ULTRASTRUCTURAL STUDIES OF VASOPRESSIN EFFECT ON ISOLATED PERFUSED RENAL COLLECTING TUBULES OF THE RABBIT

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ABSTRACT

Isolated cortical collecting tubules from rabbit kidney were studied during perfusion with solutions made either isotonic or hypotonic to the external bathing medium. Examination of living tubules revealed a reversible increase in thickness of the cellular layer, prominence of lateral cell membranes, and formation of intracellular vacuoles during periods of vasopressin-induced osmotic water transport. Examination in the electron microscope revealed that vasopressin induced no changes in cell structure in collecting tubules in the absence of an osmotic difference and significant bulk water flow across the tubule wall. In contrast, tubules fixed during vasopressin-induced periods of high osmotic water transport showed prominent dilatation of lateral intercellular spaces, bulging of apical cell membranes into the tubular lumen, and formation of intracellular vacuoles. It is concluded that the ultrastructural changes are secondary to transepithelial bulk water flow and not to a direct effect of vasopressin on the cells, and that vasopressin induces osmotic flow by increasing water permeability of the luminal cell membrane. The lateral intercellular spaces may be part of the pathway for osmotically induced transepithelial bulk water flow.

INTRODUCTION

The mammalian renal collecting tubule is a unique structure capable of altering the passive flow of water across its walls. In the absence of the antidiuretic hormone vasopressin, bulk movement of water along an osmotic gradient across the tubule wall is minimal. When vasopressin is present in blood in sufficient concentration, osmotic flow of water out of the collecting tubule is greatly increased. Much of our present understanding of the mechanisms of bulk water transport across living tissues is derived primarily from studies on vasopressin-sensitive epithelial membranes of amphibia (1, 2). Water was originally believed to flow directly through the cells via aqueous channels or pores in the apical and basal cell membranes. Acceleration of osmotic flow was thought to be accomplished by a vasopressin-induced increase in the size of the channels on the apical surface alone. However, electron microscope studies of isolated amphibian urinary bladder have shown that the hormone, in addition to causing cell swelling which is interpreted as evidence of an increase in the permeability to water of the apical surface, also produced dilation of intercellular spaces (3, 4).

This last result could conceivably reflect net flow of water between the cells as well.

Direct study of renal collecting tubules is technically difficult, and reliance on in vivo situations does not allow sufficient control of experimental conditions to permit detailed analysis of the mechanisms of transport. Recent techniques have been devised, however, which permit direct in vitro studies of isolated perfused renal cortical collecting tubules of rabbit (5, 6). It was the purpose of the present experiments to examine collecting tubules in vitro by phase-contrast and electron microscope techniques, during control conditions and following treatment with vasopressin, in order to determine the effect of the hormone on the size of the cells and the intercellular spaces.

