Adenosinetriphosphatase Activity in the Cell Membranes of Kidney Tubule Cells*

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ABSTRACT

This cytochemical study demonstrates high levels of apparent ATPase activity in the infolded cell membranes of the proximal tubules (dog, rat, human, mouse, monkey, and opossum) and ascending loops of Henle (dog, rat, human and, to a variable degree, mouse). Electron microscopy has shown (see Rhodin (1)) that these membranes separate adjacent cells where they interlock with one another by multiple cytoplasmic lamellae containing oriented mitochondria. The significance of the high ATPase activity is considered in relation to possible movements of the membranes and to "active transport" believed to occur there.

In the rat, enzyme activity in the proximal tubule membranes does not survive formol-calcium fixation, and it is therefore necessary to use unfixed sections for its demonstration. However, in edematous rats with experimental nephrosis (induced by the injection of aminonucleoside or with antikidney serum) marked ATPase activity is exhibited in these membranes even after formol-calcium fixation.

When proximal tubule or Henle loop cells of the dog acquire an altered metabolism, as indicated by accumulated lipide spheres or by "droplets," the infolded ATPase-rich membranes are no longer demonstrable.

INTRODUCTION

In their electron microscopic study of the proximal tubule, Sjöstrand and Rhodin (2) described "double membranes" which incompletely divided the cytoplasm into compartments containing mitochondria. These were shown by Rhodin (3) to be deep infoldings of the plasma membrane where it borders the basement membrane. Pease (4) demonstrated that this membrane system is even more highly developed in the distal tubule. Pease (4) and Ruska *et al.* (5) emphasized that the invaginated plasma membranes move apart as fluid accumulates in the extracellular space between cytoplasmic compartments. The mechanical aspect of this suggestion does not appeal to Rhodin (1) who, nonetheless, considers it likely that the "lamellated cytoplasm is necessary for resorptive processes, possibly with the intercellular space serving as a pathway for fluid resorbed on the brush border side of the tubular cells." With simplified three-dimensional reconstructions, Rhodin (1) clarified the status of the infolded cell membranes in relation to the "lamellated cytoplasm": "They are thus in fact not intracellular membranes (Sjöstrand and Rhodin, 1953) but infoldings of the cell membrane (Rhodin, 1954) and represent the borders between different cells, the surface of contact where the cells meet (Rhodin, 1958 *a*, *b*)."

The cytochemical staining studies reported here reveal high levels of ATPase[1] activity in these

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[1] Abbreviations: ATP: Adenosine-5′-triphosphate. ATPase: Adenosinetriphosphatase. A5′P: Adenosine-5′-monophosphate. ADP: Adenosine-5′-diphosphate.

infolded membranes. High concentrations of this enzyme have also been demonstrated in the cell membranes lining the "extracellular channels" of a transplantable rat liver tumor (6) and in the cell membranes of hepatic cells where they form the microvilli within the bile canaliculi (7).

Materials and Methods

Dog Kidney.—Tissues were obtained from 2 presumably healthy dogs which were anesthetized with 25 mgm./kg. of intravenous nembutal. Tissues were prepared as follows: (*a*) Quick freezing of pieces 2 to 3 mm. in thickness in a beaker of isopentane surrounded by a solid carbon dioxide-isopentane mixture at −70°C. The frozen tissue was sectioned on the same day at −18°C. in a cryostat (8). Sections were cut at 5 μ, attached to slides by the warmth of a finger tip, and stored in the cryostat until ready for staining. (*b*) Fixation, overnight, in formol-calcium (9) at 3–5°C. Sections were cut into cold formol-calcium at 5 μ on a B&L freezing clinical microtome and stained immediately or within a day after sectioning.

Abnormal kidneys with hydronephrotic changes were obtained from 3 dogs with unilaterally ligated ureters[2]. The contralateral, unligated kidneys were used as "controls."

Rat Kidney.—Tissues were obtained under light ether anesthesia from Sprague-Dawley male rats, weighing 75 to 200 grams. Slices 2 mm. in thickness were cut and prepared for sectioning by the procedures used with the dog kidney.

Abnormal kidneys were obtained from 8 rats injected with daily subcutaneous doses of aminonucleoside, 5 mgm./100 gm. rat, for 10 days, and from 4 rats injected intravenously with potent anti-rat kidney antibodies.[3] These procedures produced nephrosis with edema, ascites, and marked proteinuria.

Human Kidney.—Tissues obtained from surgical specimens were fixed 15 to 60 minutes after clamping the renal pedicle and also from a postmortem specimen shortly after an accidental death. Tissue preparation procedures were those employed for the dog and rat.

Other Species.—Additional kidney tissues were obtained from the monkey (following a prolonged surgical procedure), mouse and opossum, and were prepared in the manner outlined for the rat, after formol-calcium fixation.

