

# TRANSPORT ATP<sub>ASE</sub> CYTOCHEMISTRY: ULTRASTRUCTURAL LOCALIZATION OF POTASSIUM-DEPENDENT AND POTASSIUM-INDEPENDENT PHOSPHATASE ACTIVITIES IN RAT KIDNEY CORTEX

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## ABSTRACT

A cytochemical method for the light and electron microscope localization of the K- and Mg-dependent phosphatase component of the Na-K-ATPase complex was applied to rat kidney cortex, utilizing *p*-nitrophenylphosphate (NPP) as substrate. Localization of K-NPPase activity in kidneys fixed by perfusion with 1% paraformaldehyde-0.25% glutaraldehyde demonstrated that distal tubules are the major cortical site for this sodium transport enzyme. Cortical collecting tubules were moderately reactive, whereas activity in proximal tubules was resolved only after short fixation times and long incubations. In all cases, K-NPPase activity was restricted to the cytoplasmic side of the basolateral plasma membranes, which are characterized in these nephron segments by elaborate folding of the cell surface. Although the rat K-NPPase appeared almost completely insensitive to ouabain with this cytochemical medium, parallel studies with the more glycoside-sensitive rabbit kidney indicated that K-NPPase activity in these nephron segments is sensitive to this inhibitor.

In addition to K-NPPase, nonspecific alkaline phosphatase also hydrolyzed NPP. The latter could be differentiated cytochemically from the specific phosphatase, since alkaline phosphatase was K-independent, insensitive to ouabain, and specifically inhibited by cysteine. Unlike K-NPPase, alkaline phosphatase was localized primarily to the extracellular side of the microvillar border of proximal tubules. A small amount of cysteine-sensitive activity was resolved along peritubular surfaces of proximal tubules. Distal tubules were unreactive. In comparative studies, Mg-ATPase activity was localized along the extracellular side of the luminal and basolateral surfaces of proximal and distal tubules and the basolateral membranes of collecting tubules.

It is now firmly established that the active transport of sodium and potassium across the plasma membranes of most cells is mediated, at least in part, by a ouabain-sensitive, sodium- and potassium-dependent adenosine triphosphatase (Na-K-ATPase). Although biochemical and physiological studies have demonstrated repeatedly the intimate association between Na-K-ATPase and

coupled Na and K exchange across the plasma membrane, the relationship between this enzyme, membrane specialization, and the transepithelial and transcellular routes of Na transport across osmoregulatory epithelia is poorly understood. This is particularly evident in structurally complex tissues such as the mammalian kidney, where the heterogeneity of the cell types comprising the tubular epithelium further complicates interpretation of biochemical and physiological data. Accordingly, the need for a valid cytochemical method for the *in situ* localization of Na-K-ATPase is apparent.

Previous attempts to localize transport ATPase activity by modifications of the Wachstein and Meisel procedure (62) for Mg-ATPase have been unsuccessful (18, 32, 46, 61). Furthermore, the validity of the procedure itself has been challenged (see references 14, 17, 45, and 52 for pertinent discussion and references). Recently we described a new cytochemical approach to this problem, utilizing the K-dependent, ouabain-sensitive phosphatase component of the Na-K-ATPase enzyme complex to localize this transport enzyme in avian salt glands (14, 15). Biochemical studies (14), employing *p*-nitrophenyl phosphate (NPP) as substrate, indicated that this cytochemical procedure was kinetically sound, and cytochemical results at the ultrastructural level (15) revealed ouabain-sensitive, K-dependent NPPase activity along the basolateral plasma membranes of this secretory epithelium. The purpose of the present investigation is to show the applicability of this procedure to the localization of the transport ATPase complex in rat kidney cortex. Cytochemical results indicate that the basolateral plasma membranes of cortical distal and, to a lesser extent, collecting tubules are major sites of K-NPPase activity, whereas the proximal segments are only weakly reactive. A preliminary report of these studies was presented previously (16).

## MATERIALS AND METHODS

### *Preparation of Tissue*

Initial cytochemical experiments were conducted on rat kidney cortex fixed by immersion for 45 min with 3% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.5. Previous studies (14, 17) indicated that this fixation procedure maintained reasonable structural preservation with only slight effect on Na-K-ATPase and K-NPPase activities. Although useful cytochemical data were obtainable with immersion-fixed kid-

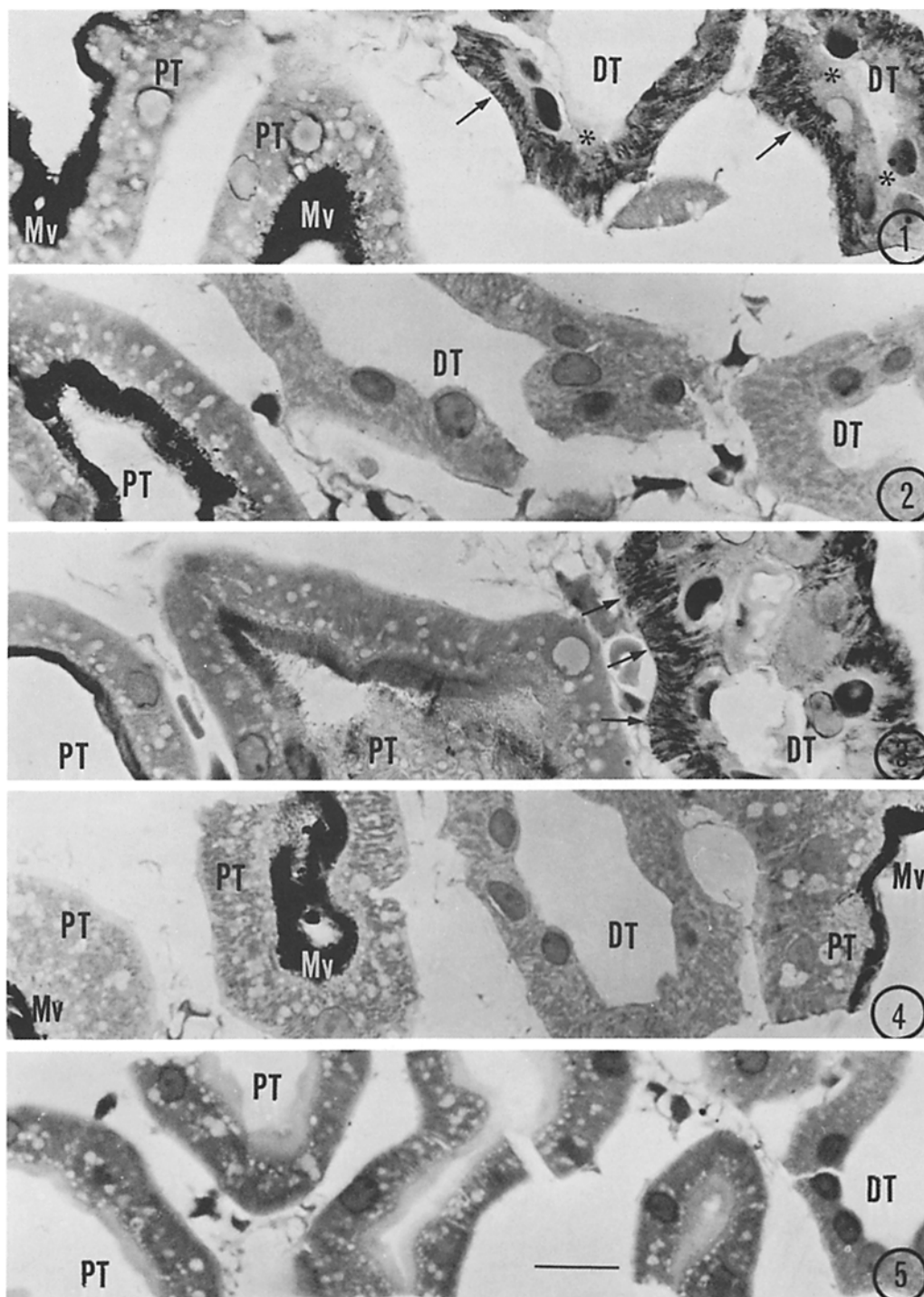
neys, structural preservation was quite poor. Renal perfusion with formaldehyde resulted in only a marginal improvement in preservation. Preliminary studies (16) indicated, however, that good structural and enzymatic preservation could be obtained by brief perfusion of kidneys with a mixture of 1% paraformaldehyde-0.25% glutaraldehyde.

Albino rats of either sex (200–300 g) were anesthetized with Nembutal, and the abdominal cavity was exposed. A retrograde perfusion (42) was initiated via the abdominal aorta by using a model 907 Holter Infusion Pump (Extracorporeal Medical Specialities, Inc., King of Prussia, Pa.) with a flow rate of 1.7 ml/min. Immediately after cannulation of the abdominal aorta, the inferior vena cava was cut and the aorta was clamped above the origin of the renal arteries. The renal artery and vein of the right kidney were then ligated. The left kidney was perfused for 1 min with cold physiological saline followed by a 10-min perfusion with cold 1% paraformaldehyde-0.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5. In some experiments, kidneys of albino rabbits (4–5 kg) were fixed for 10 min by perfusion via the renal artery. Fixed kidneys were quickly excised, and portions of the cortex were cut into small blocks (3 mm<sup>3</sup>), rinsed in cacodylate and Tris-HCl buffers as described previously (15), and frozen in an International cryostat (International Equipment Company, Needham Heights, Mass.) for immediate use.

### *Cytochemical Procedures*

Cryostat sections (50  $\mu$ m) of kidney cortex were incubated in the phosphatase medium described previously for the cytochemical localization of K-NPPase activity in the avian salt gland (15). The incubation medium, which was modified somewhat for kidney cytochemistry, contained (final concentrations): 5–20 mM disodium or diTris NPP, 20 mM MgCl<sub>2</sub>, 10–30 mM KCl, 5–40 mM SrCl<sub>2</sub> and 250 mM Tris-HCl buffer, pH 9.0. Incubations were performed at room temperature and at 30°C for 15–60 min.

After the incubation, the sections were rinsed with 0.1 M Tris buffer, pH 9.0, and then treated with 2% Pb(NO<sub>3</sub>)<sub>2</sub> to convert precipitated strontium phosphate to lead phosphate for visualization in the electron microscope. The sections were then washed and postfixed for 15 min with 1% OsO<sub>4</sub> as described previously (15). Some sections were treated after osmication with 1% (NH<sub>4</sub>)<sub>2</sub>S to convert lead phosphate to lead sulfide for examination in the light microscope. Osmicated sections were dehydrated in ethanol and embedded in the low viscosity resin of Spurr (60). Sections for light microscopy (1  $\mu$ m) were cut on a Porter-Blum MT-2 ultramicrotome (DuPont Instruments, Sorvall Operations, Newtown, Conn.) and lightly stained with methylene blue. Sections for electron microscopy were cut with diamond knives and examined, either unstained or double stained with uranyl acetate



and lead citrate (51), in a Philips 300 or a JEOL 100 B electron microscope at an operating voltage of 40 or 60 kV.