METHODS

Methods of dissection and perfusion of renal cortical collecting tubules have been described in detail (5, 6). Female New Zealand white rabbits were exsanguinated, and the left kidney was rapidly removed. Individual collecting tubules were dissected from the of kidney slices while immersed in oxygenated media (NaCl 115 mM, KCl 5 mM, $NaHCO_3$ 25 mM, NaAcetate 10 mM, NaH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.0 mM, 5% v/v calf serum (Microbiological Associates, Bethesda, Md.), and dextrose 5.5 mM, 290 milliosmolal, pH 7.4). Individual tubule segments were then transferred in small amounts of medium to a glass-bottomed chamber filled with an identical solution which was mixed by bubbling with gas (95% oxygen and 5% carbon dioxide). The chamber was mounted on the stage of a Unitron inverted phase-contrast microscope. The lumen was cannulated by introducing a glass micropipet filled with infusion solution into one of the broken ends of the tubule. The pipet was connected to a microsyringe pump permitting delivery of fluid into the tubule lumen at a constant rate (ca. 4 nl/min/mm tubule length). The other end of the tubule was immobilized by another glass micropipet. Two infusion solutions were used: one was isotonic (290 milliosmolal) to that bathing the peritubular or blood border of the cells and contained NaCl 150 mM, K_2HPO_4 2.5 mM, $MgSO_4$ 1.2 mM, and $CaCl_2$ 1.0 mM, the pH being adjusted to 7.4 with 0.15 M HCl; the other, an hypotonic solution (125 milliosmolal), was identical in composition to the isotonic infusion fluid except that the NaCl concentration was reduced to 60 mM. Throughout the experiments, the tubules were periodically observed and photographed through a 100× oil immersion phase-contrast objective. Tubules were fixed by replacing the external bathing medium with 0.9% glutaraldehyde in a 67% concentration of the usual bathing medium, but without addition of the calf serum. The osmolality of this solution, measured with an Aminco-Bowman osmometer, was 290 milliosmolal. The tubules were observed and photographed through the phase-contrast microscope during the initial fixation period which lasted 10 min. The tubules were then disengaged from the pipets and transferred in a small amount of liquid to a bottle containing about 30 ml of the glutaraldehyde solution. Fixation was continued for an additional 3 hr. The tubules were rinsed twice with a 0.1 M phosphate-buffered 7.5% sucrose solution and then postfixed for 15–30 min in a phosphate-buffered solution of 1% osmium tetroxide (7). After a water rinse, the tubules were dehydrated by passage through a graded ethanol series and were finally washed in propylene oxide. Araldite 502 (Ciba Products, Inc.) was used for embedding. Thin sections from several areas of each tubule were cut with glass or diamond knives on a Porter-Blum or LKB ultramicrotome. The sections were mounted on Formvar-coated copper grids and, after staining with uranyl acetate and lead citrate (8), were examined in an RCA-EMU 3G or a Philips EM 200 electron microscope.

Data obtained during preliminary and previous studies (6) allow a generalization of the permeability response of collecting tubules in the control state and following treatment with vasopressin (Fig. 1). Tubules were perfused with hypotonic solution at a known steady rate. Net water absorption (osmotic permeability to water per unit tubule area) was calculated from the difference between the rate of infusion and the volume collected per unit time.

Freshly dissected collecting tubules initially were always highly permeable to water (Fig. 1, Period I). However, after approximately 200 min, the permeability decreased and reached a stable lower value in virtually all studies (Period II). Addition of vasopressin, 25 μU ml^{-1} (Parke-Davis Pitressin), to the blood surface of the tubules caused a marked increase in the permeability to water (Period III) which was reversed when hormone was removed (Period IV). Period V merely indicates the response of the tubules to a second exposure to vasopressin. In the present study, tubules were fixed in the presence of a transcellular osmotic gradient at times corresponding to each of the first four periods shown in Fig. 1.

Period I, two tubules; Period II, two tubules; Period III, four tubules; Period IV, two tubules.

In order to determine the effects of vasopressin in the absence of a transcellular osmotic gradient, we perfused two tubules with isotonic solution for a suitable control period, following which vasopressin was added. The tubules were fixed in the presence of vasopressin at times corresponding to Period III (Fig. 1). In one of the studies, lumen volume was