Staining Procedure for ATPase.—Generally we employed the method of Wachstein and Meisel (10). The pH of the reaction is 7.2 and lead ions are used to trap phosphate hydrolyzed from the ATP by enzyme in the section. The method of Padykula and Herman (11) was also employed; although it gave similar staining of the infolded cell membranes, we are not reporting the results because the reaction is performed at alkaline pH. The specificity of the visualized enzyme was examined by: (1) substituting adenosine-5′-phosphate and glycerophosphate for ATP in the Wachstein-Meisel medium, and (2) staining sections for alkaline phosphatase (pH 9.4) and acid phosphatase (pH 5.0) according to the methods of Gomori (12). Controls were always used in which substrate was omitted from the incubation mixture. Additional controls sometimes employed were: (*a*) incubation of heat-inactivated sections in direct contact with unheated active sections; (*b*) incubation of heat-inactivated sections in the complete substrate mixture with the addition of liver homogenate.

Neutral lipide was stained by 0.5 per cent Oil Red O in 60 per cent triethylphosphate for 10 minutes (12). This was frequently done following visualization of the sulfide in the phosphatase methods. Phospholipide was stained by Baker's acid-hematin method (13).

Sections were mounted in aqueous glycerogel medium except for the acid-hematin and hematoxylin-eosin preparations, which were mounted in permount.

RESULTS

As stressed by Wachstein (14), striking differences in histochemical staining reactions were observed in presumably normal kidneys of different species.

Dog Kidney.—Table I summarizes the results for ATPase staining in two dog kidneys. In dog A, infolded membranes of proximal tubule cells had slightly more enzyme activity than those of dog B. In both animals, the infolded membranes were more numerous and tortuous in the ascending loops of Henle than in the proximal tubules (Fig. 1).

Proximal tubule cells possessed spherical and rod-shaped "droplets" of varying size which gave a strong reaction for ATPase. In some tubules their elongate nature and size suggested a transformation from mitochondria (15). Where such "droplets" were present in large numbers, ATPase-stained membranes were not present (Table I). In dog B, some cells of the ascending loop of Henle showed marked accumulations of neutral lipide. In such lipide-filled cells, ATPase-rich membranes could not be detected; adjacent lipide-poor cells showed the usual membranes with ATPase activity

[2] We are indebted to Dr. Bruno Volk of the Department of Pathology who performed the surgery, with the assistance of students in the Sophomore Pathology course.

[3] Nephrotic rats injected with antikidney antibody were generously furnished by Dr. Beatrice Seegal of Columbia University, College of Physicians and Surgeons.

TABLE I

Staining Reactions in Dog Kidney

Tissue preparation method	Proximal tubule		Ascending loop of Henle		Distal tubule	Capillaries	Glomeruli
	Infolded cell membranes	Droplets	Infolded cell membranes	Lipide	Cell membranes	Endothelium	Basement membranes
Formol-calcium fixation							
Dog A	+++–0	0–++	+++	0	0	>+++	+
Dog B	++–0	0–++	++–0	0–+++	0	>+++	+
Cryostat sections							
Dog A	++*–0	0–++	++*	0	0	>+++	+
Dog B	+*–0	0–++	+*–0	0–+++	0	>+++	+

ATPase staining gradations in dog kidney:
0 = No staining.
+ = Faint brown precipitate.
++ = Dark brown precipitate.
+++ = Opaque brown precipitate.
Lipide staining is graded from 0–+++ on the basis of the number and size of lipide droplets present.
* = Cryostat sections showed some morphologic damage to, and decreased staining intensity of the infolded cell membranes.

TABLE II

Staining Reactions in Rat Kidney

Tissue preparation method	Proximal tubule		Ascending loop of Henle	Distal tubule	Capillary walls	Glomeruli
	Infolded cell membranes	Droplets	Infolded cell membranes	Cell membranes	Endothelium	Basement membrane
Formol-calcium fixation						
(a) Normal rats	0	0	++++	0	++++	++++
(b) Edematous rats (aminonucleoside)	+++	0	++–++++*	0	++++	++++
(c) Edematous rats (antikidney serum)	++	0–+	++–++++*	0	++++	++++
Cryostat sections						
(a) Normal rats	+–++	0–+	++++	0	++++	++++

ATPase staining in the rat kidney.
Staining gradations are:
0 = No stain.
+ = Faint brown precipitate.
++ = Darker brown precipitate.
+++ = Brownish black, opaque precipitate.
++++ = Opaque black precipitate.
* = Atrophic tubules had attenuated membranes with decreased ATPase activity.

(Fig. 2). In the atrophic tubules of hydronephrotic kidneys, stained membranes were not observed.

