

Ultrastructural Localization of Na^+, K^+ -ATPase in Rat and Rabbit Kidney Medulla

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ABSTRACT Na^+, K^+ -ATPase was localized at the ultrastructural level in rat and rabbit kidney medulla. The cytochemical method for the K^+ -dependent phosphatase component of the enzyme, using *p*-nitrophenylphosphate (NPP) as substrate, was employed to demonstrate the distribution of Na^+, K^+ -ATPase in tissue-chopped sections from kidneys perfusion-fixed with 1% paraformaldehyde-0.25% glutaraldehyde. In the outer medulla of rat kidney, ascending thick limbs (MATL) were sites of intense K^+ -dependent NPPase (K^+ -NPPase) activity, whereas descending thick limbs and collecting tubules were barely reactive. Although descending thin limbs (DTL) of short loop nephrons were unstained, DTL from long loop nephrons in outer medulla were sites of moderate K^+ -NPPase activity. In rat inner medulla, DTL and ascending thin limbs (ATL) were unreactive for K^+ -NPPase. In rabbit medulla, only MATL were sites of significant K^+ -NPPase activity. The specificity of the cytochemical localization of Na^+, K^+ -ATPase at reactive sites in rat and rabbit kidney medulla was demonstrated by K^+ -dependence of reaction product deposition, localization of reaction product (precipitated phosphate hydrolyzed from NPP) to the cytoplasmic side of basolateral plasma membranes, insensitivity of the reaction to inhibitors of nonspecific alkaline phosphatase, and, in the glycoside-sensitive rabbit kidney, substantial inhibition of staining by ouabain.

The observed pattern of distribution of the sodium transport enzyme in kidney medulla is particularly relevant to current models for urine concentration. The presence of substantial Na^+, K^+ -ATPase in MATL is consistent with the putative role of this segment as the driving force for the countercurrent multiplication system in the outer medulla. The absence of significant activity in inner medullary ATL and DTL, however, implies that interstitial solute accumulation in this region probably occurs by passive processes. The localization of significant Na^+, K^+ -ATPase in outer medullary DTL of long loop nephrons in the rat suggests that solute addition in this segment may occur in part by an active salt secretory mechanism that could ultimately contribute to the generation of inner medullary interstitial hypertonicity and urine concentration.

Na^+, K^+ -ATPase is widely considered to be the driving force for active salt and water movement across electrolyte-transporting epithelial tissues. In such tissues, specific activities for the enzyme are usually elevated far above the level required for maintenance of normal cellular ionic homeostasis. Accordingly, in structurally complex organs like the kidney that are characterized by marked cell and tissue heterogeneity, utilization of microscopic methods to map the distribution of Na^+, K^+ -ATPase affords an opportunity to identify morphologically distinct sites that possess the requisite enzymatic machinery for active transepithelial transport. The importance of visualizing epithelial sites of Na^+, K^+ -ATPase is particularly apparent in

renal medulla where striking morphological differences, which are probably transport-related, exist not only among the various classes of medullary tubules, but even within individual nephron segments themselves (4, 5, 37, 44, 63, 64). With particular reference to the descending thin limb of rodent outer medulla, for example, the cytoarchitecture of the tubular cells differs markedly when segments from superficial and juxta-medullary nephrons are compared (4, 5, 44, 63, 64), as does the spatial relationship of these segments to ascending thick limbs and vascular bundles (44, 45). Many of these differences appear to be species-specific (44).

Whereas the general role of medullary tubules in the pro-

duction of a concentrated urine by countercurrent multiplication seems well-established, the specific transport properties of individual nephron segments of various species remain controversial (3, 7, 35). Localization of Na^+, K^+ -ATPase among the various classes (and subclasses) of these nephron segments in specific regions of the medulla has particular relevance, therefore, to current models for urine concentration (8, 42, 53, 66) in which the contribution of active (and passive) transport processes to solute concentration and dilution in descending and ascending thin limbs, respectively, is critical. Among the techniques available for the localization of Na^+, K^+ -ATPase, the ultracytochemical method for the localization of the ouabain-sensitive, K^+ -dependent nitrophenylphosphatase (K^+ -NPPase) component of the enzyme (17, 18) has been used extensively (see discussion and references in Ernst and Mills [22], and Ernst and Hootman [20]). A previous paper from this laboratory (19) employed this method to localize the enzyme in rat and rabbit renal cortex. The present report extends this study to the medulla and demonstrates that, in addition to the localization of Na^+, K^+ -ATPase to ascending thick limbs in rat and rabbit, significant levels of activity also are associated with outer medullary descending thin limbs from long loop nephrons in the rat. In contrast, little if any activity could be resolved in the outer medullary thin limbs of the rabbit, or in the inner medullary descending or ascending thin limbs of either species.

MATERIALS AND METHODS

Adequate preservation of renal medulla for cytochemical experiments was obtained by modification of the procedures described in the previous study on the localization of cortical K^+ -NPPase (19). For this purpose, a number of preliminary experiments were carried out in which medullary enzymatic and structural preservation was assessed as a function of variation in the fixation procedure. The following protocol was established as most satisfactory. Albino rats (150–250 g) of either sex were anesthetized with Nembutal and their kidneys fixed by retrograde perfusion via the abdominal aorta after sequential severance of the vena cava and ligation of the aorta above the origin of the renal arteries. Kidneys were perfused with a mixture of 1% paraformaldehyde/0.25% glutaraldehyde (19) in 0.1 M cacodylate buffer (pH 7.5) for 5 min at room temperature. Flow rate was maintained at 5 ml/min with a model 911 Holter infusion pump (Extracorporeal Medical Specialties, Inc., King of Prussia, Pa.). Kidneys of albino rabbits (4–5 kg) were fixed under the same conditions except that the fixative was perfused directly into the renal artery and allowed to drain by cutting the renal vein.

After fixation, kidneys were excised, and slices (2–3 mm thick) were cut with a razor blade perpendicular to the long axis of the kidney. After removing most of the overlying cortex, the slices were rinsed in 0.1 M cacodylate buffer and then sectioned with a Smith-Farquhar TC-2 tissue chopper (DuPont Instruments-Sorvall, Dupont Co., Newtown, Conn.) to produce 100- μm -thick sections containing kidney tubules extending from juxtamedullary cortex to papilla. In some experiments, 50- μm -thick sections were prepared with an International cryostat (International Equipment Co., Needham Heights, Mass.). Tissue sections were incubated for 15–90 min at room temperature in the cytochemical medium utilized in the previous study of renal cortex (19). This medium allows for the K^+ - and Mg^{2+} -dependent hydrolysis of NPP by Na^+, K^+ -ATPase; inorganic phosphate is precipitated at the site of substrate hydrolysis by strontium and converted in a postincubatory step to electron-dense lead phosphate for subsequent viewing in the electron microscope. Biochemical characterization of this cytochemical medium and other methodological considerations were presented and discussed in detail previously (17–20). Incubations were conducted routinely in the presence of 10 mM cysteine (19) to inhibit nonspecific alkaline phosphatase, which also hydrolyzes NPP. For comparative purposes, 0.5 mM bromotetramisole oxalate (Aldrich Chemical Co., Milwaukee, Wisc.), an alternative specific inhibitor of alkaline phosphatase (9), was employed either in place of, or in addition to cysteine. Neither of these compounds causes significant inhibition of K^+ -NPPase (19, 26, 30). The specificity of the cytochemical reaction for the phosphatase component of Na^+, K^+ -ATPase was assessed further by deletion of K^+ or addition of 10 mM ouabain to the incubation medium.