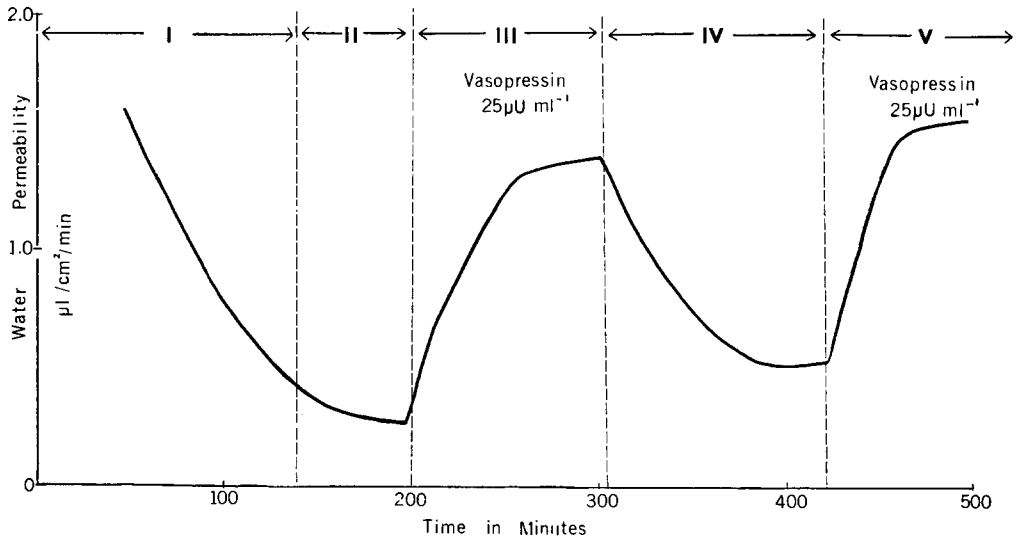

FIGURE 1 Net water absorption (plotted against time) of isolated tubules perfused with hypotonic solution. This is a mean response curve, derived from measurements of several tubules, in the presence and absence of vasopressin in the external bathing medium.

decreased rapidly by the reduction of the rate of infusion by one-half during the vasopressin period (Period III).

RESULTS

Light Microscopic Observations

During the initial period of infusion with an hypotonic solution, the tubule cells decreased in thickness (i.e., the distance between the apical and basal cell borders decreased) as they became relatively impermeable to water (Period II). Addition of vasopressin to the medium bathing the tubule produced an increase in cell thickness which was apparent within 5–10 min (Fig. 2). Vacuoles also appeared in many of the cells, and the lateral cell borders became more distinct. Removal of vasopressin from the external bathing medium produced a gradual decrease in cell thickness and vacuole size. Within 100–140 min, when the rate of water transport had declined to the initial control value, the cells appeared flatter and the vacuoles either were greatly reduced in size or had disappeared (Fig. 2).

Projections of phase-contrast photographs of the cellular layers of four tubules taken during the control period (Period II) of low water transport and following treatment with vasopressin (Period III) were traced on paper of uniform thickness. The weights of the cut-out tracings in the period of high water transport (Period III) were 28 ± 8 (SEM) % greater than those in the control period (Period II) of low water transport.

Average cell thickness was also estimated from the difference between the outside diameter of the tubule (base-to-base) and the lumen diameter (apex-to-apex) measured at numerous places along the projected image of the tubule. Following treatment with vasopressin the lumen diameter decreased, whereas the outer diameter was unchanged. The average increase in tubule wall thickness was about 23%.

The cells of tubules perfused with a solution isotonic to the bathing medium appeared similar to those relatively impermeable to water (Period II) during the infusion of hypotonic fluid. Addition of vasopressin to the external bathing medium at a time corresponding to Period III of Fig. 1 had no visible effect on the tubules. In none of the experiments did addition of glutaraldehyde solution to the perfusion chamber change the volume of the cell layer (Periods I–IV) or the size of the vacuoles of permeable tubules (Period III).

Electron Microscopic Observations

The renal cortical collecting tubule is lined by a single layer of cells some of which appear to overlap (Fig. 3). As reported previously (9), in the rat two