In the acid-hematin preparations, the mitochondria appeared as elongate black threads of varying length in the basal portion of the cells of the proximal and distal tubules. By careful focusing, they could be seen to lie within compartments outlined by *unstained* membranes (Figs. 3 and 4).

Capillaries stained heavily for ATPase activity, while glomeruli were weakly stained. The stain appeared to be in the endothelium of the former and in the basement membrane of the latter. The brush borders of the proximal tubules were lightly stained.

In the renal medulla, membranes of the collecting duct cells were unstained. In striking contrast, all capillaries adjacent to these ducts were strongly positive.

Rat Kidney.—ATPase staining in rat kidney is summarized in Table II.

In this species, ATPase activity was seen in the infolded cell membranes of the ascending loops of Henle and occasionally in some segments of the distal tubule. As in the dog, the collecting ducts in the medulla were unreactive, while the adjacent capillaries reacted strongly (Fig. 7).

Proximal tubule cells did not show stained membranes after formol-calcium (Fig. 5), but did so in cryostat sections of unfixed tissue (Fig. 6). If such cryostat sections were treated with cold formol-calcium for 5 minutes prior to staining, enzyme activity was lost in the proximal tubule membranes, but not in the cell membranes of the ascending loops of Henle. Brush borders, capillary endothelium, and glomerular basement membranes were strongly positive in both unfixed and fixed sections.

The outstanding alteration noted in rats made edematous by injection of aminonucleoside or of antikidney antibody was the intense ATPase activity in the infolded membranes of proximal tubule cells, even in sections prepared from formol-calcium–fixed tissue (Figs. 8 to 10). In the atrophic tubules of the nephrotic rats the membranes appeared attenuated and showed less ATPase activity.

Human Kidney.—High ATPase activity was

present in the infolded cell membranes of the proximal tubule and in the ascending loop of Henle (Fig. 12). Capillaries stained strongly, whereas glomeruli were unstained.

Other Species.—In the opossum (Fig. 11), monkey, and mouse, staining for ATPase activity occurred primarily in the infolded membranes of proximal tubule cells. Mouse kidney showed slight activity in some cells of the ascending loops of Henle. Capillary walls were stained in all species, as were the brush borders of the proximal tubules.

Measurements on sections stained for ATPase activity or by the acid-hematin procedure permits an approximation of the width of the interlocking cytoplasmic compartments. It is about 0.8 μ in the rat, 0.5 μ in the mouse, and 1.0 μ in the dog. Based on but few measurements, this can be used only to suggest an order of magnitude. As such, it compares not unfavorably with the distance measured in electron micrographs, 0.4 to 1.1 μ in the mouse (from the micrographs of Rhodin (1)), and 0.5 to 1.0 μ in the rat (from the micrographs of Ruska *et al.* (5)).

Sections incubated with A5′P substituted for ATP at pH 7.4 (Fig. 15), or with glycerophosphate at pH 7.4 and 9.4 (Figs. 13 and 14) gave staining only in the brush borders of the proximal tubules. Cell membranes were also negative in all species with glycerophosphate at pH 5.0; in the dog and rat cytoplasmic "droplets" and occasional nuclei were stained (Fig. 16). Sections incubated without substrate did not show staining. Heat-inactivated sections (rat kidney) incubated in direct contact with active sections did not adsorb stain, nor did staining occur when heat-inactivated sections (rat kidney) were placed in buffered ATP medium to which liver homogenate was added as enzyme source.

DISCUSSION

Phosphatase activity, with ATP as substrate, has been localized in the infolded plasma membranes of cells in several segments of the renal tubules of a number of mammalian species. With A5′P or glycerophosphate, no such staining was obtained, at pH 7.2, 9.4 and 5.0. This suggests that the enzyme visualized with ATP as substrate is a specific ATPase. Novikoff *et al.* (16) have examined more extensively the substrate-specificity of the phosphatase revealed in bile canaliculi of rat liver with ATP as substrate, and have concluded it probably is ATPase which is visualized by the method employed, rather than non-specific phosphatase (see also (7)).

The apparent ATPase activity shows considerable variation both in localization and intensity among the six species studied. To what extent the physiologic state of the animal affects this activity is not known. Failure to demonstrate enzyme activity by this staining method cannot be taken to mean *absence* of enzyme. Thus, the plasma membranes may have physiologically significant levels of ATPase activity *in vivo* and yet be unstained in our preparations because the initial level is too low to produce sufficient reaction product in the presence of inhibitory lead, following freezing. On the other hand, darkly stained areas of the plasma membrane may confidently be said to have high enzyme concentration in the living cell; this is especially true if the stain shows in fixed tissue since fixation may markedly diminish ATPase activity (16). Whether the enzyme actually functions as a hydrolase in the cell is uncertain (17); in isolated mitochondria of liver and heart, the evidence suggests a role in coupling phosphorylation to oxidation (18).