After incubation, sections were rinsed in 0.1 M Tris buffer (pH 9.0), treated with 2% $\text{Pb}(\text{NO}_3)_2$, rinsed in 0.1 M cacodylate buffer (pH 7.5), and postfixed for 30 min with 1% OsO_4 (19, 20). The osmolarity of all rinses was elevated to near

isotonicity with plasma (300–320 mosM) by addition of sucrose. In the case of fixatives, preservation of renal ultrastructure was improved significantly by adjusting the osmolarity of the buffer, independent of the osmotic contribution of aldehyde or OsO_4 , to isotonicity (usually with NaCl). A similar conclusion was reached in the morphological studies of Maunsbach (51). Osmolarity was determined with a model 3L Advanced Osmometer (Advanced Instruments, Inc., Newton Highlands, Mass.).

Before dehydration and embedment in Spurr resin, various regions of the medulla (outer and inner stripes of outer medulla, inner medulla) were identified under a dissecting microscope and isolated by judicious cutting with a razor blade for favorable orientation in plastic for microtomy. Distribution of reaction product was analyzed without counterstaining with lead or uranyl salts in ultrathin sections with Philips 201 and 400 electron microscopes.

RESULTS

Distribution of Medullary Tubules

The distribution of the various renal tubular segments within specific regions of the rat medulla is well-established (39, 43, 45) and is described here briefly for purposes of orientation. The medulla is divided macroscopically into outer and inner portions. Straight portions of cortical proximal tubules, or pars recta, enter the outer medulla as descending thick limbs. At approximately the same medullary depth, these undergo an abrupt transition to descending thin limbs (DTL), thereby subdividing the outer medulla into outer and inner stripes. DTL of long loop nephrons extend into the inner medulla where they loop back towards the outer medulla as ascending thin limbs (ATL). At the border between inner and outer medulla, ATL undergo abrupt transitions to form ascending thick limbs (MATL), which traverse both stripes of the outer medulla to reenter the cortex. Collecting tubules (CT) extend throughout the entire medulla. DTL of short loop nephrons turn back in the outer medulla as MATL without entering the inner medulla. In the current study therefore, inner stripe could be distinguished from outer stripe in the outer medulla by the presence of DTL in addition to MATL, while inner medulla could be recognized easily by the absence of thick limbs.

Cytochemical Localization of K^+ -NPPase in Outer Medulla

The most highly reactive site for K^+ -NPPase in rat renal medulla is in MATL, where enzymatic activity is localized along the highly folded basolateral plasmalemmal surfaces that characterize this nephron segment (Figs. 1 and 3). Deposition of reaction product, which is more intense in inner stripe (Fig. 3, 30-min incubation), as compared with that in the outer stripe (Fig. 1, 90-min incubation), is restricted to the cytoplasmic side of the plasmalemma (Fig. 2). In contrast, only fine K^+ -dependent deposits could be resolved along the mostly flat abluminal surfaces of the thick descending limb of the outer stripe, and these were apparent only after extended incubations (Fig. 1). The K^+ -independent hydrolysis of NPP by alkaline phosphatase, which was shown in the previous study (19) to stain microvillar borders of cortical proximal tubules intensely even at short incubation times, was reduced greatly or abolished completely in descending thick limbs by inclusion of 10 mM cysteine (Fig. 1), or 0.5 mM bromotetramisole oxalate (data not shown), in the incubation medium. In the presence of these inhibitors of alkaline phosphatase, the low levels of residual microvillar staining (Fig. 1) were K^+ -independent and therefore not attributable to K^+ -NPPase activity. Neither of these reagents (together or separately) inhibited the K^+ -NPPase reaction in MATL (Figs. 1–3), or in other segments reactive for the cation-specific phosphatase (see below).

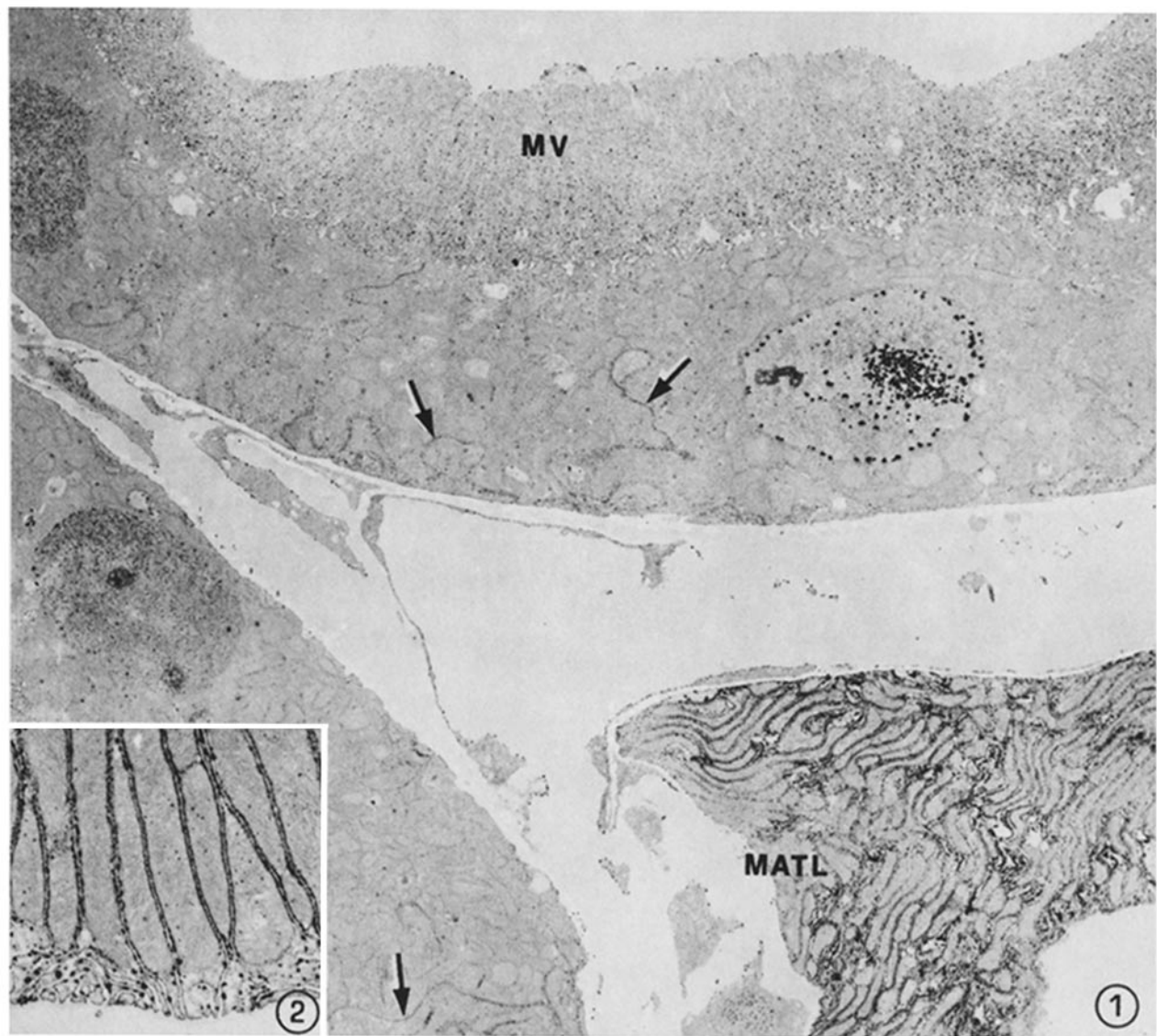


FIGURE 1 This electron micrograph shows the distribution of K^+ -NPPase activity in the outer stripe of rat outer medulla after a 90-min incubation. Reaction product is associated preferentially with the highly folded basolateral plasmalemma of ascending thick limbs (MATL). Enzymatic activity along basolateral surfaces in descending thick limbs is barely detectable (arrows). Cysteine (10 mM) was included in the incubation medium to inhibit the nonspecific alkaline phosphatase activity, which is particularly abundant along the microvillar border (MV) of the descending thick limb. Residual microvillar staining was K^+ -independent. The variable stain deposits in nuclei are artifactual (19). $\times 7,000$.