### Cytochemical Controls

The following controls were carried out to substantiate the specificity of the cytochemical procedure.

(a) Since the phosphatase component of the Na-K-ATPase complex has an obligatory requirement for K and Mg (14), the effect of deletion of either of these cations from the medium was assessed.

(b) The effect of addition of 10 mM ouabain to the phosphatase medium was tested. This cardiac glycoside is known to inhibit K-NPPase activity specifically (see reference 14).

(c) Since kidney cortex contains appreciable quantities of nonspecific alkaline phosphatase, an enzyme which is also capable of hydrolyzing NPP, it was important to differentiate clearly staining due to this enzymatic activity from that derived from the specific phosphatase. Such a differentiation was achieved by showing that sites of alkaline phosphatase activity were insensitive to the absence of K or to the presence of ouabain in the medium and were sensitive to the addition of 10 mM cysteine to the medium. Cysteine is a potent inhibitor of alkaline phosphatase (24, 47). Finally, K-independent, cysteine-sensitive alkaline phosphatase activity was selectively demonstrated by substituting  $\beta$ -glycerophosphate for

NPP, since glycerophosphate is not a substrate for the specific phosphatase (14, 20).

(d) In order to compare sites of enzymatically produced precipitates with those produced nonenzymatically, substrate was deleted from the medium. In addition, the artifact experiments originally suggested by Gillis and Page (22) were employed as described previously (15). Sections were soaked in a substrate-deficient medium containing the heavy metal capture ion and subsequently incubated after addition of inorganic phosphate (or vice versa). Postincubatory treatment was the same as that described above for tissues incubated in the complete medium.

(e) For comparative purposes, sections of kidney cortex were incubated in the Wachstein-Meisel medium (62) for the cytochemical location of Mg-ATPase. In some of these experiments, Mg was deleted from the medium or was replaced by calcium or strontium ions.

## RESULTS

### Light Microscopy

In the presence of K, NPPase activity was localized to the microvillar border of cortical proximal tubules, whereas staining in distal tubules was restricted to the basal portions of the tubular epithelium (Fig. 1). Close examination of Fig. 1 indicates that distal tubule staining exhibits a

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FIGURES 1-5 Light microscope localization of phosphatase activity in rat kidney cortex, fixed by perfusion for 10 min with 1% paraformaldehyde-0.25% glutaraldehyde. Cryostat sections (50  $\mu$ m) were incubated for 30 min at room temperature in a medium containing 20 mM NPP (Figs. 1-3) or 20 mM  $\beta$ -glycerophosphate (Figs. 4, 5), 20 mM MgCl<sub>2</sub>, 30 mM KCl, 20 mM SrCl<sub>2</sub>, and 250 mM Tris-HCl buffer, pH 9.0. In some cases, K was deleted from the medium or 10 mM cysteine was added to the medium. After incubation, the sections were rinsed in buffer, treated with 2% Pb(NO<sub>3</sub>)<sub>2</sub>, rinsed and osmicated as indicated in the text. For light microscope visualization, precipitated PbPi was converted to PbS by treatment of osmicated sections with 1% (NH<sub>4</sub>)<sub>2</sub>S. The tissue was then embedded in the resin of Spurr (60), and 1- $\mu$ m sections were prepared and counterstained lightly with methylene blue. PT, proximal tubules; DT, distal tubules. Scale marker for micrographs is shown in Fig. 5 and equals 10  $\mu$ m.

FIGURE 1 In the presence of K, NPPase activity is localized to the microvillar border (*Mv*) of PT and to the basal region of the epithelial cells comprising DT. The striated appearance of DT staining (arrows) is apparent. With the exception of the microvillar border, PT appear unstained as do the apical cytoplasm (asterisks) and the luminal surfaces of DT.  $\times$  1,200.

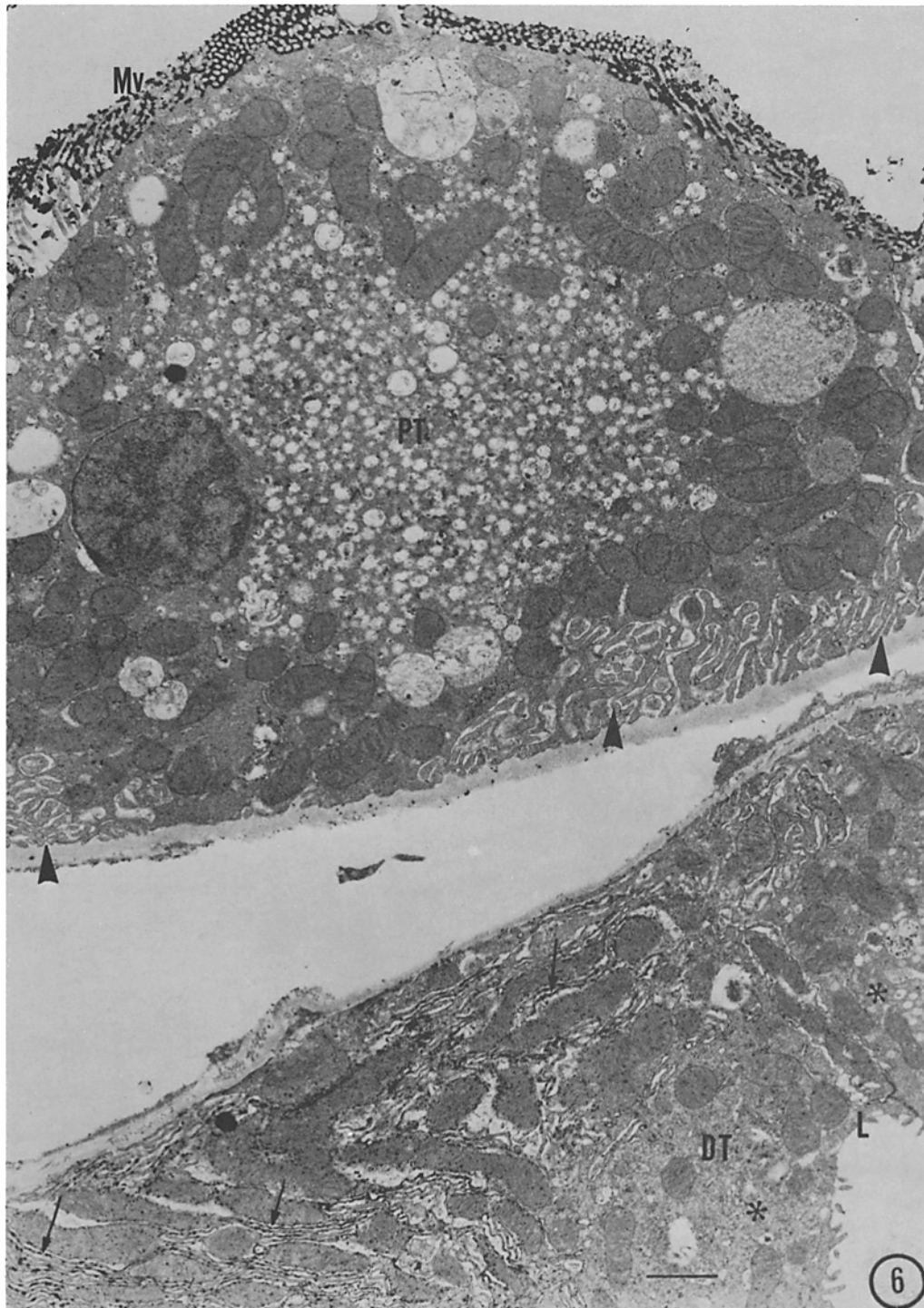
FIGURE 2 When K is deleted from the medium, NPPase activity of DT is abolished, whereas staining of PT is unaffected.  $\times$  1,200.

FIGURE 3 When cysteine is added to the K-containing medium, staining of PT is greatly reduced whereas the striated appearance (arrows) of the K-dependent DT staining is unaffected.  $\times$  1,200.

FIGURE 4 Only the microvillar border (*Mv*) of PT stains when  $\beta$ -glycerophosphate is substituted for NPP in the K-containing medium.  $\times$  1,200.

FIGURE 5 The phosphatase activity of PT seen in Fig. 4 with  $\beta$ -glycerophosphate as substrate is inhibited when cysteine is added to the medium.  $\times$  1,200.





striated pattern, reflecting a localization to the highly folded basal plasmalemma which produces the striated appearance of these tubules when viewed in conventional light microscope preparations. Distal tubules were unstained when K was deleted from the medium, whereas proximal tubule staining was unaffected (Fig. 2). In contrast, when 10 mM cysteine was added to the K-containing medium in order to inhibit alkaline phosphatase, microvillar staining of proximal tubules was greatly reduced, while staining of distal tubules was unaffected (Fig. 3). The small amount of residual proximal tubule phosphatase activity observed in the presence of cysteine was not affected further by deleting K from the medium, whereas the distal reaction was abolished. When  $\beta$ -glycerophosphate (a substrate for alkaline phosphatase, but not for the specific K-dependent phosphatase) was substituted for NPP in the K-containing medium, only proximal tubules were stained (Fig. 4). Deletion of K from this medium had no effect on stain deposition, whereas addition of cysteine (Fig. 5) completely inhibited microvillar staining. These results indicate, therefore, that at the level of the light microscope, Na-K-ATPase, as reflected by its K-dependent, cysteine-insensitive phosphatase activity, is restricted to the basal region of distal tubules and may be differentiated from the K-independent, cysteine-sensitive alkaline phosphatase activity associated with the microvillar border of proximal tubules.