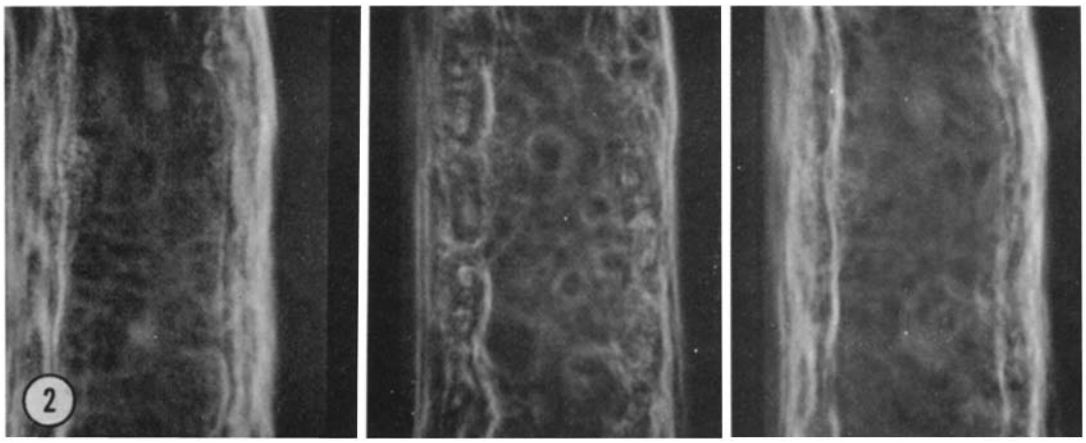

FIGURE 2 Phase microscope photographs of a living tubule during perfusion with hypotonic solution. The focal plane is at the center of the tubule lumen. At the left, the tubule is in Period II of relatively low water permeability. In the center is shown the same portion of the same tubule after addition of vasopressin to the external bathing medium while the tubule is in Period III of high water permeability. Compared to the cells in the photograph on the left, the cells are swollen and bulge slightly into the lumen. The lateral cell borders are more distinct. On the right is the same tubule after removal of vasopressin when water permeability has again become relatively low (Period IV). The vasopressin-induced changes have reversed, and the tubule has the same appearance it had in Period II. An electron micrograph of this tubule is shown in Fig. 9. × 1000.

cell types can be distinguished in cortical collecting tubules: ordinary lining cells and intercalated cells. Ordinary lining cells have an ovoid nucleus generally located near the base of the cell. Small numbers of elongated mitochondria are scattered throughout the cytoplasm which contains sparse amounts of rough and smooth endoplasmic reticulum. Occasional cytosomes containing dense membranous material, multivesicular bodies, and cytoplasmic vacuoles are present. In many cells, the centrioles are differentiated into a single apical cilium with accompanying basal body (Fig. 5). The other cell type (Fig. 3), characterized by large numbers of mitochondria, the presence of numerous small apical vacuoles, and a heavy coat of extracellular filamentous material on the lumen surface, is believed to represent the intercalated cells described by other investigators. No cilia were seen on these cells, and their centrioles, when present in the plane of section, were deep in the cytoplasm adjacent to the nucleus. The lateral membranes of all cells were joined at their apical portions by a typical tight junctional complex (Fig. 4). Numerous interdigitations were formed by folds of the lateral cell membranes, and these folds were occasionally joined by desmosomes in the mid- and basilar portions of the cells. Surrounding the cells was an intact basement membrane to which adhered collagen fibrils and fragments of capillaries.

Tubules Relatively Impermeable to Water (Period II); Hypotonic Infusion

In the control state of low permeability to water, the cytoplasm of tubule cells appeared moderately dense (Fig. 3). The apical cell membranes formed a smooth surface with little bulging into the lumen of the tubule. The cytoplasmic vacuoles were inconspicuous, and the lateral cell membranes of adjacent cells were closely apposed (Figs. 3, 4).

Tubules Highly Permeable to Water Induced by Vasopressin (Period III); Hypotonic Infusion

The addition of vasopressin to tubules in the presence of a transcellular osmotic gradient produced striking changes in the appearance of the cells (Fig. 6). All cells appeared swollen and the innermost borders bulged slightly in the tubule lumen. Large membrane-limited vacuoles appeared in the cytoplasm. There was prominent dilatation of the lateral intercellular spaces, with formation of large communicating lakes surround-