FIGURE 2 Reaction product deposition resulting from K^+ -NPPase activity is restricted to the cytoplasmic side of the highly folded plasma membranes of MATL. This inner stripe segment from rat outer medulla was incubated for 30 min in the presence of 10 mM cysteine and 0.5 mM bromotetramisole. $\times 12,000$.

Whereas K^+ -NPPase activity was localized invariably along basolateral plasmalemmal surfaces, heavy metal precipitates sometimes were seen associated with nuclear chromatin or with cisternae of the nuclear envelope. As illustrated in Figs. 1 and 3, these deposits appeared in an apparently random and highly variable pattern with nuclei in the same or neighboring tubules exhibiting opposite extremes of nuclear precipitation. These loci were shown in previous studies with this method (18, 19) to be sites of artifactual deposits. In addition, a poorly defined staining sometimes was seen along luminal surfaces of K^+ -NPPase-reactive cells (e.g., in MATL). Often such deposits were absent or greatly reduced on apical surfaces of adjacent cells, or even adjacent apical surfaces of the same cell. These deposits were particularly apparent after prolonged incubation

times, or in poorly fixed cells where apical regions exhibited high amplitude swelling and consequent disruption of structural integrity. Although this staining is considered artifactual, the presence of a minor apical Na^+,K^+ -ATPase activity, as suggested previously in immunocytochemical studies of canine cortex by Kyte (46, 47), cannot be ruled out unequivocally.

In the inner stripe of the outer medulla of rat kidney, DTL of long loop nephrons are associated preferentially with MATL and are characterized morphologically by pronounced cellular interdigitation and plasma membrane amplification (44, 45, 64; see also Figs. 3–6). The basolateral surfaces of this segment were sites of significant K^+ -NPPase activity, which ranged in intensity from light (Fig. 3) to moderate (see below) as compared with adjacent MATL. Reaction product deposition in

DTL and MATL was sensitive to fixation. The extent of activity in both of these segments was proportionately reduced in local tissue areas where structural preservation was particularly enhanced over that generally encountered with the fixation protocol used in this study. Fig. 4 shows the transition region between a descending thick limb and DTL. Whereas the basolateral surfaces of the thick limb are unreactive, focal deposits appear along this interface in the initial portions of the DTL as membrane folding begins to be enhanced. Fig. 5 shows at higher magnification a moderately heavy localization in a more highly specialized segment of a DTL after incubation for 30 min. In Fig. 6, pronounced K^+ -NPPase activity in a DTL may be compared with the considerably lighter staining of an adjacent CT after a 90-min incubation. Although prin-

cipal cells (55) of outer medullary CT often had some activity associated with their shallow basolateral folds, they appeared consistently less reactive than most DTL, and many were devoid of significant activity altogether. Generally those portions of DTL exhibiting extensive cellular interdigitations were most reactive, whereas those with thinner walls and reduced membrane plications showed less activity. Figs. 3 and 5 illustrate the extent of the range of activity encountered in these outer medullary DTL segments of the rat. Staining in the DTL was restricted to the cytoplasmic side of the plasmalemma and was not inhibited by bromotetramisole or cysteine (Figs. 3-6).

In contrast to the K^+ -NPPase activity in the DTL of long loop nephrons in the outer stripe (Figs. 3-6), similar activity could not be resolved in the corresponding thin segments of

FIGURE 3 Descending thin limbs (DTL) of long loop nephrons in the rat are closely associated with ascending thick limbs (MATL). Focal deposits of reaction product are present along the folded basolateral plasma membranes of the DTL shown in this micrograph (arrows). These surfaces in the adjacent MATL are intensely reactive. Incubation was for 30 min in the presence of 10 mM cysteine and 0.5 mM bromotetramisole. $\times 9,000$.