Modern electron microscopy suggests that the cell membranes, in the kidney as in other tissues, are actively changing; such changes can be expected to involve utilization of ATP energy. Cytoplasmic vesicles which appear to arise by invagination of cell membranes much like the pinocytosis vacuoles demonstrated in the cinematographic studies of Lewis (19), Gey (20), and Frederic and Chèvremont (see (21)), have been demonstrated by Moore and Ruska (22), Palade (23), and Bennett (24). The possibilitv has been raised by Bennett (24) that, even without intake of fluid, motion of the cell membrane may occur and carry in molecules bound to receptor sites.

Christensen *et al.* (25) have pointed to the prodigious amount of pinocytosis activity that would be required (in the ascitic cells studied) to account for the glycine uptake from the medium, and have concluded that pinocytosis transfer could not account for such uptake. They also stress that even where pinocytosis occurs, a mechanism is still required to transfer the molecules *across* the cell membrane so long as it remains intact.

The high ATPase activity we have observed in specialized areas of the plasma membrane may be associated with either or both types of membrane activity, membrane movement and "active transport" of molecules across the membrane. As al-

ready indicated, lower enzyme activity might be present elsewhere in the cell membrane and may not be revealed by our methods.

In the kidney, the proximal tubule is the presumed site of active transfer of large quantities of solute and water. Here there exists an intimate association of infolded cell membranes and mitochondria. Indeed, electron micrographs indicate virtual contact between the two, particularly when the "labyrinth space" is distended by fluid (5). This would bring the membranes with high ATPase activity in contact, or near contact, with the ATP-synthesizing mitochondria over a wide area. In these regions of the cells there is an enormous area where intracellular space and extracellular space are separated only by the plasma membrane.

Rats with nephrotic edema and ascites, experimentally induced by aminonucleoside or kidney antibody, manifested an apparent increase in ATPase activity in the infolded membranes of the proximal tubule cells. We do not know whether this represents a different form of ATPase (more resistant to fixation) or an increased level of the ATPase already present.

On the other hand, the ATPase-rich infolded membranes seem to disappear from tubule cells in which gross alteration of metabolism occurs. In the dog, when large numbers of "droplets," perhaps arising from mitochondria (*cf.* (15)), were present in the *proximal tubules* or when large numbers of fat droplets were present in the cells of the *ascending Henle limbs*, the infolded membranes were no longer visible.

In three species studied (dog, rat, human) it is in the ascending loop of Henle that the infolded cell membranes show the highest level of ATPase activity. This is the portion of the tubule in which the interlocking cytoplasmic lamellae reach their highest state of complexity (Rhodin (1)). Here, too, the mitochondria show the greatest concentration of inner membranes (1) and the highest levels of oxidative enzyme activity (26). It may be expected that ATP is synthesized at a high rate by the mitochondria of these cells.

Wirz (27) has compared the arrangement of tubules and capillaries in the renal medulla to a counter-current distribution system in which the osmotic gradient is considered to be maintained by an active process. It is therefore of interest that ATPase activity is so high in the capillaries of this region (Fig. 4).

Thus, the results we have described place ATPase activity in areas of potential importance in molecular transport, in the infolded membranes lying close to the mitochondria and in the capillary bed. Recent observations on other tissues, with the same methods as employed here, are consistent with a role of the enzyme in active molecular transport (6, 7).

We wish to acknowledge the assistance of Mr. Jay Walker with the photomicrography, and Mr. Vincent Drapiewski with the preparation of sections of the rat kidneys.