#### Electron Microscopy

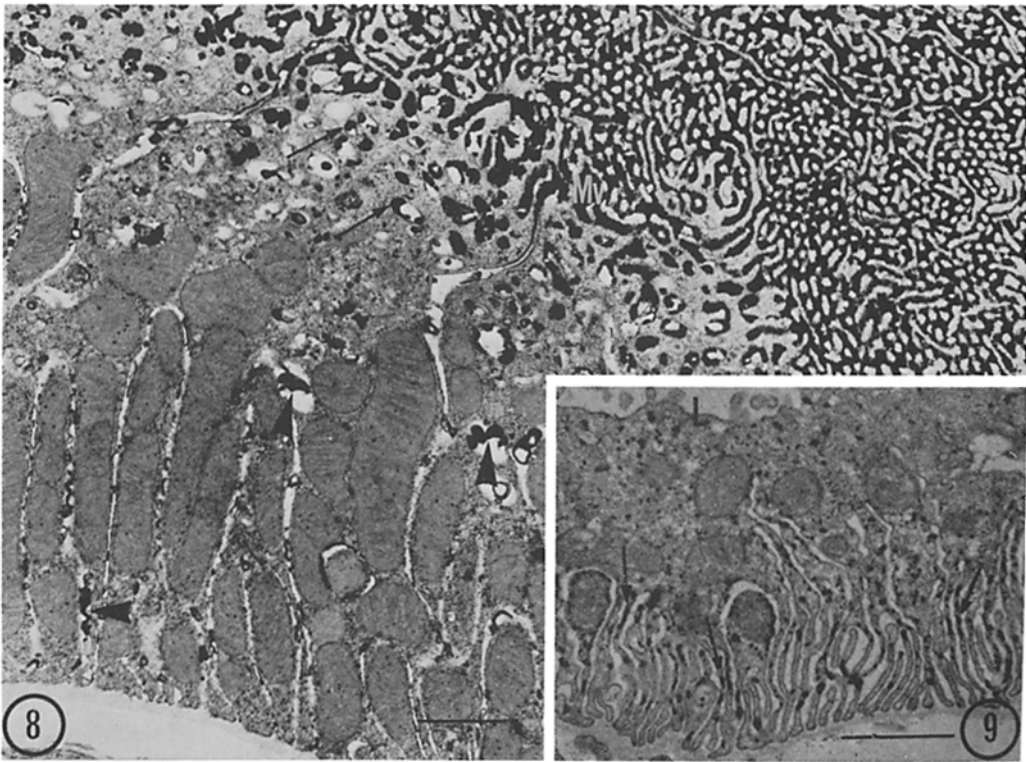
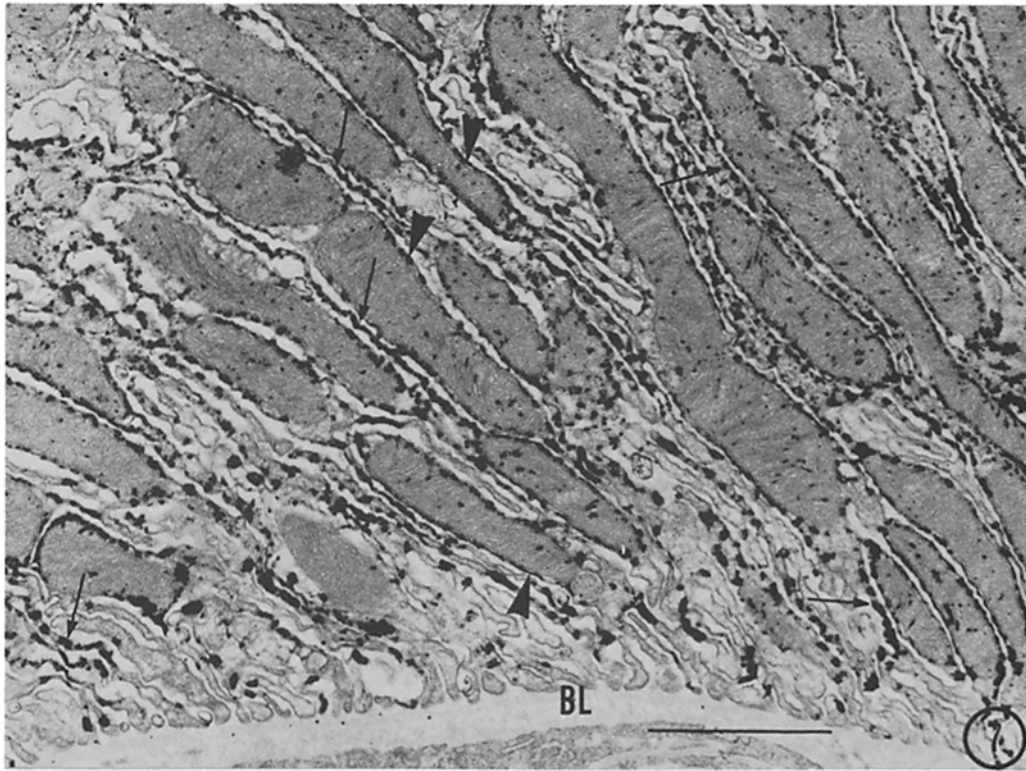
The pattern of stain deposition seen at the level of the electron microscope paralleled that observed by light microscopy. In the presence of K, NPPase

activity was localized differentially to the microvillar border of proximal tubules and to the basolateral folds of distal tubules (Fig. 6). All segments (pars convoluta, pars radiata) of cortical proximal and distal tubules were stained in a similar manner, although some variation in density of proximal staining was observed. Reaction product lined the cytoplasmic side of the basal plasma membrane folds of distal tubules (Fig. 7) and of cortical collecting tubules (Fig. 9) and the extracellular side of the microvillar border of proximal tubules (Fig. 8). Some collecting tubules were unreactive, particularly in cells where basal membrane amplification was poorly developed or absent. The luminal surfaces of distal (Figs. 6 and 12) and collecting (Fig. 9) tubules were generally unstained. Little reaction product was associated with the basal folds of the proximal tubular epithelium at short incubation times (15 min) with 20 mM NPP at room temperature (Fig. 6) or 5 mM NPP at 30°C (Fig. 8). Incubation with 20 mM NPP at 30°C, however, did reveal some precipitate along these surfaces, and this staining could be increased by incubation for longer periods (45–60 min) at room temperature (see below).

The rate of stain deposition along the microvillar border of proximal tubules was greater than that observed along the basal membranes of distal segments (Fig. 6). Accordingly, the most precise localization of proximal tubule staining was obtained with short incubations (15 min) under conditions ensuring a relatively low rate of substrate hydrolysis (e.g. 5 mM NPP at room temperature or 30°C; 20 mM NPP at room temperature). Distal tubule staining was seen to greater advan-

FIGURES 6–22 Electron microscope localization of phosphatase activity in rat kidney cortex. Except as otherwise noted, 50- $\mu$ m cryostat sections of kidney cortex, fixed by perfusion for 10 min with 1% paraformaldehyde-0.25% glutaraldehyde, was incubated in a standard cytochemical medium (20 mM NPP, 20 mM MgCl<sub>2</sub>, 30 mM KCl, 20 mM SrCl<sub>2</sub>, and 250 mM Tris-HCl buffer, pH 9.0) at the temperature and timed indicated. Additions to and deletions from the medium are noted. Except for treatment with (NH<sub>4</sub>)<sub>2</sub>S, postincubatory procedures were the same as described in the legend for Figs. 1–5. Thin sections were counterstained with lead citrate (51) and uranyl acetate. PT, proximal tubules; DT, distal tubules. Scale marker indicates 1  $\mu$ m.

FIGURE 6 In the presence of K, NPPase activity is restricted to the microvillar border (Mv) of PT and the basal plasma membrane folds (arrows) of DT. The apical cytoplasm (asterisks) and luminal surfaces (L) of the DT epithelium are devoid of precipitate. Cytoplasmic areas and basal plasma membrane folds (arrowhead) of PT are unstained. The staining pattern seen here at the electron microscope level mirrors that observed with light microscope preparations (see Fig. 1). 40 mM SrCl<sub>2</sub>; incubation was for 15 min at room temperature.  $\times$  10,000.



tage with longer incubations (30–60 min) at either temperature and with higher substrate concentrations (20 mM NPP). With the longer incubation times, microvilli of proximal tubules were generally overstained. Precise deposition of reaction product was somewhat better resolved in both nephron segments with 20–40 mM strontium than with lower concentrations. Substitution of the disodium salt of NPP for the diTris salt had little effect on staining. Varying the K concentration in the medium from 10 to 30 mM also had no observable effect.

Stain deposition in proximal tubules was independent of K (Fig. 10). In contrast, staining of distal tubules (and collecting tubules) was reduced sharply by deleting K from the phosphatase medium (Fig. 10). Similarly, removal of Mg from the medium led to a loss of distal tubule staining (Fig. 11), but had little effect on the proximal tubule reaction. Alternatively, addition of cysteine to the complete phosphatase medium resulted in differential inhibition of stain deposition along the microvillar border of proximal tubules, but was without effect on distal tubule staining (Fig. 12). As expected, phosphatase activity was greatly reduced or absent in all nephron segments after incubation in a K-deficient medium containing cysteine. In the absence of cysteine, long incubation times (45–60 min) at room temperature led to a rather poorly localized deposition of precipitate associated with proximal tubular basal folds and to overstained microvilli (Fig. 13). However, staining at both sites was markedly reduced or eliminated by cysteine (Fig. 14).

The absence of appreciable K-dependent

NPPase activity in proximal tubules was surprising considering that biochemical studies (53) have shown that Na-K-ATPase is present in this nephron segment, although at relatively low levels of specific activity. It seemed likely, therefore, that the enzyme was not present in sufficient amounts to be resolved by the phosphatase procedure. Since fixation with glutaraldehyde markedly inhibits Na-K-ATPase activity (17), an attempt was made to maintain higher levels of the phosphatase activity by modifying the method of fixation. Initial experiments indicated that some K-dependent, cysteine-insensitive activity could be demonstrated in proximal tubules of kidneys fixed by immersion in 1%–3% paraformaldehyde, a fixative which maintains high levels of Na-K-ATPase and NPPase activities (14, 17). However, structural preservation was inadequate to permit unequivocal interpretation of cytochemical data. In contrast, reduction of the perfusion time with 1% paraformaldehyde-0.25% glutaraldehyde from 10 min to 2.5 min revealed moderate K-dependent, cysteine-insensitive phosphatase activity along the basal folds of the proximal tubules (Figs. 15, 16). The flattened peritubular surfaces of the more terminal proximal segments were, however, generally unstained. Distal and collecting tubules were heavily stained. Since structural preservation was remarkably good, this fixation protocol is preferable to the longer perfusion times.