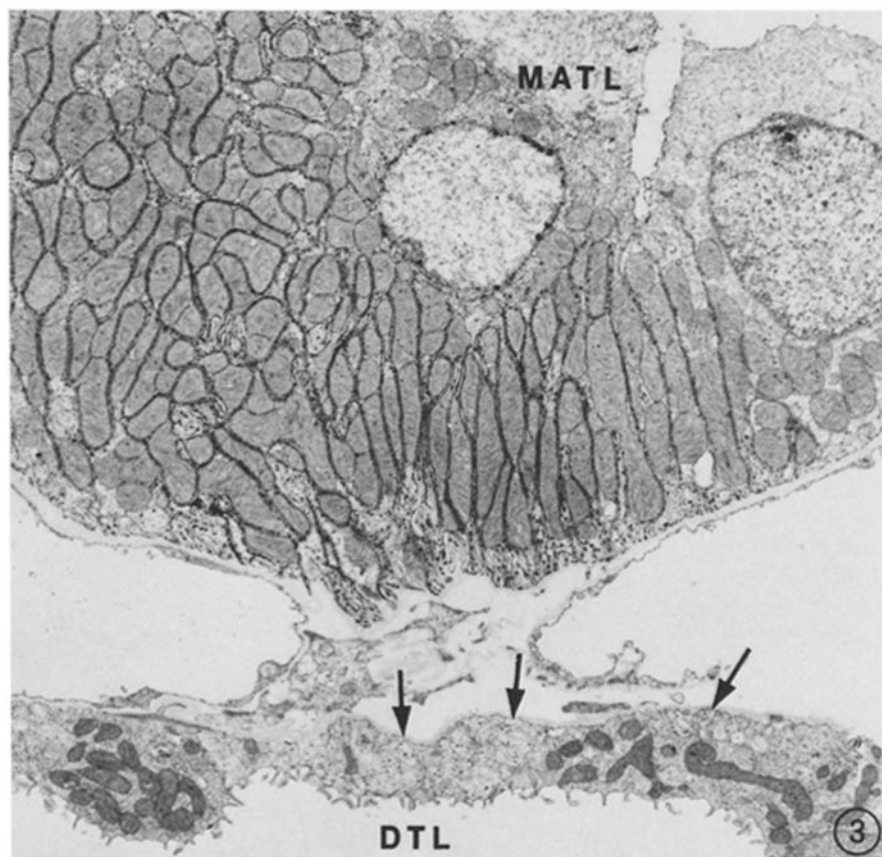
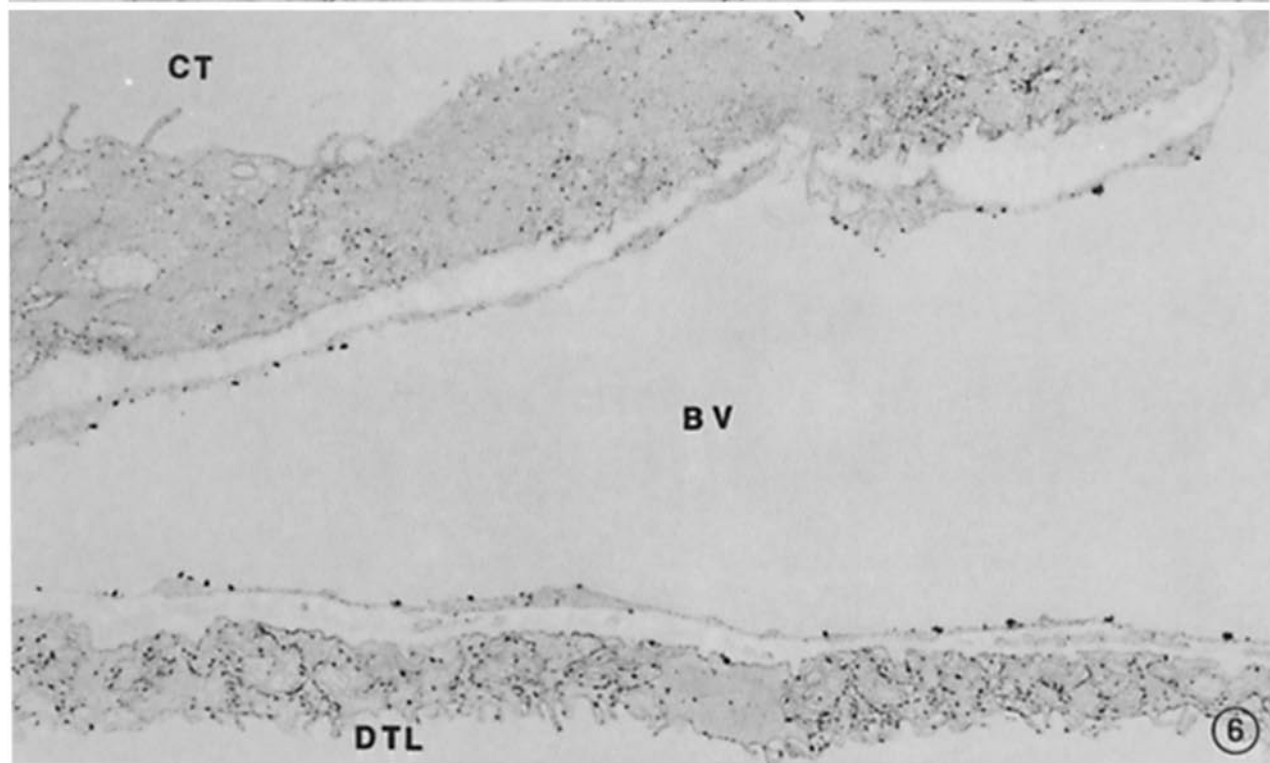
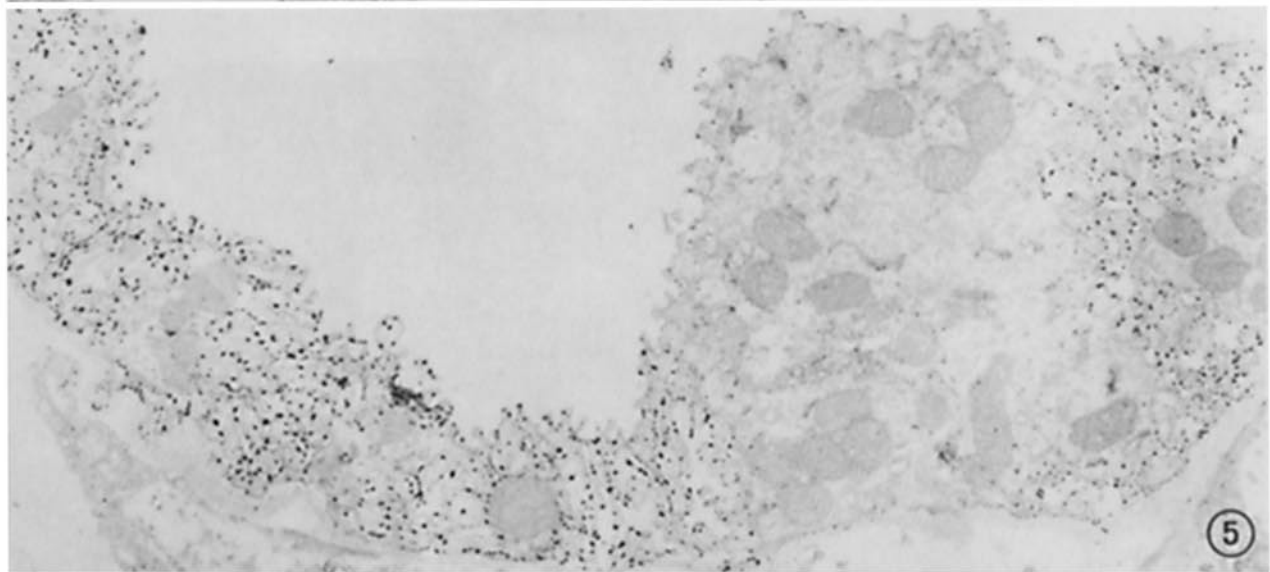
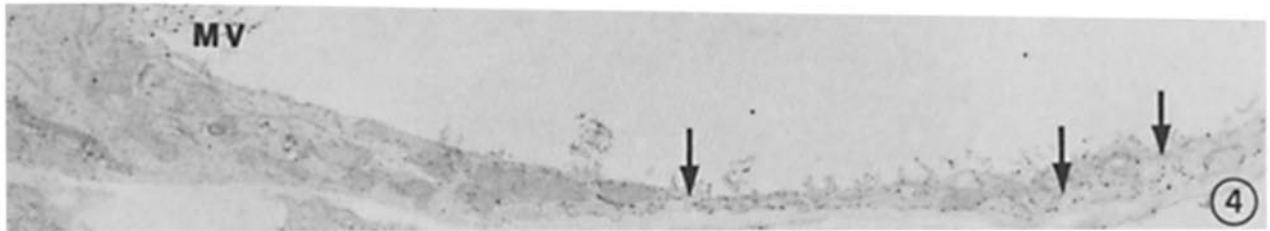


FIGURE 4 This micrograph shows the transition region between a descending thick and thin limb at the border between outer and inner stripes of rat outer medulla. Little if any K^+ -NPPase activity is present in the thick limb, whereas the thin limb exhibits fine deposits of reaction product along the basolateral plasmalemma (arrows). Incubation was for 30 min in the presence of 10 mM cysteine. A few deposits remain along the microvillar (MV) surfaces of the thick limb because of residual alkaline phosphatase activity. $\times 4,000$.

FIGURE 5 This micrograph shows a long loop DTL from rat outer medulla that exhibits moderate K^+ -NPPase activity along its highly amplified basolateral surfaces. Note that reaction product is restricted to the cytoplasmic side of the plasmalemma. In general, activity was most pronounced in DTL with highly folded basolateral plasma membranes. Incubation was for 30 min in the presence of 10 mM cysteine. $\times 19,000$.

FIGURE 6 The moderate K^+ -NPPase activity in the long loop outer medullary DTL shown here contrasts with the low level of activity seen in a neighboring collecting tubule (CT). Medullary collecting tubules were often unreactive. A few nonspecific heavy metal deposits are adsorbed to the endothelial surfaces of the peritubular blood vessel (BV). Incubation was for 90 min in the presence of 10 mM cysteine. $\times 14,000$.

FIGURE 7 In contrast to the K^+ -NPPase activity in rat outer medullary DTL from long loops (Figs. 5 and 6), the comparable short loop DTL segments, which exhibit little surface amplification, were unreactive. Incubation was for 30 min in the presence of 10 mM cysteine. $\times 11,000$.



short loop nephrons (Fig. 7). These segments differ from the long loop DTL in that they exhibit little cellular interdigitation, and are sequestered in positions remote from MATL where they associate preferentially with the vascular bundles of the vasa recta (44, 45, 64).