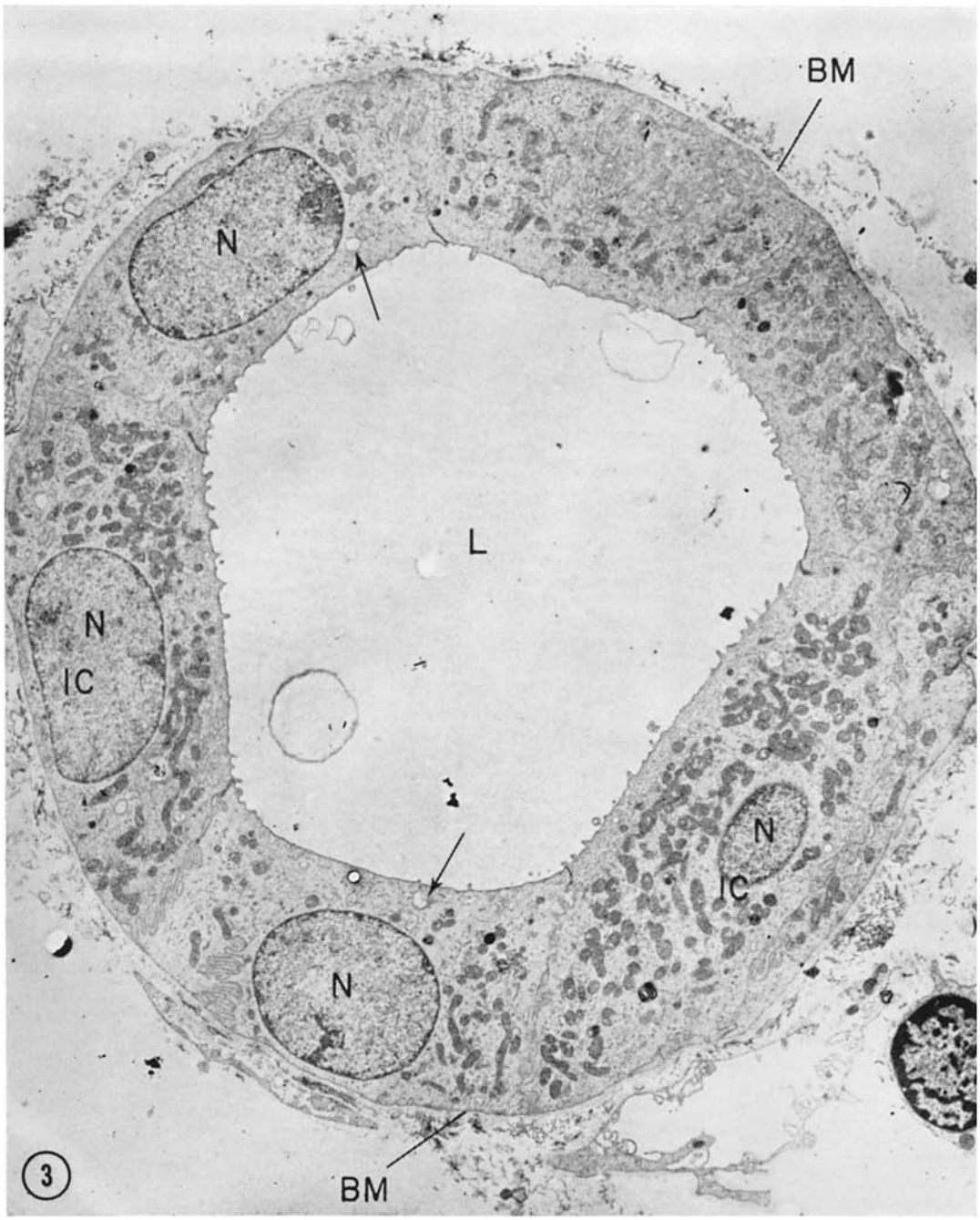

FIGURE 3 Low-power electron micrograph of a cross-section of a tubule relatively impermeable to water (Period II); hypotonic perfusion. Two intercalated cells (*IC*) contain numerous mitochondria and have many small vacuoles in their apical cytoplasm. The ordinary lining cells have a relatively small number of mitochondria and occasional cytoplasmic vacuoles (arrows). The tubules are surrounded by a basement membrane (*BM*) to which adhere collagen fibrils and capillary fragments. *N*, nucleus; *L*, tubule lumen. × 5300.