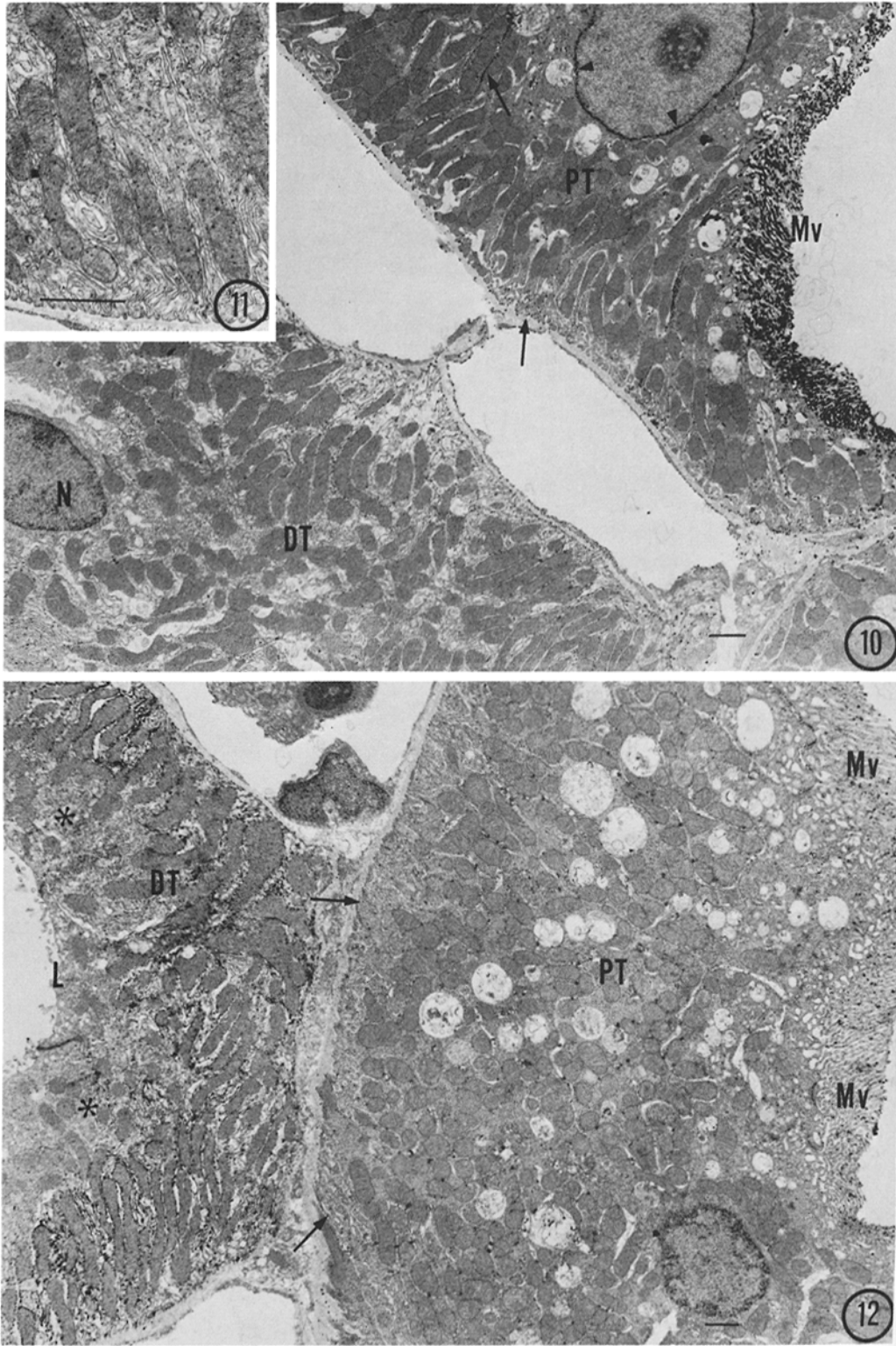
K-dependent NPPase activity in other tissues is sensitive to ouabain when assayed biochemically (14, 20, 66) or cytochemically (11, 15, 36). Repeated attempts, however, to show that the K-NPPase reaction product in rat kidney cortex was

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FIGURE 7 Higher magnification of the basal area of a DT similar to that shown in the preceding figure. Reaction product is localized almost exclusively to the cytoplasmic side of the highly folded plasmalemma (arrows). Note that except for a few focal deposits, precipitate is excluded from the mitochondria even where plasma membrane deposits are closely applied to the outer mitochondrial membrane (arrowheads). *BL*, basal lamina. Incubation was for 15 min at 30°C.  $\times 24,300$ .

FIGURE 8 In the presence of K, NPPase activity of PT is restricted to the extracellular side of the microvillar border (*Mv*) and to the apical pits (arrows). Little, if any, stain deposition occurs along basal folds of the plasma membrane. The materials marked by arrowheads are myelin figures and should not be confused with reaction product. 5 mM NPP, 10 mM  $MgCl_2$ ; incubation was for 15 min at 30°C.  $\times 10,900$ .

FIGURE 9 This micrograph shows a portion of a cortical collecting tubule reacted for NPPase activity in the presence of KCl. Selective staining of the cytoplasmic side of the basal folds of the plasma membrane (arrows) is identical to that observed in DT; however, the amount of reaction product is less than that observed in DT. A few focal deposits are present in the apical cytoplasm; the luminal surface (*L*) is unreacted. 10 mM KCl; incubation time was for 60 min at room temperature.  $\times 14,900$ .



sensitive to ouabain gave inconsistent results or were unsuccessful altogether. Negative results were obtained even in the presence of low concentrations of K and of Sr (cations known to reduce inhibition of Na-K-ATPase or K-NPPase by ouabain in other systems [14, 58]) or in tissues preincubated with ouabain before incubation in the phosphatase medium. Inhibition of K-NPPase by ouabain in cortex fixed by immersion in 3% paraformaldehyde was also inconsistent. Since rat kidney is notably insensitive to cardiac glycosides (2), a comparative study was initiated with the more glycoside-sensitive rabbit kidney (3). As was the case with the rat kidney, the basal folds of the rabbit distal tubular cells were highly reactive sites for NPPase activity (Figs. 17, 18). When 10 mM ouabain was included in the K-containing phosphatase medium, deposition of reaction product in distal tubules (and collecting tubules) was markedly reduced (Fig. 19). As expected, ouabain had no effect on microvillar staining of proximal tubules.

The high degree of membrane specificity of the K-dependent and ouabain-sensitive phosphatase is particularly well demonstrated in Fig. 17. In this micrograph, several unreacted intercalated cells with flattened basolateral plasma membranes are seen interspersed between the highly reactive principal cells which comprise the bulk of the early distal convoluted tubular epithelium. Distal tubular intercalated cells, first described in the rat (26), were observed more frequently in the rabbit. Intercalated cells with well-defined basal folds were often moderately reactive in both species.

When  $\beta$ -glycerophosphate was substituted for NPP in the K-containing phosphatase medium, only proximal tubules stained (Fig. 20). The microvillar border stained heavily, whereas a lighter reaction product was localized along the basal folds. The latter staining pattern parallels that observed after long incubation times with NPP as substrate (Fig. 13). Deletion of K or Mg from the glycerophosphate-substituted medium had no effect on proximal tubule staining, whereas the addition of cysteine to the medium abolished this activity (Fig. 21).

Since mitochondria and particularly nuclei showed varying amounts of precipitate with the NPPase medium (see Figs. 10, 12, 18), it was important to determine whether these sites were capable of binding nonenzymatically produced precipitate. Few deposits were seen when substrate was deleted from the medium. When sections were preincubated in a substrate-deficient medium containing 20 mM Sr and then postincubated after addition of 10 mM  $\text{KH}_2\text{PO}_4$ , fine deposits were seen in mitochondria and nuclei and heavier staining was observed within the nuclear envelope of many, but not all, nuclei and within the cisternae of the endoplasmic reticulum (Fig. 22). Few deposits were associated with the plasma membrane in contrast to the preferential staining of membranes produced by enzymatic hydrolysis of substrate. Accordingly, the variable staining of nuclei and nuclear membranes seen in the presence of substrate (e.g., Figs. 10, 12, and 18) is probably artifactual. A similar conclusion was reached in the previous study with this method (15) and is

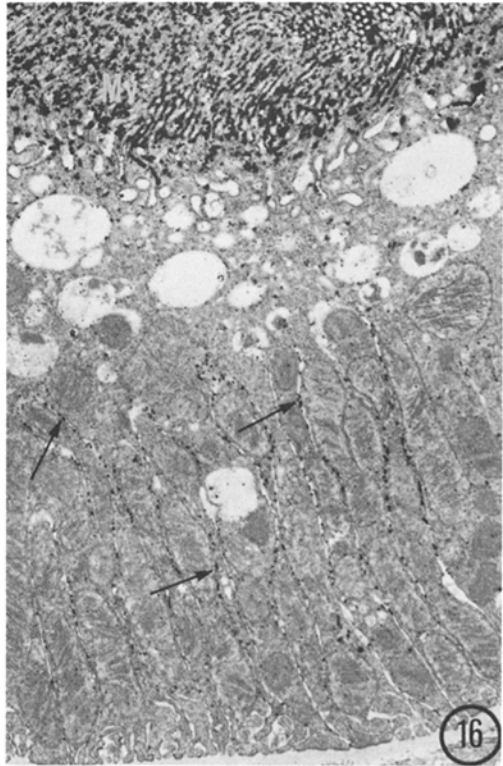
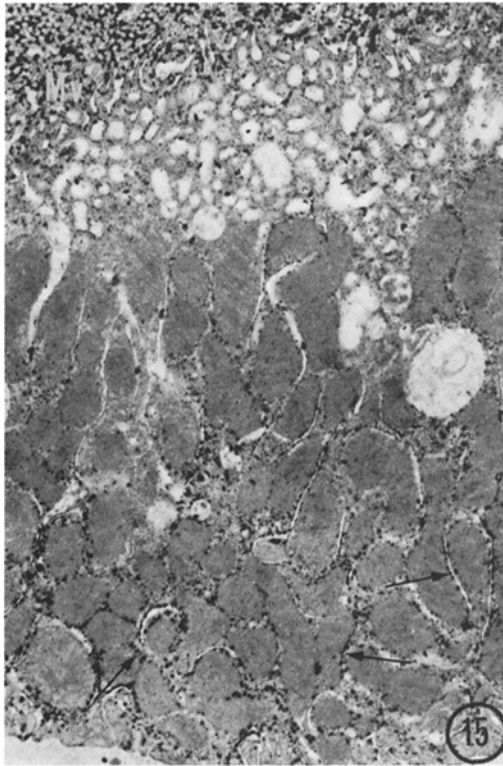
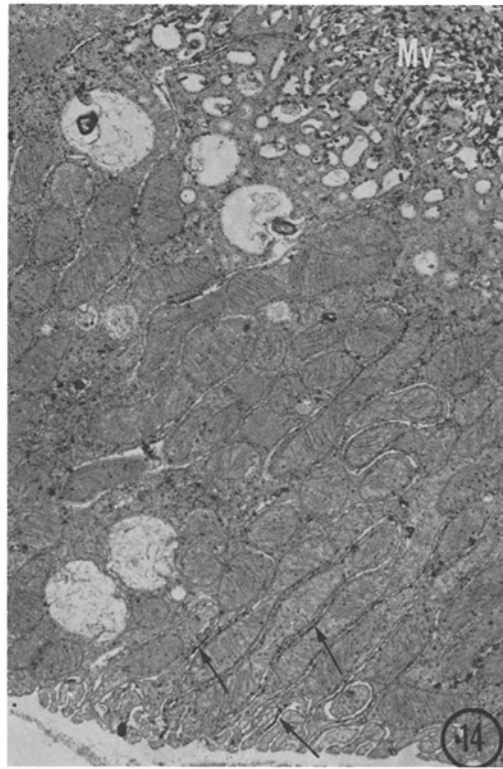
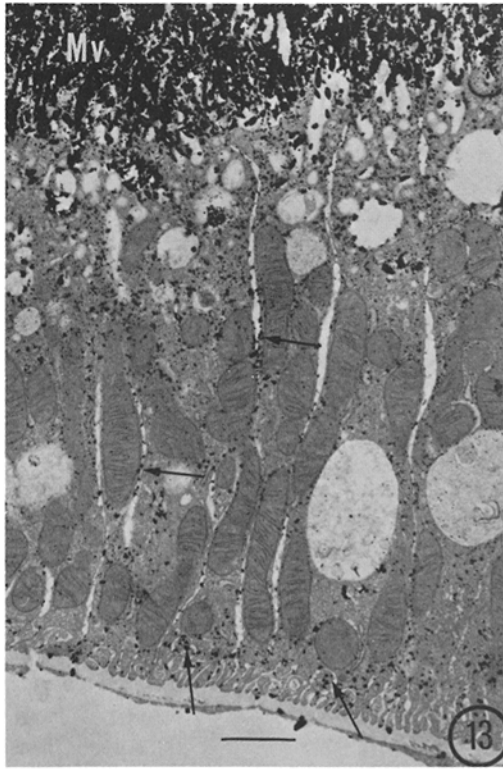
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FIGURE 10 This micrograph shows the effect of deleting K from the medium on the localization of NPPase activity. Microvillar (Mv) staining of PT is unaffected (compare with Figs. 6 and 8). In contrast, stain deposition in DT is markedly reduced (compare with Figs. 6 and 7). These results are identical to that seen with the light microscope (Fig. 2). In this micrograph, a few K-independent deposits are associated with the basal folds of the PT plasmalemma (arrows). Nuclear chromatin of the DT nucleus (N) is lightly peppered with precipitate whereas, in the PT, precipitate has accumulated in the cisterna of the nuclear envelope (arrowheads). Both types of nuclear deposits are likely to be artifactual in nature (see Fig. 22). Incubation was for 15 min at 30°C.  $\times$  4,900.