When incubations were carried out in the absence of K^+ , deposition of basolateral plasmalemmal reaction product in MATL and DTL was greatly reduced or absent (Fig. 8). As in the earlier study of rat cortical K^+ -NPPase (19), attempts to inhibit the K^+ -dependent reaction in the medulla with ouabain gave inconsistent results, with some slight diminution in reactivity. Similar results were obtained with strophanthidin. As discussed previously (19, 20), lack of substantial inhibition by ouabain in rat kidney is apparently attributable to the marked insensitivity of rodent kidney to cardiac glycosides (2), and to a reduction in affinity of the glycoside for its receptor in the presence of the high strontium concentrations (10–20 mM) employed in the procedure (17). In the glycoside-sensitive rabbit kidney (2), however, K^+ -dependent reaction product also is localized intensively to MATL (Fig. 9), but is strongly inhibited by ouabain (Fig. 10). In contrast to the outer medullary long loop DTL of the rat, little if any K^+ -NPPase activity could be resolved in rabbit DTL, even after prolonged incubation (Fig. 9). Morphologically, rabbit DTL resemble the short loop DTL of the rat (37), which were also unreactive.

Cytochemical Localization of K^+ -NPPase in Inner Medulla

Fig. 11 shows the abrupt transition between a highly reactive MATL and an ATL at the border between outer and inner medulla in the rat. This transitional area is shown in another tubule at higher magnification in Fig. 12. The ATL exhibits no enzymatic activity. ATL in deeper regions of the inner medulla are unreactive as well, even after prolonged incubation (Fig. 13). Similarly, DTL of long loop nephrons in rat inner medulla are unreactive (Figs. 11 and 13). These segments differ from their K^+ -NPPase-reactive DTL counterparts of the outer medulla (Figs. 3–6) in exhibiting little cellular interdigitation (44, 64). Collecting tubules in inner medulla showed little or no activity (Fig. 11). Nephron segments in rabbit inner medulla were also unreactive for K^+ -NPPase.

DISCUSSION

Localization of Na^+,K^+ -ATPase activity among the various renal tubules comprising rat and rabbit medulla was accomplished using a cytochemical method (17, 18) for the ultrastructural localization of K^+ -NPPase activity. Detailed consideration of the method, including discussion of criteria establishing its validity, and description of its application to a wide variety of transporting tissues from various species, has been the subject of several recent reviews (13, 20, 22, 24, 25). Earlier studies with the method in rat and rabbit kidney cortex (19) showed that enzymatic activity was present in abundance in

distal convoluted tubules, whereas late distal and cortical collecting tubules and initial (but not later) portions of proximal tubules were less reactive. In general, the distribution of enzymatic activity among cortical nephrons paralleled that determined by biochemical assays of microdissected tubules (38, 61, 62), and is confirmed by other studies in renal cortex where Na^+,K^+ -ATPase was localized with the K^+ -NPPase procedure (23, 59) and its modifications (6, 52), or by immunocytochemistry (46, 47). In the present study of rat and rabbit renal medulla, basolateral surfaces of MATL in both outer and inner stripes (Figs. 1–3, 9, 11, and 12) are highly reactive for Na^+,K^+ -ATPase, although the activity in the inner stripe segment is characteristically more intense than that in the outer stripe (cf. Figs. 1 and 3), or that in cortical straight and convoluted segments. In contrast, descending thick limbs (pars recta), like cortical straight proximal tubules (19), contain little activity (Fig. 1). These results are consistent with assays of the enzyme in microdissected segments (38, 61, 62) with the exception of distal convoluted tubules where Katz et al. (38) found surprisingly higher activities than in medullary (or cortical) ascending thick limbs.

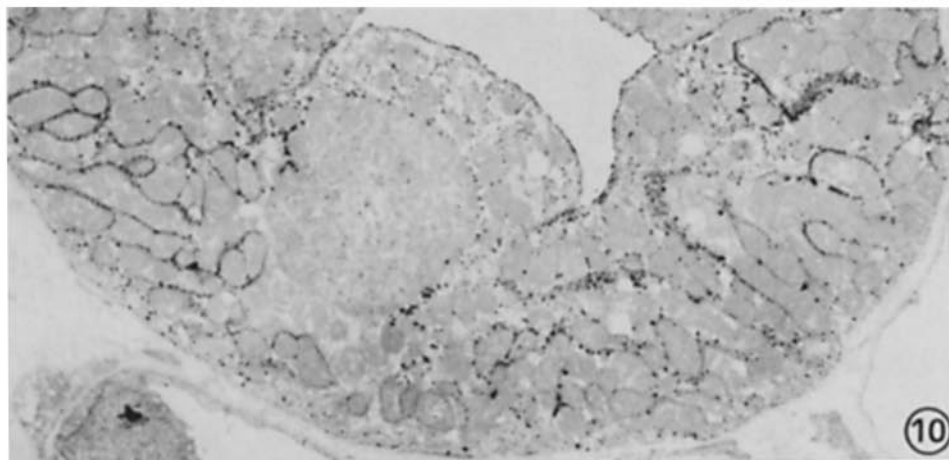
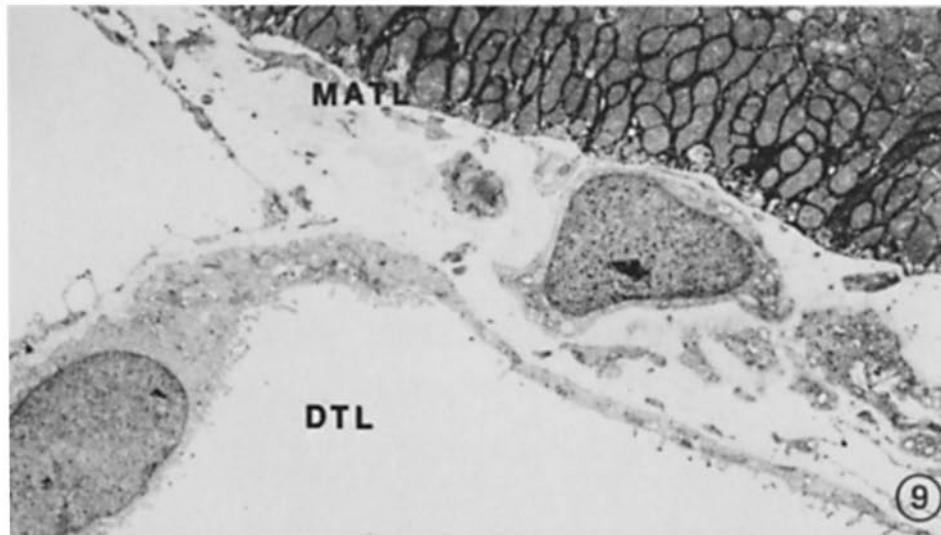
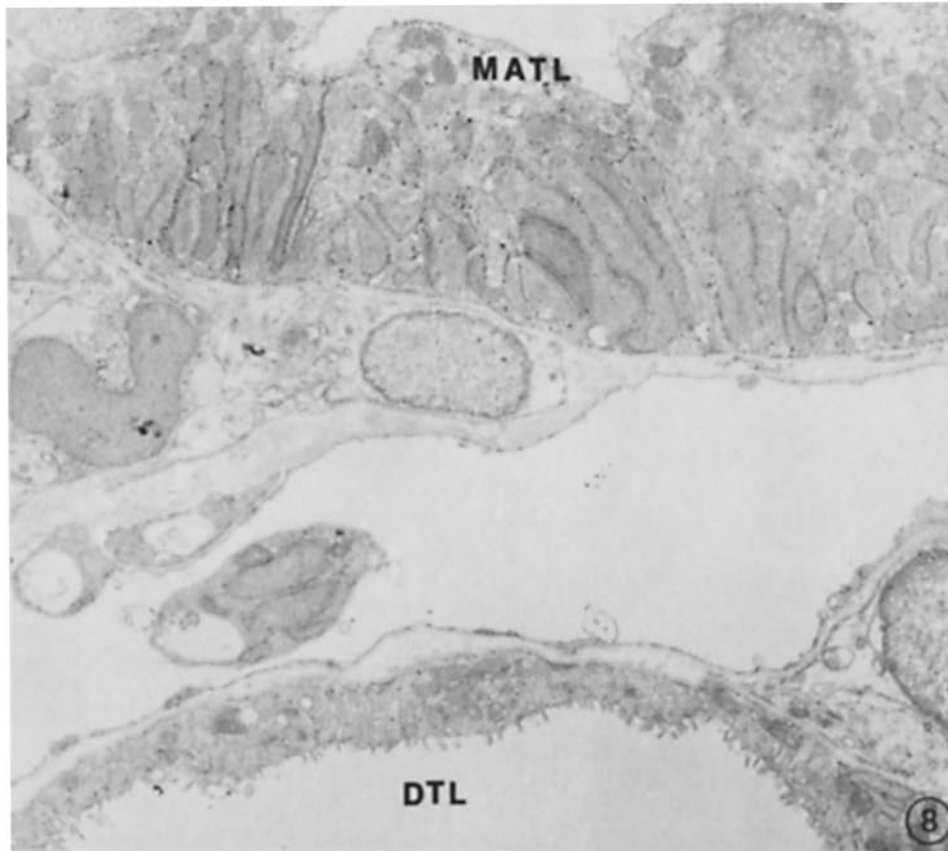
In addition to the highly reactive MATL, an initially unexpected finding in the outer medulla of the rat was a repeatable localization of comparatively low-to-moderate K^+ -NPPase activity along basolateral plasmalemmal surfaces of DTL of long loop nephrons in the inner stripe (Figs. 3–6). Deposition of reaction product exhibited all of the characteristics of the localization seen in renal distal segments (Figs. 1–3) and in other salt-transporting epithelia, namely, restriction to cytoplasmic surfaces of the plasma membrane (Figs. 5 and 6), dependence on the presence of K^+ (Fig. 8), and insensitivity to inhibitors of nonspecific (alkaline) phosphatase activity such as bromotetramisole (Fig. 3) and cysteine (Figs. 4–6). Inhibition of the staining reaction could not be demonstrated in an unequivocal and repeatable fashion with ouabain because of the relative insensitivity of rat renal Na^+,K^+ -ATPase to the glycoside (2), which is potentiated in the K^+ -NPPase cytochemical medium (17, 19). However, K^+ -dependent NPPase activity in tissues from glycoside-sensitive species are invariably ouabain-sensitive with this cytochemical method (17, 18, 20), and there is accordingly no reason to suspect that the K^+ -dependent activity of the rat DTL is attributable to an enzyme other than Na^+,K^+ -ATPase. Thus, whereas K^+ -NPPase of rat MATL is not inhibited substantially by ouabain, the identical K^+ -dependent activity in MATL of the glycoside-sensitive rabbit kidney (Fig. 9) is inhibited when ouabain is included in the medium (Fig. 10). In contrast to those of rat medulla, DTL of rabbit medulla were devoid of cytochemically demonstrable K^+ -NPPase activity (Fig. 9).