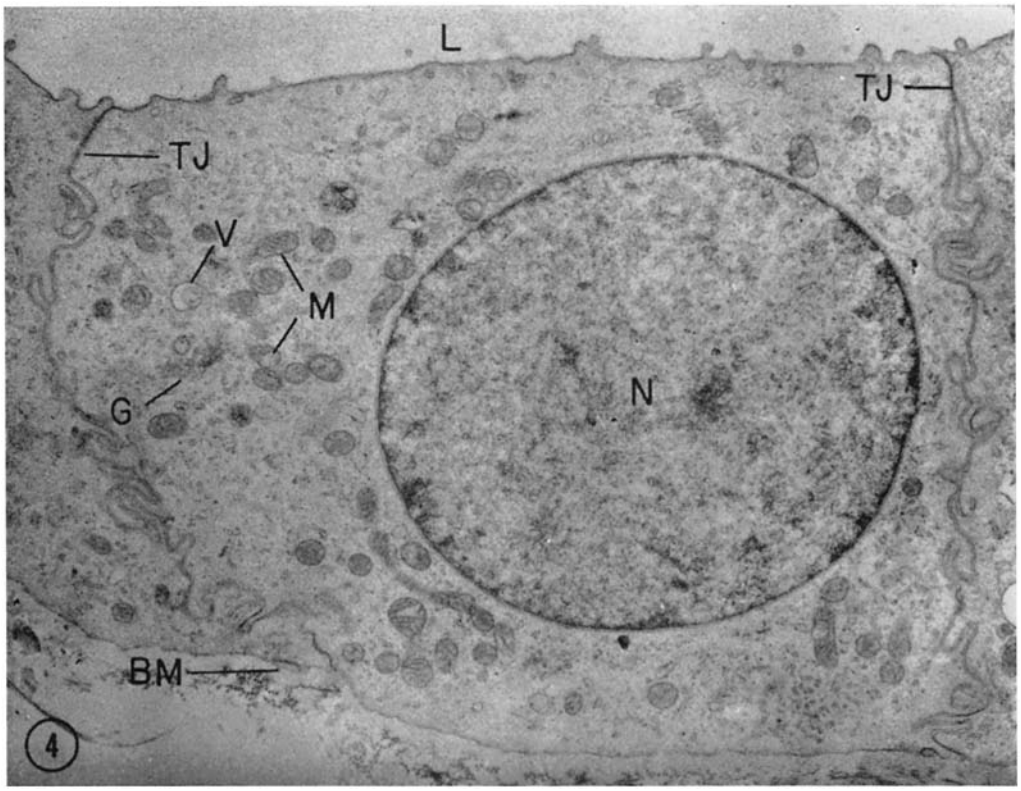

FIGURE 4 Lining cell of a tubule relatively impermeable to water (Period II); hypotonic infusion. A few microvilli project into the tubular lumen (*L*). Adjacent cells are joined by a tight junctional complex near the lumen (*TJ*). The lateral cell membranes have numerous interdigitating folds. *N*, nucleus; *M*, mitochondria; *V*, vacuole; *G*, Golgi region; *BM*, basement membrane. × 12,500.

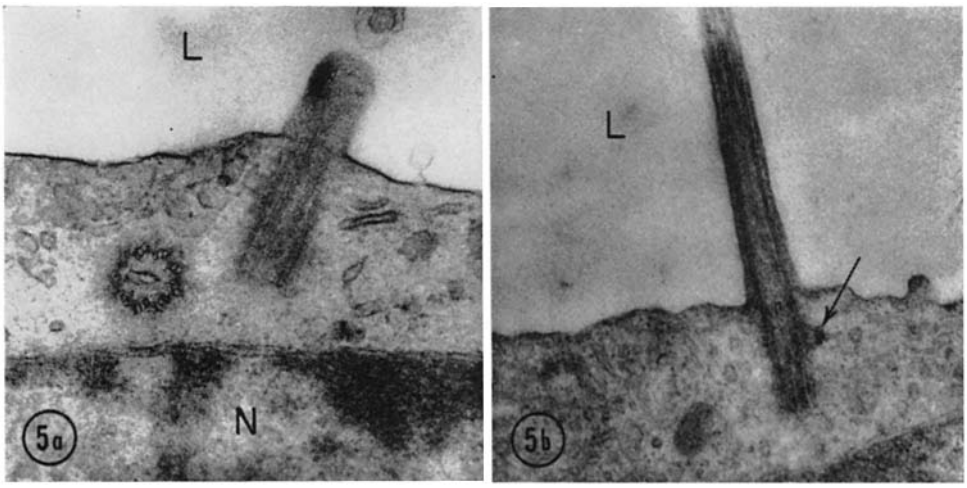

FIGURE 5 Details of single cilia which were frequently observed on the lining cells. Fig. 5 *a* shows the base of a cilium and its associated basal body. Fig. 5 *b* shows a cilium with a ciliary rootlet (arrow). *L*, lumen; *N*, nucleus; *a*, × 48,000. *b*, × 39,000.