FIGURE 11 When Mg is deleted from the K-containing medium, basal membrane staining in DT is abolished. Incubation was for 30 min at room temperature.  $\times$  12,400.

FIGURE 12 This micrograph shows the effect of adding cysteine to the K-containing medium. The K- and Mg-dependent NPPase activity of DT (see Figs. 10 and 11) is insensitive to this sulfhydryl-containing amino acid. DT reaction product is confined to the basal plasmalemma; apical cytoplasm (asterisks) and luminal membranes (L) are unstained. In contrast, the K-independent staining of PT, as seen in Fig. 10, is greatly reduced by cysteine. Only a few deposits are seen along the microvillar border (Mv) and basal folds (arrows) are completely unreacted. Identical results are seen in light microscope preparations (see Fig. 3). 10 mM cysteine, 40 mM Sr; incubation was for 15 min at 30°C.  $\times$  4,900.





consistent with earlier studies on artifactual heavy metal deposits localized in nuclei with other phosphatase procedures (8).

For comparative purposes, Mg-ATPase activity was demonstrated by the Wachstein-Meisel procedure (62). With this procedure, all Na-K-ATPase activity is inhibited by the capture ion (lead) employed to precipitate hydrolyzed inorganic phosphate (reference 32; see also reference 17 for pertinent discussion and references). In contrast to the localization of K-NPPase activity along the cytoplasmic side of the basolateral plasma membranes of distal and collecting tubules (Figs. 7, 9), Mg-ATPase reaction product was restricted to the extracellular side of these membranes (Figs. 23, 24). Moreover, unlike the K-NPPase localization, Mg-ATPase was also present along the luminal surface of distal tubules (Fig. 23). In addition, both the microvillar and basolateral surfaces of proximal cells were sites of ATPase activity (Figs. 25, 26). In general, both membrane sites appeared more heavily reacted in initial portions of proximal tubules (Fig. 25) than in the middle portion (Fig. 26). The basolateral membranes of the terminal

portion were generally unstained, whereas the microvillar borders were quite reactive. In addition, cytoplasmic granules or lysosomes in the middle segment were heavily stained (Fig. 26). The staining pattern shown here is similar to that for the three proximal segments ( $P_1$ ,  $P_2$ ,  $P_3$ ) described in detail by Jacobsen et al. (34) using a modified Wachstein-Meisel procedure at the level of the light microscope.

Deletion of Mg from the ATPase medium abolished staining in distal tubules but had only a small effect on proximal tubule staining. Substitution of Sr for Mg partially restored distal tubule staining whereas Ca substitution completely restored distal tubule reactivity. Moreover, in the presence of Ca, staining of both the microvillar and basolateral membranes in proximal tubules appeared heavier than that observed in either the presence or absence of Mg.

#### DISCUSSION

The demonstration of Na-K-ATPase activity in the present study is based on the cytochemical localization of the K-dependent phosphatase com-

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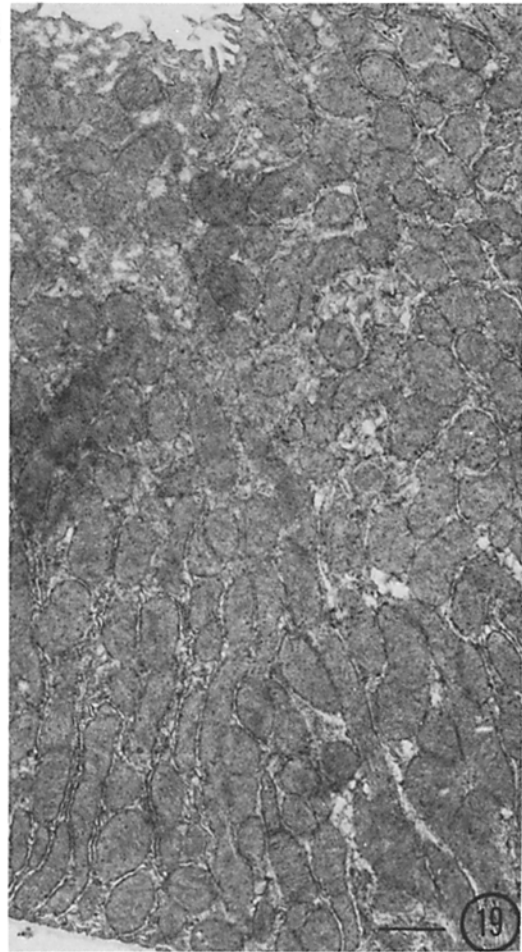
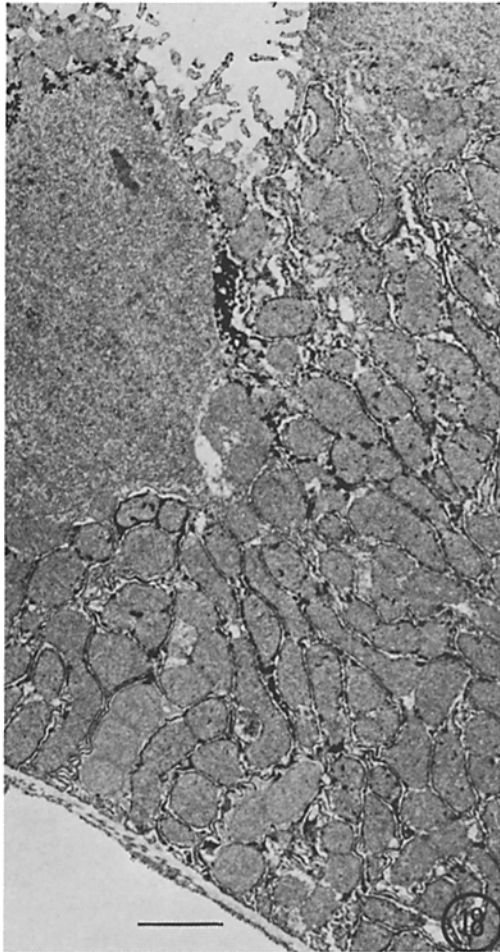
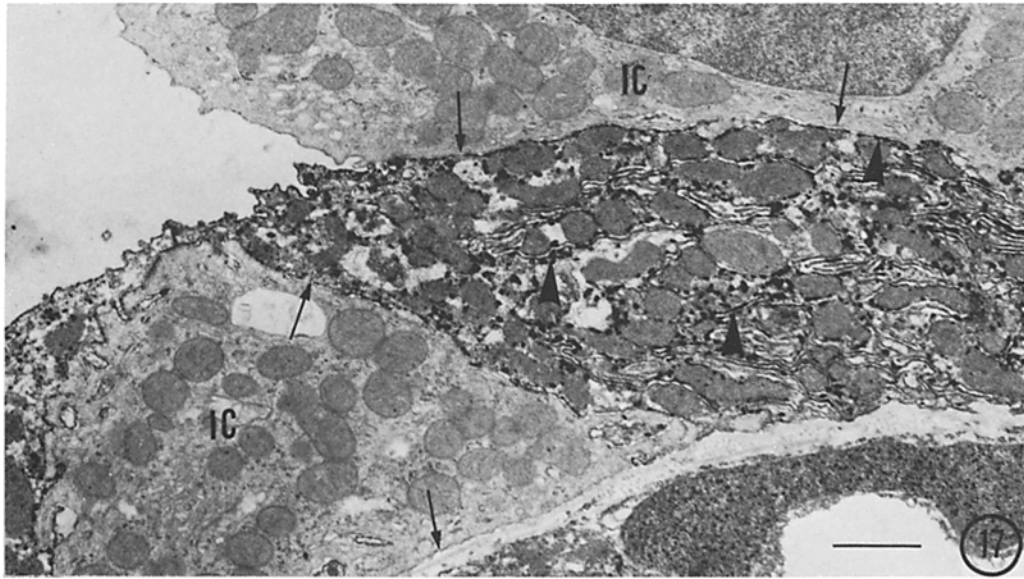
FIGURE 13 This micrograph shows the effect of long incubation times (30–60 min) on NPPase activity in PT in the presence of K. The microvillar border (*Mv*) is overstained and reaction product appears to have diffused into the apical cytoplasm. In addition, varying amounts of precipitate are associated with the basal folds of the plasma membrane (arrows). These deposits, which were generally small and focal in nature, were seen on the intracellular as well as the extracellular side of the membrane. Incubation was for 45 min at room temperature.  $\times 9,400$ .