Although the observed activity in the DTL might merely function in the regulation of cellular ionic content, experience shows that the sensitivity of the cytochemical method (as well as the alternative light microscopic autoradiographic technique

FIGURE 8 When K^+ is deleted from the incubation medium, NPPase activity in rat outer medullary ascending thick limbs (MATL), and long loop descending thin limbs (DTL), is greatly reduced. Incubation was for 30 min in the presence of 10 mM cysteine. $\times 5,500$.

FIGURE 9 The basolateral surfaces of MATL in outer medulla of rabbit kidney are sites of intense K^+ -NPPase activity. Little if any activity is present, however, in adjacent DTL segments. Incubation was for 60 min in the presence of 10 mM cysteine. $\times 5,000$.

FIGURE 10 K^+ -NPPase activity in rabbit MATL was greatly reduced when incubations were conducted in the presence of 10 mM ouabain (compare with Fig. 9). Incubation was for 60 min in the presence of 10 mM cysteine. $\times 8,000$.



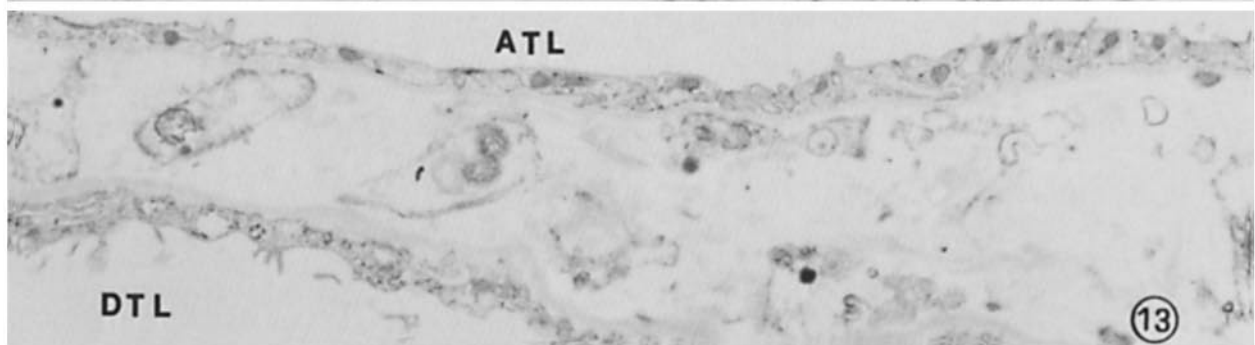
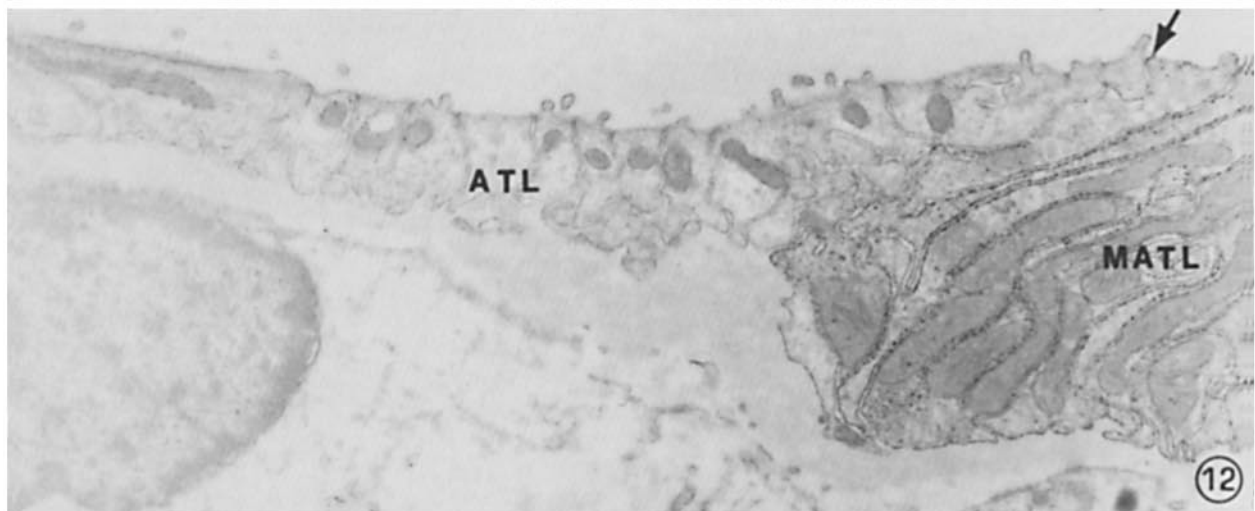
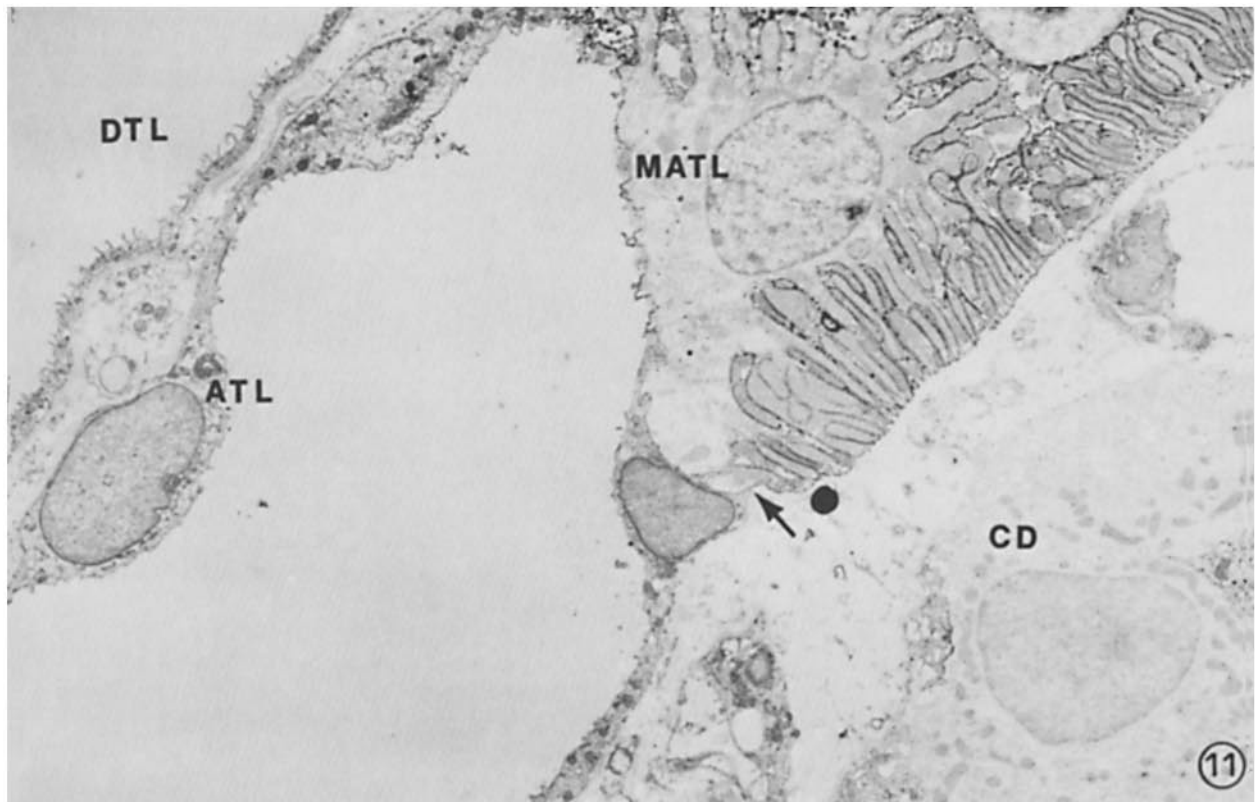