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Explanation of Plates

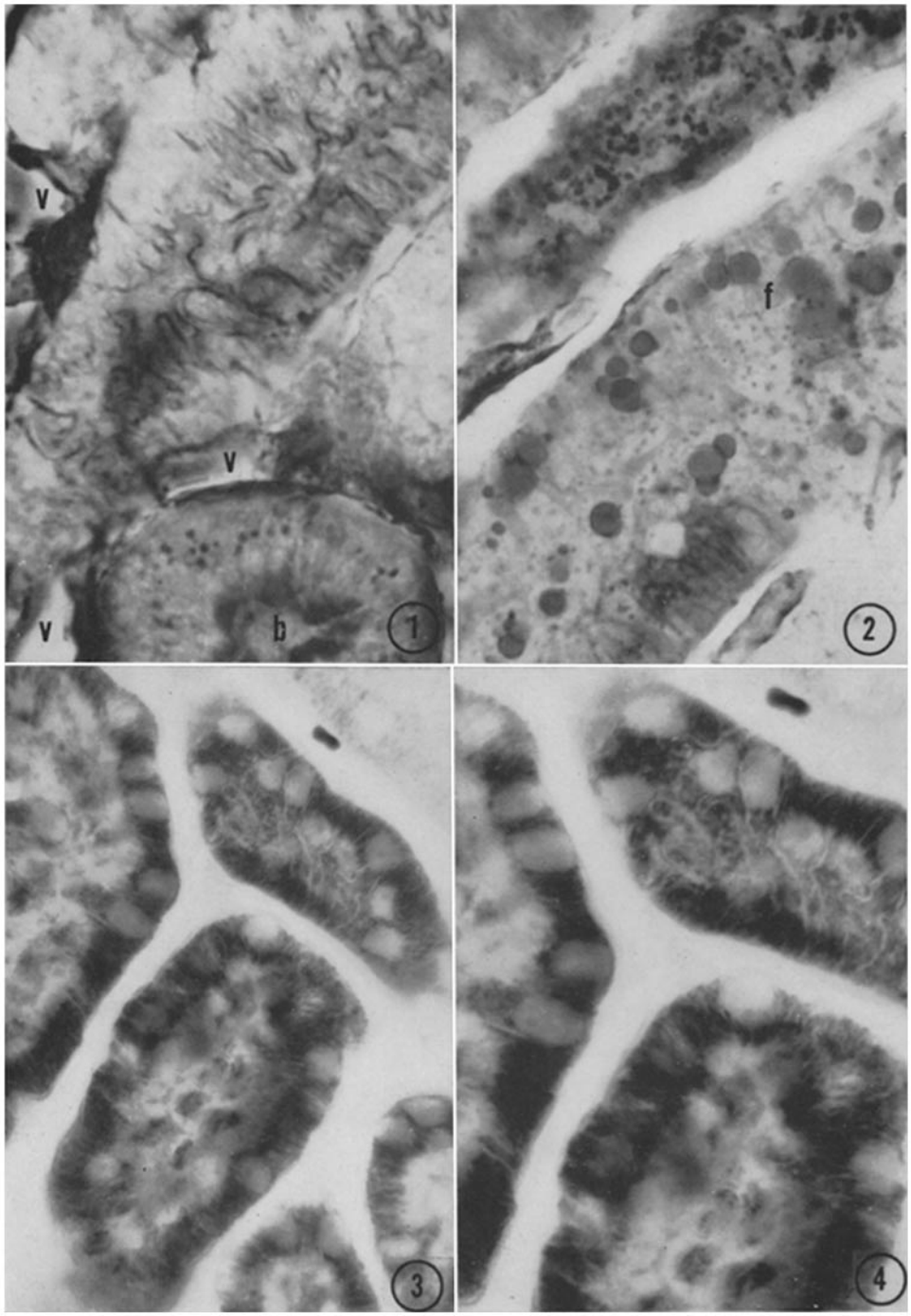
Plate 388

Fig. 1. Dog kidney, formol-calcium fixation, incubated 15 minutes with ATP at pH 7.4. The ascending loop of Henle has numerous infolded membranes with high ATPase activity. In the adjacent proximal tubule, stained brush border (*b*), and ATPase-stained "droplets" are present. Blood vessels (*v*) exhibit ATPase activity. Magnification, 1,200.

Fig. 2. Dog kidney, formol-calcium fixation, incubated 15 minutes with ATP at 7.4 and then stained for lipide with Oil Red 0. Note that where tubule cells contain large quantities of fat (*f*), there are no ATPase-rich membranes. At lower part of figure, the tubule cells have fewer lipide droplets and here the membranes are stained. Magnification, 1,200.

Fig. 3. Dog kidney, acid-hematin stain. The infolded membranes are clearly seen, as unstained lines. In areas the darkly stained mitochondria may be seen in compartments delimited by the membranes (see Fig. 4). Magnification, 1,200.

Fig. 4. Dog kidney, acid-hematin stain. Portion of Fig. 3 enlarged to show details of infolded membranes. Magnification, 1,800.



(Spater *et al.*: ATPase activity in cell membranes)

PLATE 389

FIG. 5. Rat kidney, formol-calcium fixation, incubated 15 minutes with ATP at pH 7.4. Proximal tubule, upper left, shows stained brush border (*b*); cell membranes are unstained. The ascending loops of Henle are densely packed with stained membranes which show clearly in some areas (arrow). Beneath Henle's loop is a deeply stained capillary (*v*). Magnification, 1,200.

FIG. 6. Rat kidney, unfixed cryostat section, incubated 15 minutes with ATP at pH 7.4. Proximal tubule with brush border (*b*). Stained infolded membranes are clearly evident (arrows). Blood vessels (*v*) stain very darkly. Magnification, 1,200.