FIGURE 14 The heavy microvillar (*Mv*) staining of PT seen in the previous micrograph after prolonged incubation is greatly reduced when cysteine is added to the medium. In addition, the reaction product associated with the basal plasmalemma of uninhibited controls (Fig. 13) is almost completely inhibited. Occasional residual deposits were seen, however, along the cytoplasmic side of the basal cell surface (arrows). Although no basal membrane deposits were evident in PT incubated in a cysteine-containing medium deficient in K, the cysteine-insensitive deposits seen here in the presence of K were not sufficiently reproducible to conclude that K-NPPase activity is present in PT. 10 mM cysteine; incubation was for 45 min at room temperature.  $\times 8,000$ .

FIGURE 15 When the time of renal perfusion with fixative was reduced from 10 min to 2.5 min, a moderately heavy deposition of reaction product appeared in early segments of PT after incubation in the presence of K and cysteine. Reaction product was primarily localized to the cytoplasmic side of the basal plasmalemma (arrows). Some evidence of diffusion of reaction product into adjoining cytoplasm is apparent. The light microvillar (*Mv*) staining was similar to that seen after perfusion for 10 min with fixative and incubation in the presence of cysteine (compare with Fig. 14). 10 mM cysteine, 10 mM KCl; incubation was for 60 min at room temperature.  $\times 9,700$ .

FIGURE 16 The PT shown here was treated the same as that shown in Fig. 15 except that K was deleted from the incubation medium. Microvillar (*Mv*) staining is unaffected by K deletion, whereas basal membrane staining is greatly reduced (compare with Fig. 15). Some K-independent NPPase activity along the basal folds of PT (arrows) was apparent in some, but not all early segments of PT. 10 mM cysteine; incubation was for 60 min at room temperature.  $\times 10,200$ .





ponent of the transport ATPase complex. This is accomplished by the heavy metal precipitation of inorganic phosphate which is hydrolyzed in a K-dependent step from NPP (15). Evidence supporting the view that this specific phosphatase activity represents the terminal dephosphorylation step in the transport ATPase reaction was summarized in a previous paper (14). The cytochemical medium employed in the initial studies with the avian salt gland was shown to be kinetically sound (14) and the ultrastructural localization of K-NPPase activity (15) correlated closely with the quantitative data derived from the kinetic experiments. In recent studies, the K-NPPase procedure was applied successfully to such diverse tissues as rat cornea (36), frog skin (23), and iguana salt gland (11). In a paper published during the preparation of this manuscript, Firth (19) described a localization of K-NPPase activity in rat distal tubules and an L-tetramisole-sensitive alkaline phosphatase activity in proximal tubules. These results are consistent with our preliminary studies in rat cortex (16) and agree in many respects with the more extensive observations reported in this paper. In Firth's study, however, K-NPPase was not apparent at all at the light microscope level and was restricted, at the electron microscope level, to distal tubules alone.

The results reported in the present investigation indicate that the transport ATPase complex is associated with all segments of cortical kidney tubules. However, cytochemical staining of these tubules for K-NPPase activity indicates striking quantitative differences in enzyme activity among the different cortical segments. All segments of

cortical distal tubules stained strongly (Fig. 1, 3, 6, 7, 12) whereas collecting tubules were only moderately reactive (Fig. 9). In contrast, proximal tubular K-NPPase activity could be demonstrated only in kidneys fixed for much shorter periods of time, and even then, stain deposition was relatively light (Fig. 15). These quantitative differences in K-NPPase activity among the various tubular segments are consistent with the biochemical experiments of Schmidt and Dubach (53). These investigators assayed Na-K-ATPase activity in microdissected segments of the rat nephron. The specific activity of the enzyme in cortical distal tubules was three to five times greater than the low activity present in the corresponding proximal segments. In a single determination, the specific activity of collecting duct Na-K-ATPase was approximately 80% of that measured for the distal tubule.

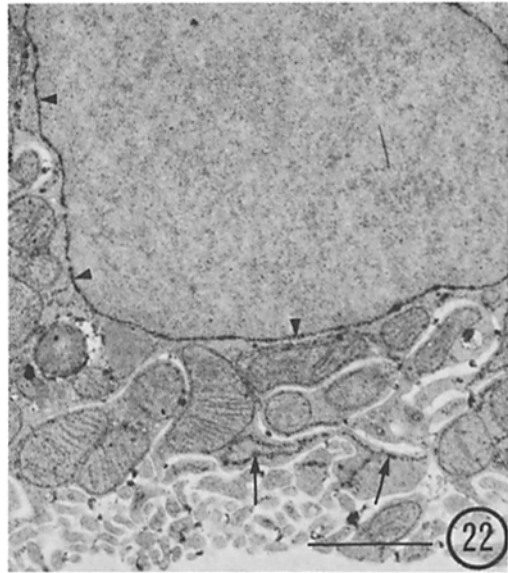
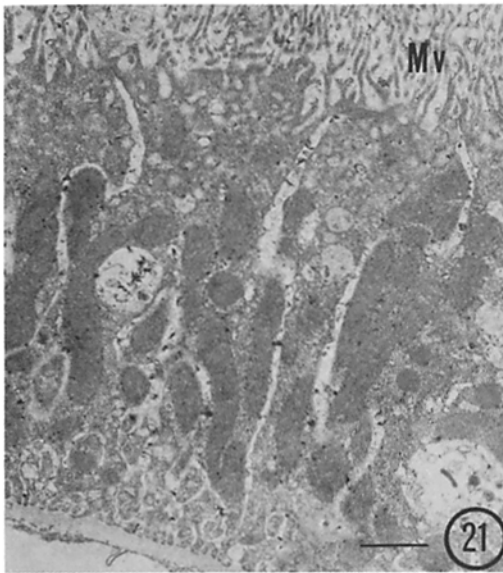
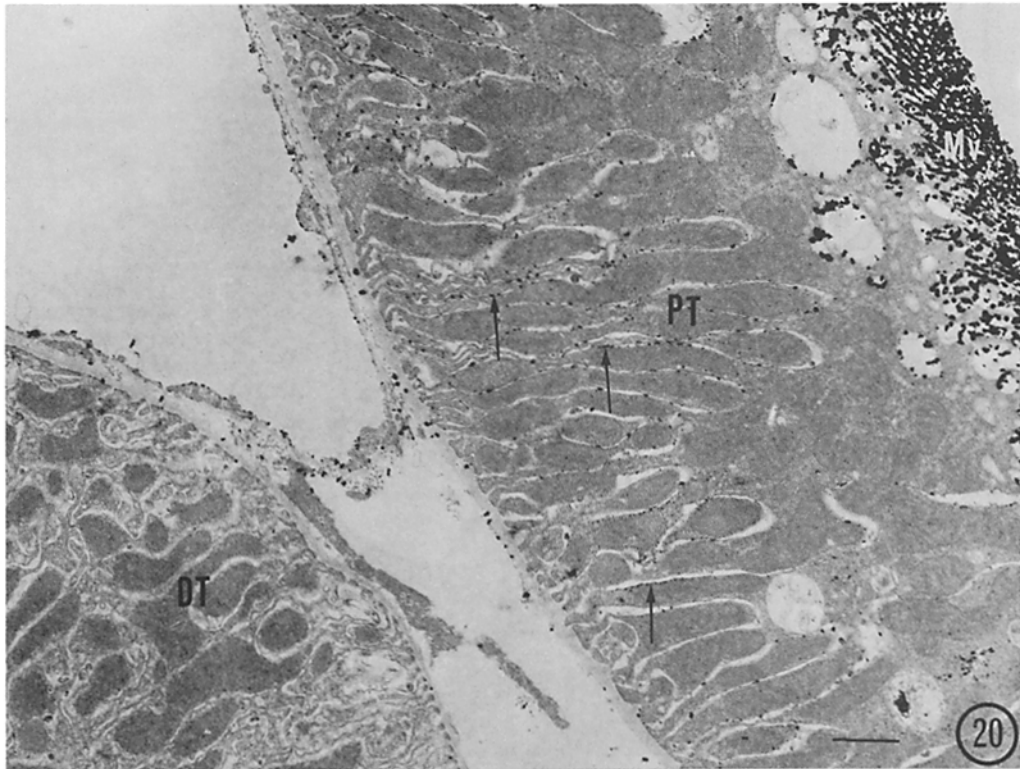
K-dependent reaction product in all three segments was restricted to the cytoplasmic side of the basolateral plasma membranes (Figs. 7, 9, 15) and was K- and Mg-dependent (Figs. 2, 10, 11, 16) and insensitive to cysteine (Figs. 3, 12, 15). Localization of K-NPPase activity to the cytoplasmic side of the plasma membrane correlates with the demonstration of internal release of hydrolyzed phosphate by the Na-K-ATPase of red cell membranes (56) and is identical to that observed in the previous study with the avian salt gland (15) and with the localization in iguana salt gland (11). Other studies with this phosphatase procedure (36), however, demonstrated K-NPPase activity along the extracellular surface of the plasmalemma. Although the reason for these conflicting

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FIGURE 17 This micrograph shows a segment of a DT from rabbit kidney after a 10-min perfusion with fixative and incubation in the standard NPPase medium employed in rat kidney cortex. This portion of the DT contains two intercalated cells (IC) interposed between two typical DT principal cells (extreme left and right center). The IC seen here are similar to the dark cells of collecting tubules (26). The absence of basal membrane amplification was seen in some, but not all, IC. Reaction product is restricted to the intracellular side of the basal and lateral plasmalemma of the DT cells (arrowheads). The flat surfaces of both the basal and lateral plasmalemma of the IC (arrows) are devoid of reaction product. 10 mM KCl; incubation was for 45 min at room temperature.  $\times 11,800$ .

FIGURE 18 This micrograph shows the localization of NPPase activity in a rabbit DT at higher magnification. The pattern of staining along the cytoplasmic sides of the basal plasma membrane is similar in all respects to that observed in rat distal tubules (see Fig. 7). 10 mM KCl; incubation was for 45 min at room temperature.  $\times 11,000$ .

FIGURE 19 When ouabain is added to the K-containing medium, the membrane staining of rabbit DT seen in Figs. 17 and 18 is markedly reduced or absent. 10 mM KCl, 10 mM ouabain; incubation was for 45 min at room temperature.  $\times 8,500$ .



results is not clear, it seems likely that the disparity is due to variations in the inorganic phosphate trapping and precipitation mechanism rather than to differences in enzyme sites (for a more complete discussion of this point, see Leuenberger and Novikoff [36]).

K-dependent reaction product was preferentially associated with those portions of the basolateral cell surfaces which exhibit marked surface amplification in the form of membrane folds. Little stain deposition was observed along unfolded portions of these membranes, a morphological feature of the descending straight portion of the proximal tubule (33, 43) and of some localized areas in cortical collecting tubules and in intercalated cells (see Fig. 17). The localization of the transport ATPase complex along the basal surfaces of the tubular epithelium is consistent with the biochemical experiments of Schmidt and Dubach (55) which showed that microdissected fragments of the basal portion of proximal tubules had a specific activity for Na-K-ATPase which was seven times greater than that of the intact proximal segment. Activity in brush border fragments was negligible.

