inhibited flux of  $\text{K}^+$  from the lumen to the serum in the distal tubule. Since the tight junction in distal tubule is of a high resistance type (32) and  $(\text{Na}^+ + \text{K}^+)$  ATPase never transports  $\text{K}^+$  out of cells (50, 51), it follows that the luminal surfaces of the distal tubule cells must contain  $(\text{Na}^+ + \text{K}^+)$  ATPase. Presumably this is the antigen detected by antiholoenzyme antibody. Since  $\text{K}^+$  fluxes across the tubule into the serum are much less significant than  $\text{Na}^+$  fluxes in this direction, the concentration of  $(\text{Na}^+ + \text{K}^+)$  ATPase on the luminal surface is much less than on the basal portions of the cell (Figs. 12 and 15).

This discussion has concerned itself with the logical connections between the patterns of ferritin which are observed on these specifically stained thin sections of kidney tubules and the various cation fluxes which have been shown to occur across these epithelia. It has been argued that these two sets of information are consistent with each other. The next manuscript in this pair (29) will discuss observations of the distribution of  $(\text{Na}^+ + \text{K}^+)$  ATPase over the plasma membrane of the

proximal tubule cell. Many of the points raised in the present discussion will contribute to an understanding of these latter results.

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