FIGURE 11 This micrograph shows the transition region (arrow) between an ascending thin limb (ATL) and its corresponding ascending thick limb (MATL) at the border between inner and outer medulla of the rat kidney. Although the MATL exhibits intense K^+ -NPPase activity, the ATL is unreactive. An inner medullary descending thin limb (DTL) and collecting duct (CD) are unstained. Incubation was for 30 min in the presence of 10 mM cysteine. $\times 4,500$.

FIGURE 12 The abrupt transition region (arrow) between a rat ascending thick (MATL) and thin (ATL) limb is shown at high magnification. K^+ -NPPase activity is present along the amplified basolateral surfaces of the MATL, whereas these surfaces in the ATL are unreactive. Incubation was for 30 min in the presence of 10 mM cysteine. $\times 14,000$.

FIGURE 13 Even after prolonged incubation (90 min), descending (DTL) and ascending (ATL) thin limbs of rat inner medulla are unreactive for K^+ -NPPase activity. Incubation was in the presence of 10 mM cysteine. $\times 8,500$.

with [^3H]ouabain) is such that successful results have only been obtained in transporting tissues where the specific activity of the enzyme is relatively high (13, 20, 22). Moreover, the absence of appreciable K^+ -dependent activity in other cortical (19) and medullary (Figs. 1, 4, 6, 7, and 11–13) segments argues against such an interpretation. Although Na^+, K^+ -ATPase activity has been demonstrated biochemically in microdissected rat outer medullary DTL (38), the observed activity was at the absolute limit of sensitivity of the method and was interpreted as being close to zero (15, 38). One explanation for the discrepancy between the biochemical and the cytochemical data may reside in tissue sampling. Although Katz et al. (38) did not state the type of outer medullary DTL assayed in their study, proximal segments were derived from superficial cortex and identified by their continuity with DTL. It seems likely, therefore, that DTL were also microdissected from superficial nephrons which, in contrast to the DTL of long loop juxtamedullary nephrons (Figs. 4–6), have no cytochemically demonstrable K^+ -NPPase activity (Fig. 7).

Although outer medullary DTL of rat long loop nephrons were sites of substantial K^+ -NPPase activity, their inner medullary counterparts were not reactive (Figs. 11 and 13). Similarly, ATL in the inner medulla were unreactive even after prolonged incubation (Fig. 13), while K^+ -NPPase activity at short incubation periods was pronounced in their tubular continuation as MATL at the transition region between inner and outer medulla (Figs. 11 and 12).

Functional Correlations

MEDULLARY ASCENDING THICK LIMBS: It is well-established that the medullary loop of Henle plays an important role in the development of a corticomedullary interstitial osmotic gradient that permits concentration of the urine in collecting ducts by equilibration with the hypertonic medullary interstitium (reviewed by Berliner [7]). Since fluid obtained from the bend of the loop in the papillary region is hypertonic, whereas early distal tubular fluid is hypoosmotic to plasma (29), the ascending limb is commonly assumed to be a major site of active transport. The rich basolateral localization of K^+ -NPPase in MATL is consistent with the view (cf. Jorgensen [36]) that the driving force for the countercurrent multiplication system, at least in outer medulla, is mediated by Na^+, K^+ -ATPase. Such a role is inferred, in part, from studies showing that renal infusion of ouabain elicits marked inhibition of medullary Na^+, K^+ -ATPase accompanied by a substantial decrease in free water clearance, free water absorption, and loss of the ability to produce either a concentrated or dilute urine (16, 50). Studies with isolated perfused MATL from rabbit kidneys show that NaCl reabsorption and transepithelial potential difference (lumen positive) are reduced by peritubular ouabain (11, 58). Although the lumen positive potential difference suggests "active" Cl^- transport from the tubular fluid, this does not preclude a direct role for Na^+, K^+ -ATPase in the transport processes in this segment. By analogy, serosal ouabain inhibits transport in other epithelia where NaCl uptake is thought to be mediated by a furosemide-sensitive NaCl cotransport system that is linked in an obligatory fashion to active Na^+ extrusion by Na^+, K^+ -ATPase (reviewed by Frizzell et al. [28]). Consonant with this view are the observations that Cl^- transport is abolished when luminal Na^+ is replaced with choline (10) and that luminal furosemide inhibits net NaCl transport and reduces the lumen positive potential difference (12). Jorgensen (36) has suggested that this potential difference

results from a local diffusion potential for Na^+ across cation-permeable tight junctions.