Amplification of basolateral cell surfaces is an essential feature of most osmoregulatory epithelia (6). The formation of such intra- and extracellular compartments by surface extension may have a functional significance in permitting the coupling of water transport to active solute transport in the elaboration of hypo-, iso- and hyperosmotic fluids by means of local standing gradients within the compartments (9, 10). The current study in kidney cortex, as well as the previous study in the salt

gland (15), lends direct support to this hypothesis by showing that the transport ATPase complex is associated almost exclusively with these extended channels.

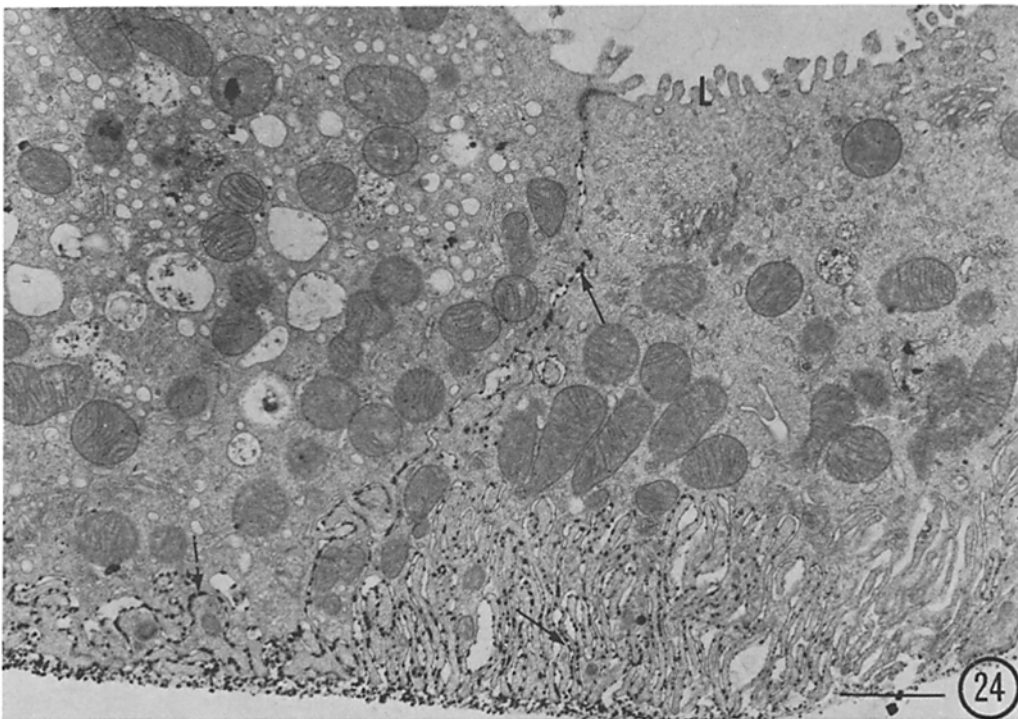
Cytochemically demonstrable K-NPPase activity in rat kidney cortex was not consistently inhibited by ouabain. Although this observation is not in agreement with the initial cytochemical studies of Firth (19) utilizing the same tissue, subsequent work by this investigator (personal communication) is now in agreement with the data on ouabain insensitivity reported here. Rat kidney Na-K-ATPase is known to be relatively insensitive to cardiac glycosides (2, 3). Moreover, the sensitivity of the transport ATPase complex to ouabain in other tissues is reduced when NPP is substituted for ATP (20, 66). In addition, we showed that the concentration of ouabain required for 50% inhibition of salt gland K-NPPase was increased 50-fold in the presence of 20 mM Sr and that only 75% of the K-dependent activity could be inhibited by 10 mM ouabain (14). The combination of these conditions apparently reduced the ouabain sensitivity of the rat enzyme to levels which could not be resolved consistently with this cytochemical method. An attempt was made to circumvent this problem by preincubation of tissue in a medium which supports ouabain binding (41) and subsequent incubation for cytochemistry in the NPPase medium also containing ouabain. The results in this case were also negative, presumably because ouabain binding in rat kidney, unlike that in sensitive tissues, is reversible (2, 3) and any ouabain bound under the optimal conditions of the preincubatory period would still

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FIGURE 20 When 20 mM NPP of the standard incubation medium is replaced by 20 mM  $\beta$ -glycerophosphate, staining of microvilli (*Mv*) and basal membrane folds (arrows) of PT is similar to that observed with NPP as substrate (compare with Fig. 13). DT, however, are totally unreactive with  $\beta$ -glycerophosphate. 10 mM KCl; incubation was for 30 min at room temperature.  $\times$  8,600.

FIGURE 21 The phosphatase activity of PT observed with  $\beta$ -glycerophosphate as substrate is completely inhibited when cysteine is added to the medium. 10 mM K, 10 mM cysteine; incubation was for 30 min at room temperature.  $\times$  8,800.

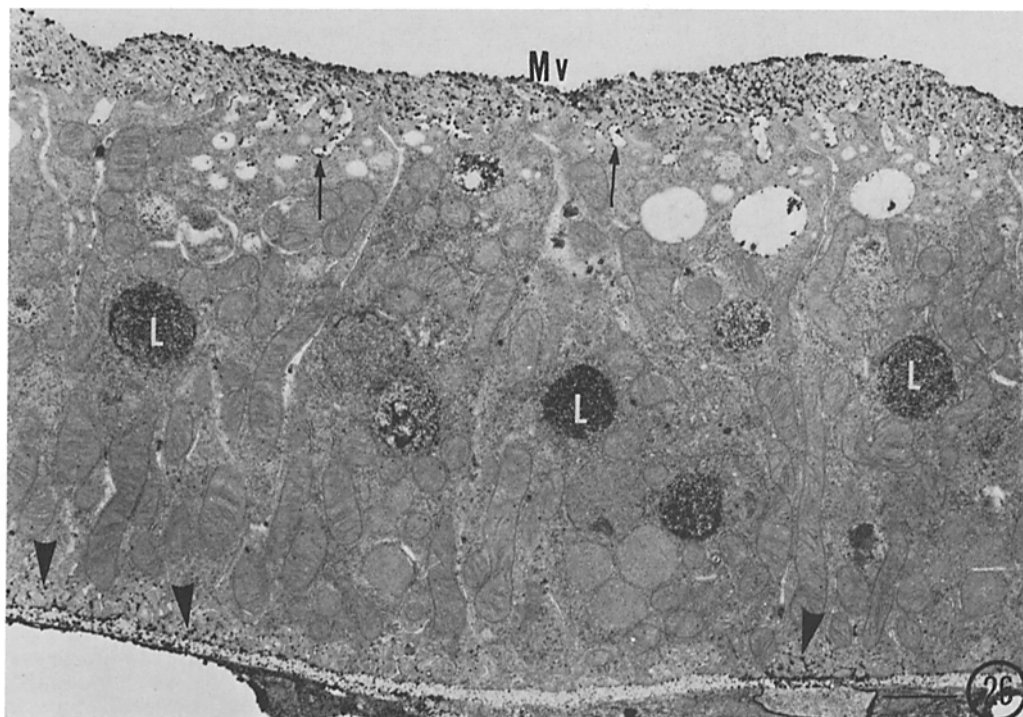
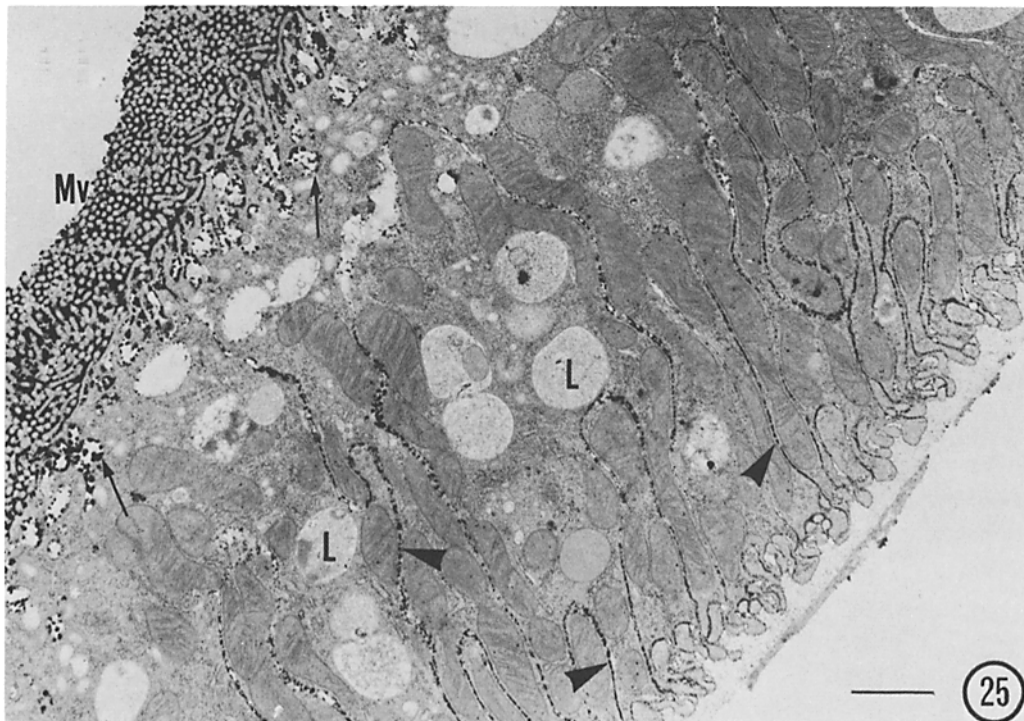
FIGURE 22 This micrograph shows the distribution of nonenzymatically produced precipitates. Sections were incubated for 15 min in the standard incubation medium containing 20 mM Sr but without NPP. The tissue was postincubated for an additional 45 min after the addition of  $K_2HPO_4$  to a final concentration of 10 mM. The tissue was subsequently processed in the routine manner employed for experimental tissue. Both in PT, as shown here, and to varying degrees in DT, precipitate was found within the cisterna of the nuclear envelope (arrowheads) and the endoplasmic reticulum (arrows), along mitochondrial membranes, and was finely distributed within the mitochondrial matrix and nucleoplasm. Precipitate was associated only rarely with the plasmalemma. Section was not counterstained with lead and uranyl salts.  $\times$  16,200.



**FIGURE 23** ATPase activity was localized in this DT using the Wachstein-Meisel (62) procedure. Reaction product is deposited heavily along the extracellular side of the luminal membrane (*L*) and along the basolateral cell surfaces where it completely fills the extracellular compartments between adjacent membrane folds (arrows). Incubation was for 30 min at room temperature.  $\times 9,800$ .