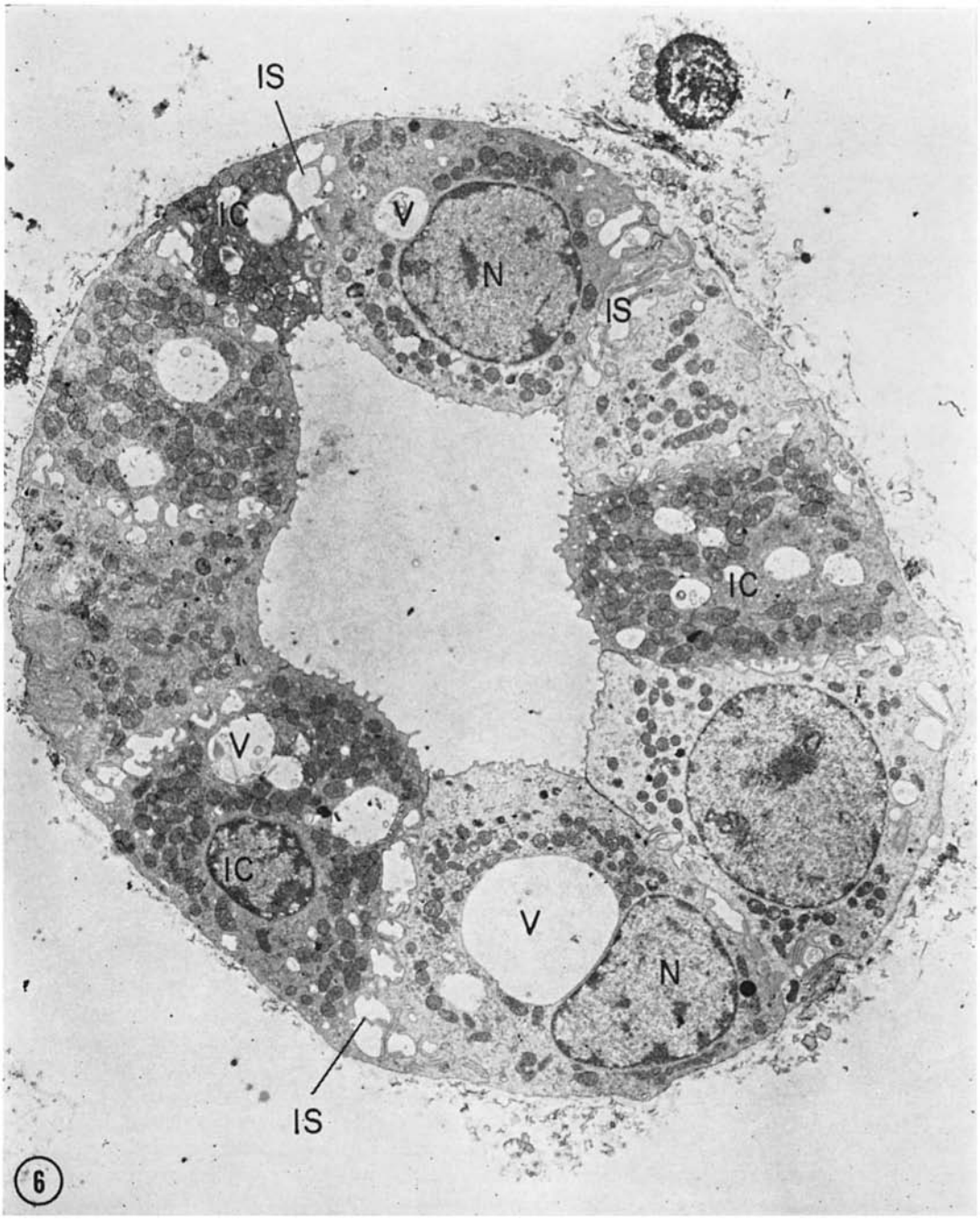

FIGURE 6 Low-power electron micrograph of a tubule highly permeable to water following vasopressin treatment (Period III); hypotonic infusion. The cells are swollen and tend to bulge into the tubule lumen (*L*). Large vacuoles (*V*) are present in the cytoplasm of both the ordinary lining cells and the intercalated cells (*IC*). The lateral cell membranes have separated to form large intercellular spaces (*IS*). *N*, nucleus. × 5600.