FIG. 7. Rat kidney, formol-calcium fixation, incubated 15 minutes with ATP at pH 7.4. Collecting ducts in the renal medulla are unstained. Adjacent capillaries show high ATPase activity. Magnification, 1,200.

FIG. 8. Nephrotic rat kidney (aminonucleoside-injected), formol-calcium fixed, incubated 15 minutes with ATP at pH 7.4. Marked enzyme activity is present in the infolded membranes of the ascending loops of Henle. Magnification, 1,200.

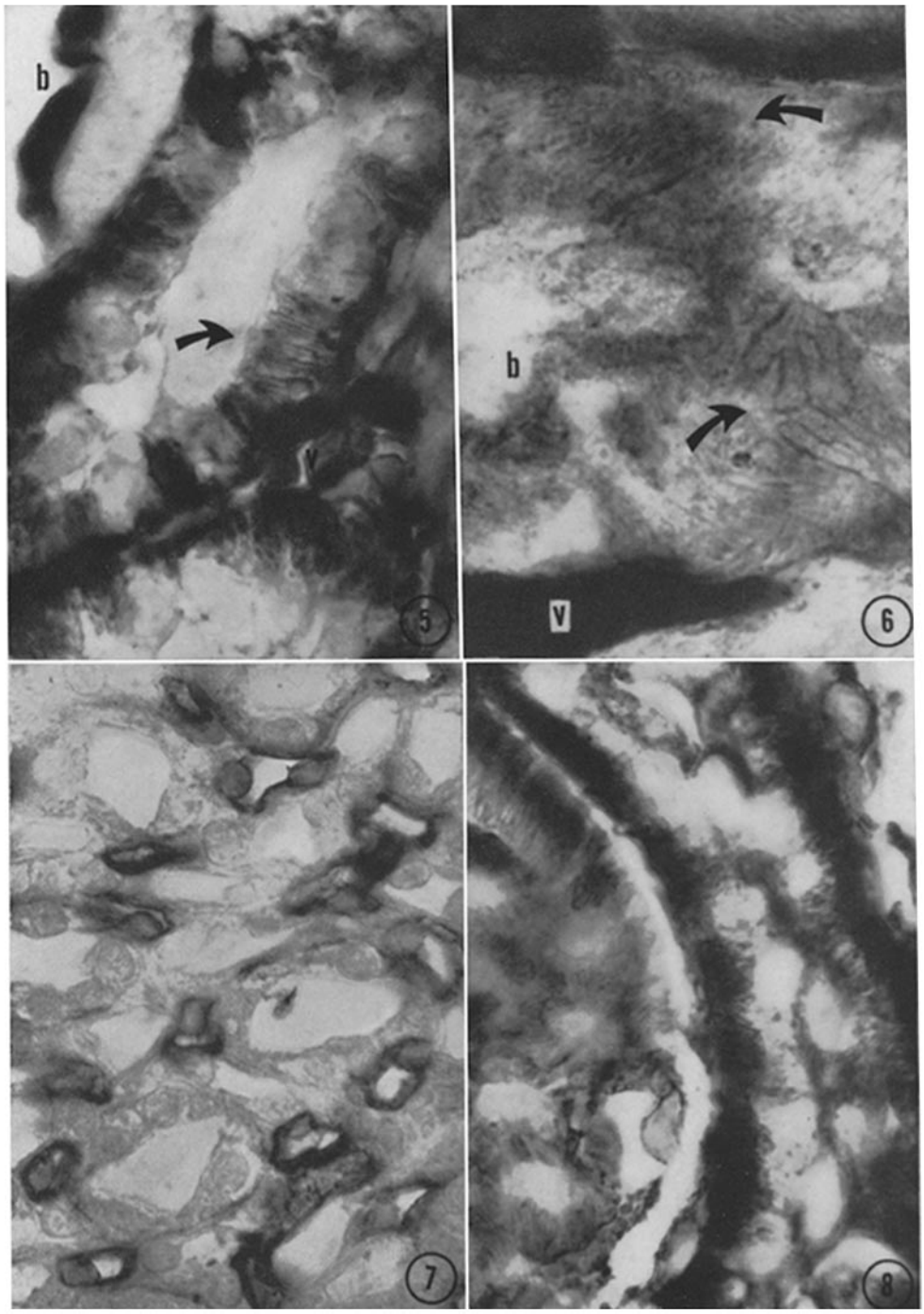


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PLATE 390

FIG. 9. Nephrotic rat kidney (aminonucleoside-injected), formol-calcium fixation, incubated 15 minutes with ATP at pH 7.4. Longitudinal section of proximal tubule is shown. Infolded membranes exhibit marked ATPase activity (see Fig. 10). Blood capillaries (*v*) and brush border (*b*) are stained; nucleus (*n*) is unstained. Magnification, 1,200.