**FIGURE 24** Localization of ATPase activity in cortical collecting tubules. Reaction product is found along the extracellular side of basal membrane folds and lateral cell surfaces (arrows), but is absent from the luminal membrane (*L*). Incubation was for 15 min at room temperature.  $\times 13,400$ .





**FIGURE 25** Localization of ATPase activity in early segments of PT. The extracellular surfaces of microvilli (*Mv*), apical pits (arrows) and basolateral plasma membranes (arrowheads) are stained. Lysosomes (*L*) in early segments were generally unreactive. Incubation was for 30 min at room temperature.  $\times 10,300$ .

**FIGURE 26** Localization of ATPase activity in the middle segments of PT. Microvilli (*Mv*), apical pits (arrows), and the shallow basal membrane folds (arrowheads) are all reactive, but to a lesser extent than was observed in the early PT segments (compare to Fig. 25). Unlike the early PT, lysosomes (*L*) of the middle segment are also sites of ATPase activity. Incubation was for 30 min at room temperature.  $\times 9,300$ .

be subject to the densitizing effects of the cytochemical medium. In a recent paper (28), Guth and Albers modified the NPPase procedure by the addition of dimethylsulfoxide (DMSO) to the medium. This solvent increases the specific activity of K-NPPase, shifts the pH optimum for the reaction to pH 9 (1), and greatly increases the sensitivity of K-NPPase to ouabain (44). Localization of K-dependent activity at the light microscope level, which was achieved in the absence of Sr, depended on the observation that at an alkaline pH, inorganic phosphate is capable of binding to tissue components (27). When this light microscope procedure was applied to rat kidney (28), distal tubules stained heavily and reaction product was K-dependent and ouabain-sensitive. These results, therefore, are entirely consistent with the present study and indicate that the observed sites of K-dependent phosphatase activity reported here are associated with the ouabain-sensitive transport ATPase. This conclusion is further substantiated by the demonstration of ouabain-sensitivity of K-NPPase activity in rabbit kidneys (Figs. 17-19), a tissue which is considerably more sensitive to ouabain than rat kidney (3). In this case, the degree of ouabain inhibition of staining was somewhat variable but always significantly less than uninhibited controls.

Physiological studies have repeatedly suggested that Na reabsorption by kidney tubules occurs, at least in part, by passive diffusion of Na across the luminal surface and active extrusion of this cation across the basolateral cell surfaces by means of Na-K exchange pumps (48), presumably mediated by the ouabain-sensitive Na-K-ATPase complex (12). The absence of substantial quantities of cytochemically demonstrable transport ATPase activity in proximal convoluted tubules is, therefore, somewhat surprising considering that this segment of the rat nephron is responsible for the iso-osmotic reabsorption of one-half to two-thirds of the filtered sodium (63). However, recent studies suggest the presence of at least two sodium pumps in kidney cortex (49, 65): (a) an Na-K exchange pump (pump B) which is sensitive to ouabain and which derives its energy from Na-K-ATPase; (b) an electrogenic pump (pump A) which extrudes Na accompanied by passive chloride and water flux, is sensitive to ethacrynic acid, and presumably operates independent of Na-K-ATPase. The differentially low level of cytochemically demonstrable K-NPPase activity in proximal convoluted tubules suggests that this segment is not a major site for

pump B. Further support for this view is obtained from measurement of canine Na excretion after renal infusion with ouabain (40). In this study, 80-90% of the filtered Na load continued to be reabsorbed despite a 75% inhibition of cortical Na-K-ATPase. Since solute-free water reabsorption was significantly depressed, at least a part of the observed natriuresis was likely to have been due to inhibition of Na-K-ATPase in the ascending limb of Henle (40). However, 10% of the filtered Na is reabsorbed in the distal convoluted tubule (64), a segment which contains high levels of K-NPPase activity. Thus, a portion of the increase in urinary Na accompanying ouabain infusion may be due to inhibition of Na-K-ATPase in this portion of the nephron as well. It is also of interest that micropuncture studies (5, 35) have indicated that Na reabsorption in the early portion of the proximal tubule may depend on a Na-linked glucose carrier at the luminal surface and an active Na pump at the basal surface. In the late portions of the proximal tubule, sodium reabsorption is passive and dependent on a passive chloride diffusion potential (5, 35). In the cytochemical studies reported here little, if any, K-NPPase is associated with the terminal segment of the proximal tubule.

In contrast to proximal tubules, cortical distal tubules were highly reactive for K-NPPase, indicating that this nephron segment is a major site for pump B. Na reabsorption in this segment is active since Na is reabsorbed against a substantial electrochemical gradient (39). Since the distal convoluted tubules are quite short, a localized high concentration of the transport ATPase may be required to attain the relatively high reabsorptive load per unit of distal convoluted tubular mass, as was suggested to be the case for the ascending thick limb of Henle (30). In addition, the distal convoluted tubule is a major site for K secretion (37). Extensive physiological experiments by Giebisch and others (see review by Giebisch [21]) suggest that K secretion across the luminal membrane may be linked to an Na-K exchange pump situated at the peritubular cell membrane. In this regard, when rats are chronically adapted to high potassium loads, Na-K-ATPase of the distal segment is markedly enhanced (57). Such an adaptation may result in an increased intracellular K pool, thereby increasing the passive flux across the luminal membrane into the urine. The strong cytochemical reaction for K-NPPase activity along the distal peritubular membrane is consistent with the postulated (21) localization of an

Na-K exchange pump at this site. Other studies (38, 39) indicated that under appropriate conditions K reabsorption also may occur in an active, ouabain-sensitive step across the luminal border. However, the luminal membrane was not reactive for K-NPPase in the present study. Active Na reabsorption and K secretion also occur in collecting tubules and are apparently linked since both fluxes are inhibited by peritubular exposure to ouabain (25). The basolateral membranes of cortical collecting tubules were moderately reactive for K-NPPase (Fig. 9).

In addition to localization of K-dependent phosphatase sites, the cytochemical procedure also demonstrated sites of nonspecific alkaline phosphatase activity, since this enzyme is capable of hydrolyzing NPP. The localization of this enzyme to the microvillar and, less strongly, to the peritubular borders of proximal tubules (Figs. 1, 2, 6, 8, 10, 13) is identical to that described by other investigators employing a variety of phosphatase procedures (50). This nonspecific enzymatic activity could be differentiated from the specific phosphatase since the nonspecific activity was independent of K (Figs. 2, 10) and Mg and was insensitive to ouabain. Furthermore, alkaline phosphatase activity was selectively inhibited by cysteine (Figs. 3, 12, 14), an amino acid which, interestingly, activates Na-K-ATPase in partially purified rabbit kidney enzyme preparations (59), but has little effect on enzyme activity in perfused kidneys (S. A. Ernst, unpublished observations). Unlike distal tubules, the basolateral membranes of proximal tubules are reactive for both K-dependent and K-independent NPPase activity (Figs. 13-16). The former is selectively resolved with short fixation times and long incubation times in the presence of cysteine (Figs. 15, 16).

Similar cysteine-sensitive sites for alkaline phosphatase were obtained when  $\beta$ -glycerophosphate was substituted for NPP (Figs. 4, 5, 20, 21). This substrate is not hydrolyzed by the transport ATPase complex (14, 20); accordingly, distal and collecting tubules were unreactive with this substrate (Figs. 4 and 20). When ATPase activity was localized by the Wachstein-Meisel procedure (62), luminal and peritubular membranes of all cortical segments were stained (Figs. 23-26), with distal and collecting tubules exhibiting the greatest reactivity. Similar observations have been reported by others (4, 13). In contrast to K-NPPase activity, ATPase activity, like that of nonspecific alkaline phosphatase, was generally restricted to the extra-

cellular side of the tubular membranes (Figs. 23-26). Although the distal and collecting tubule ATPase required Mg, staining of microvillar and, to some extent, basolateral membranes of proximal tubules was not noticeably depressed by deletion of this cation from the medium. It seems likely, therefore, that the apparent ATPase activity of proximal tubules is due, at least in part, to the wide substrate specificity of alkaline phosphatase. The distal and collecting tubule activity may be due, however, to a true Mg-dependent ATPase. In any case, Mg-ATPase activity, as resolved by the Wachstein-Meisel procedure, is unrelated to the transport ATPase complex since reaction product is independent of Na and K and insensitive to ouabain (17, 32, 52, 61). It was suggested, however, that Mg-ATPase activity may reflect a different form of the Na-K-ATPase complex (see, for example, discussion in reference 18). This now seems unlikely, since almost all Mg-ATPase activity is removed by purification of Na-K-ATPase (31). Although the function of Mg-ATPase is not resolved, it is of interest that Mg-ATPase activity of distal and collecting tubules was stimulated to the same extent when Ca replaced Mg in the medium (data not presented; see also reference 46). Sr was also stimulatory, but to a lesser extent. The apparent ATPase activity of proximal tubular microvilli was also stimulated by Ca, a finding consistent with the hypothesis (29) that alkaline phosphatase, Ca-ATPase, and much of the Mg-ATPase activity of this luminal surface may be different reflections of the same enzyme.

In summary, application of the K-NPPase procedure to renal cortex indicates that distal and collecting tubules are major sites for the transport enzyme. Moreover, this specific phosphatase is readily distinguished from the nonspecific NPPase by its substrate specificity, cation dependence, and inhibitor sensitivity. Although most portions of proximal, distal, and collecting tubule epithelium exhibited consistent patterns of staining from one tubule to the next, there is sufficient variation to warrant careful examination of the distribution of K-NPPase within different areas of the cortex and within different portions of the same nephron segment. The need for such a critical study is evident from biochemical studies showing marked regional differences in alkaline phosphatase, Mg-ATPase, and Na-K-ATPase (54) and from cytochemical studies indicating variations in localization of alkaline phosphatase and Mg-ATPase (34).



That such differences are functionally significant is reflected, no doubt, in the variation in physiological parameters which have been measured along the length of the nephron (5, 7, 35).

Finally, the close association between Na-K-ATPase and basolateral cell surface amplification stresses the importance of this cytoarchitecture in the physiology of active Na reabsorption. Although the peritubular localization in renal tissue is expected from existing physiological data, it is of interest that the same architecture is characteristic of a variety of Na secretory epithelia (6) and that ouabain-sensitive K-NPPase is similarly restricted to these folded surfaces (11, 15) even though the direction of net Na transport is from blood to lumen. Critical examination of the subcellular distribution of K-NPPase in other osmoregulatory epithelia where the mode of Na transport is questioned is, therefore, clearly needed. Such studies are currently underway in kidney medulla where recent reports suggest that thick ascending limbs of Henle may actively transport chloride by a ouabain-sensitive pump (7). Preliminary cytochemical results indicate, however, that this nephron segment, like the distal convoluted portion with which it is continuous, is highly reactive for the K-dependent phosphatase component of the Na-K-ATPase complex.

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