ASCENDING THIN LIMBS: While the contribution of MATL to outer medullary hypertonicity by active NaCl reabsorption is well-supported by the existing data, the mechanism by which solute is accumulated in the inner medulla is a subject of considerable controversy (see reviews by Berliner [7], Andreoli et al. [3], and Jamison and Robertson [35]), and constitutes a central focus in assessing recent models for urine concentration (8, 27, 42, 53, 66). Although it was assumed initially that ATL actively reabsorbed NaCl , repeated attempts by *in vivo* micropuncture and *in vitro* perfusion techniques (31, 32, 49, 54) failed to indicate active transport in this segment. Some of this data has been criticized (discussed by Marsh in Andreoli et al. [3]), and Marsh and Azen (48) have provided some positive evidence for weak active transport in ATL. However, reduction in tubular NaCl below interstitial values could be accomplished only at very low flow rates which, when coupled with the high permeability of ATL to Na^+ (31, 32, 48), suggest, as pointed out by Marsh (3), a highly inefficient active transport mechanism at best. Nonetheless, because of difficulties with strictly passive models for generating inner medullary hypertonicity (see below), and because of questions arising out of methodological approaches to *in vitro* and *in vivo* analyses of ATL transport processes, active NaCl transport in ATL is still considered necessary in many models of urinary concentration (27, 53).

In the current study, Na^+, K^+ -ATPase could not be demonstrated in ATL; the sharp transition from unreactive ATL to reactive MATL (Figs. 11 and 12) graphically illustrates the cellular specificity of the method and the precise regionalization of the enzyme's distribution. Although minor levels of Na^+, K^+ -ATPase may be present in ATL, as presumably are required for maintenance of cellular ionic homeostasis, the activity is certainly less than that of segments (e.g., descending thick limbs) where Na^+, K^+ -ATPase is barely detectable by cytochemical techniques (Fig. 1) or biochemical assay (38, 61, 62). We conclude that Na^+, K^+ -ATPase in ATL does not play a significant role in the medullary concentration process.

DESCENDING THIN LIMBS: To obviate the requirement for an active transport capability in ATL, Kokko and Rector (42) proposed a passive model for countercurrent multiplication in inner medulla. In this formulation, DTL must have low solute permeability and high water permeability. Although studies with perfused tubules from rabbits indicated that solute concentration in DTL did indeed occur primarily by removal of water (40, 41), *in vivo* and *in vitro* studies with rodent DTL have shown consistently that solute concentration occurs by a combination of solute addition and water abstraction (33, 34, 54, 56, 60). Significant addition of solute in DTL is incompatible with the passive model as discussed recently (3, 35, 53). Regardless of the mode of solute concentration, however, salt transport in this segment has been assumed to result from strictly passive processes governed by the permeability characteristics of the thin limb to salt, water, and urea. In this regard, Kokko (40) could find no evidence for active NaCl transport in isolated perfused rabbit DTL. Viewed in this context, the localization of significant Na^+, K^+ -ATPase activity in outer medullary DTL segments of rat long loop nephrons (Figs. 3–6) is of considerable interest.

Interpretation of *in vitro* and *in vivo* physiological data is complicated by the presence of two populations of DTL (short and long loops), subdivision of DTL of long loops into outer

and inner medullary segments, and the possibility of species differences in DTL permeability properties. The latter, as discussed by Berliner (7), may account for some of the contrasting data regarding the mode of NaCl concentration in this limb. Particularly relevant to the interpretation of our results is the observation that striking structural differences exist in various portions of DTL, especially in rodent kidney (14, 44, 63, 64). In particular, for juxtamedullary nephrons, the DTL in outer medulla of the rat is unique in that it is characterized by extensive interdigitations of basolateral surfaces of adjacent cells (a hallmark of ion-transporting epithelia), extremely shallow occluding junctions, and a high ratio of junctional length to apical surface area. In contrast, the inner medullary portion exhibits little basolateral plasmalemmal interdigitation and comparatively extensive occluding junctions, morphological features that it shares with the DTL of short loop nephrons. The cytochemical data presented here indicate that only the highly specialized DTL of outer medullary long loops are sites of significant Na^+, K^+ -ATPase activity.

The basolateral plasmalemmal localization in the outer medullary DTL may be associated with either reabsorptive or secretory NaCl transport since this same pattern of localization of the Na^+ pump is almost invariably seen, regardless of the direction of net salt transport (13, 20, 22). There is little evidence to support Na^+ reabsorption in this segment, whereas considerable data is available that is consistent with solute entry in rodents (33, 34, 54, 56, 60). We propose that at least a portion of the observed NaCl entry (secretion) may be active and mediated by the Na^+, K^+ -ATPase of outer medullary DTL segments of long loop nephrons that are adjacent to MATL (see Fig. 3) and therefore positioned anatomically for possible solute recycling. An active salt secretory role for outer medullary DTL also was suggested previously on the basis of elegant morphological studies by Kriz and collaborators (14, 44, 45). Although drawing physiological conclusions from strictly morphological data should be tempered with caution, it is at least intriguing that the characteristics of this segment in the rat (shallow junctions, high ratio of linear junctional length to apical surface area, elaborate basolateral membrane amplification associated with Na^+, K^+ -ATPase activity) are precisely those we have described as characteristic for hypertonic secretory epithelia (see discussion and references in Ernst et al. [21]), including the presence in DTL of a single pair of closely juxtaposed (rather than netlike) junctional strands (1, 57). In contrast to the rat, Na^+, K^+ -ATPase could not be resolved cytochemically in rabbit DTL (Fig. 9). Kaissling and Kriz (37) showed that DTL in the rabbit morphologically resemble the (unreactive) rat DTL of inner medulla (Figs. 11–13) and of short loop segments in outer medulla (Fig. 7). Consistent with the apparent absence of Na^+, K^+ -ATPase in the rabbit segment, Kokko (40) found no evidence for active salt transport in perfused DTL, and Shaver and Stirling (65) demonstrated that this segment did not bind [^3H]ouabain.

The relatively high concentration of Na^+, K^+ -ATPase in rat outer medullary DTL is not what would be predicted by the model for the renal concentration mechanism proposed by Kokko and Rector (42). Discrepancies in physiological data with regard to passive models may be explained by such things as species differences, experimental technique, or as pointed out above, by the fact that outer medullary long loop DTL of rodents differ morphologically, and perhaps physiologically, from those of rabbits. In light of these discrepancies, Bonventre and Lechene (8) recently proposed a model for urinary con-

centration that was designed to be consistent with the in vitro and in vivo micropuncture data from medullary structures of rat, hamster, and sand rat. A central and unique feature of this model is the hypothesis that the DTL fluid entering inner medulla at the outer-inner medullary junction is hypertonic to the surrounding interstitium. In an illustration of their model, they demonstrated that urine could be concentrated to 2,200 mosmol/kg with only a 33 mosmol/kg difference in osmotic pressure between DTL fluid and the interstitial compartment at the boundary of outer and inner medulla. This study assumed purely passive transport processes in the inner medulla involving solute addition and water abstraction in the DTL, and solute reabsorption in the ATL. Although direct measurement of DTL tonicity is clearly required to assess the efficacy of this particular hypothesis, the data presented here are consistent with this model in at least providing evidence for the existence of the requisite enzymatic machinery in outer medullary DTL to effect such an elevation in tubular osmolarity at the outer-inner medullary junction. In common with both the models of Bonventre and Lechene (8) and of Kokko and Rector (42), the absence of appreciable Na^+, K^+ -ATPase in inner medullary DTL and ATL would be consistent with the proposed passive nature of the transport processes in this region. If Na^+, K^+ -ATPase in outer medullary DTL does play a role in urine concentration in rodents, its apparent absence in significant quantity in rabbit DTL remains to be explained, although it should be noted that this might be correlated with the relatively poor concentrating ability of this species. Quantitative data, using alternative techniques, are required to more fully define a functional role for Na^+, K^+ -ATPase in DTL from various species.

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