FIG. 10. Nephrotic rat kidney (aminonucleoside-injected). Portion of Fig. 9 enlarged to show details of infolded membranes. Magnification, 1,700.

FIG. 11. Opossum kidney, formol-calcium fixation, incubated 15 minutes with ATP at pH 7.4. Proximal tubule shows darkly stained brush border as well as stained infolded membranes. Magnification, 1,200.

FIG. 12. Human kidney, formol-calcium fixation, incubated 15 minutes with ATP at pH 7.4. Deeply stained infolded membranes are visible at center and near bottom. Magnification, 1,200.

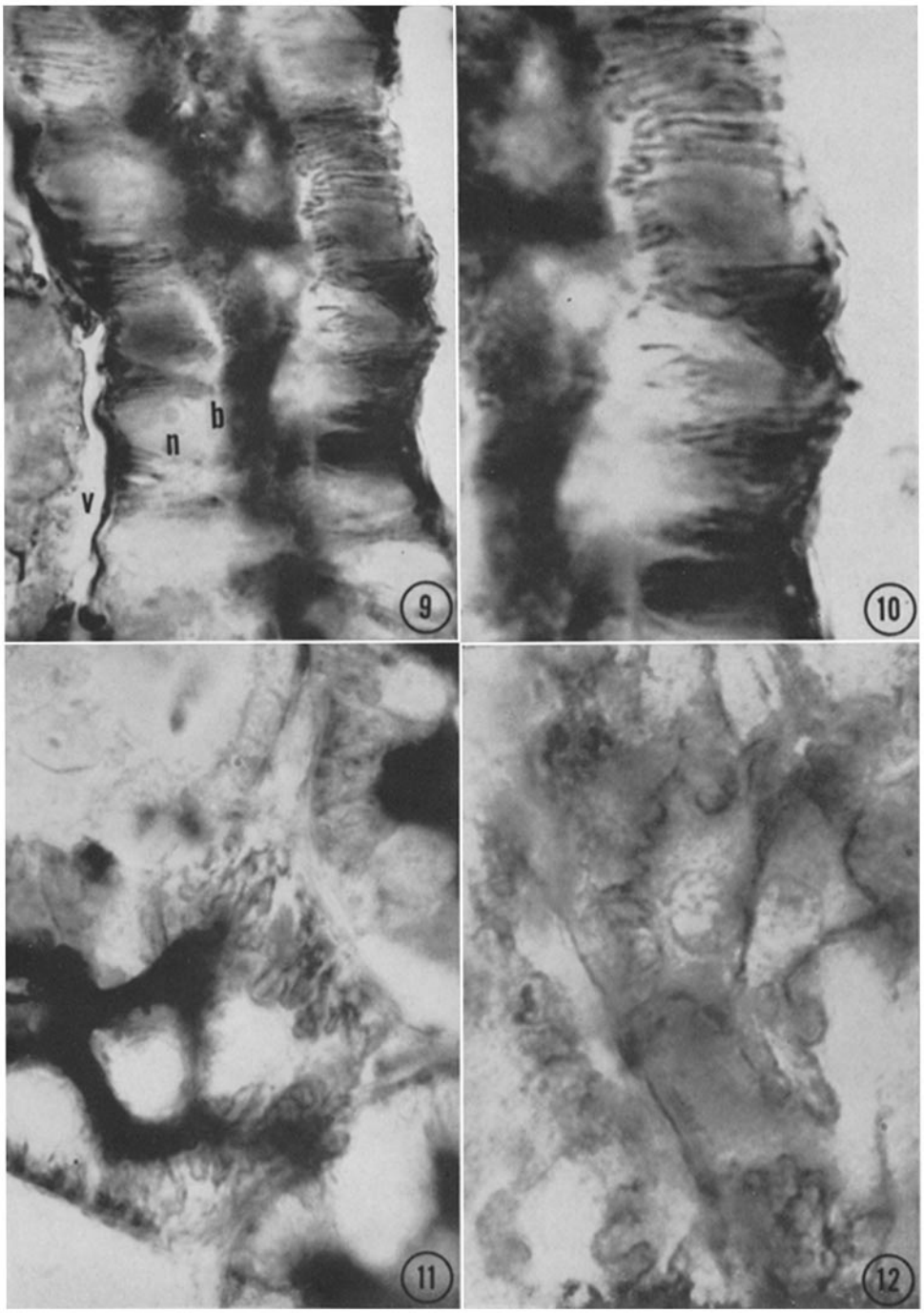

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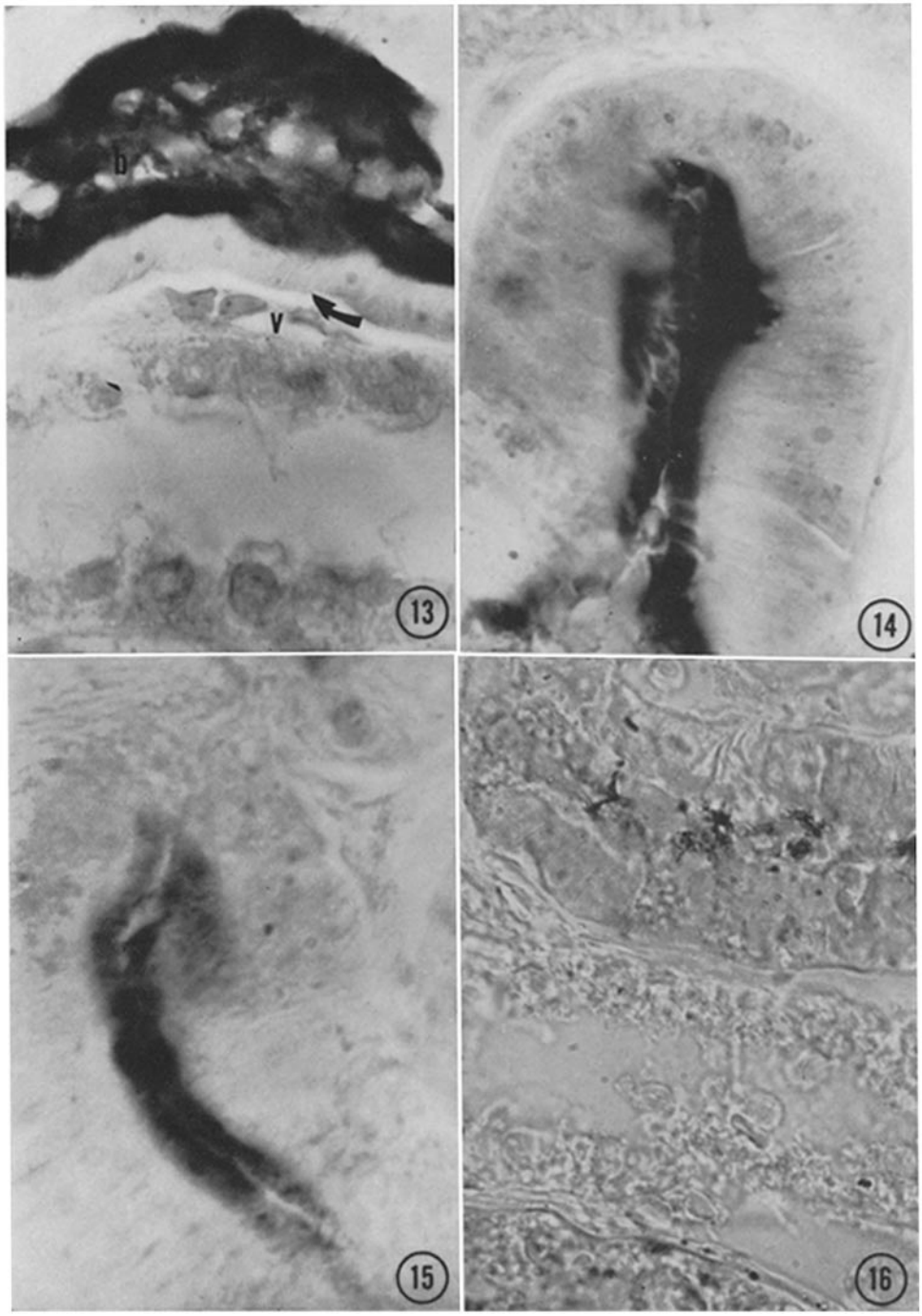
PLATE 391

FIG. 13. Dog kidney control section, formol-calcium fixation. Incubated 15 minutes with glycerophosphate at pH 9.4. Brush border (*b*) is darkly stained, but the cell membranes (arrow) and capillaries (*v*) are unstained. Magnification, 1,200.

FIG. 14. Dog kidney, formol-calcium fixation. Incubated 15 minutes with glycerophosphate at pH 7.4. Brush border is stained. There is no sign of staining in the cell membranes. Magnification, 1,500.

FIG. 15. Dog kidney, formol-calcium fixation. Incubated 15 minutes with adenosine-5′-monophosphate at pH 7.4. Brush border is stained, but the cell membranes are unstained. Magnification, 1,500.

FIG. 16. Dog kidney, formol-calcium fixation. Incubated 30 minutes with glycerophosphate at pH 5.0. There is no sign of staining in the cell membranes. Darkly stained droplets are seen at the luminal end of some of the proximal tubule cells. Magnification, 1,200.



(Spater *et al.*: ATPase activity in cell membranes)