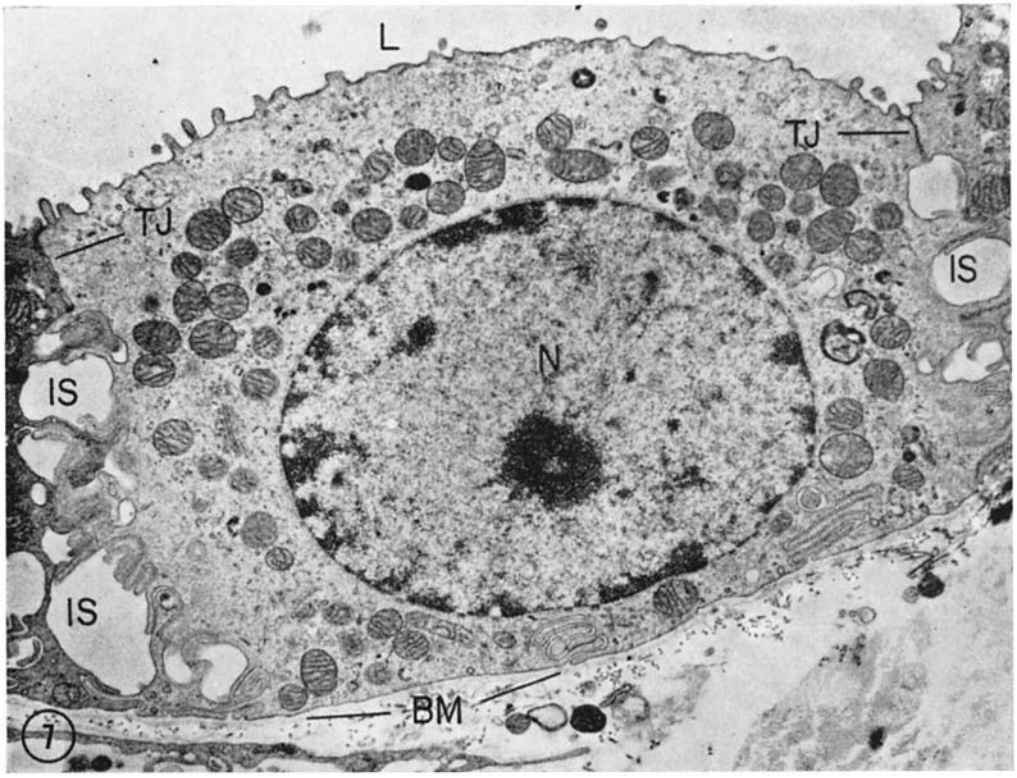

FIGURE 7 Lining cell of vasopressin-treated tubule highly permeable to water (Period III); hypotonic infusion. The tight junctions (*TJ*) near the tubular lumen (*L*) are unaltered. The cell bulges slightly into the tubule lumen. The lateral cell membranes have separated to form large intercellular spaces (*IS*). *N*, nucleus; *BM*, basement membrane. × 12,000.

ing both types of cells. The tight apical junctions were not altered, and there was no separation of the adjacent lateral cell membranes at their basilar portions near the intact basement membrane (Figs. 7, 8). Cytoplasmic vacuoles, usually larger and more numerous than those in the ordinary lining cells, were seen in intercalated cells. No significant changes were evident in the other cytoplasmic components of either the ordinary lining cells or intercalated cells.

Tubules Relatively Impermeable to Water Following Reversal of Vasopressin Effect (Period IV); Hypotonic Infusion

Tubules relatively impermeable to water after removal of the hormone from the bathing medium appeared identical with those in the initial control period (Period II) of low water permeability (Figs. 3, 9). The vacuoles and cellular swelling disappeared, and the lateral membranes of adjacent cells were closely apposed.

Tubules Initially Permeable to Water (Period I); Hypotonic Infusion

Cells of tubules removed within 20 min after initial cannulation had features intermediate between those in states of high and low permeability to water (Fig. 10). The apical cell membranes bulged slightly into the tubule lumen, and there was moderate focal dilatation of the lateral intercellular space. The cytoplasmic vacuoles were, however, inconspicuous.

Tubules Treated with Vasopressin in the Absence of an Osmotic Gradient, Corresponding to Period III; Isotonic Infusion

In the absence of a transcellular osmotic gradient, cells treated with vasopressin appeared

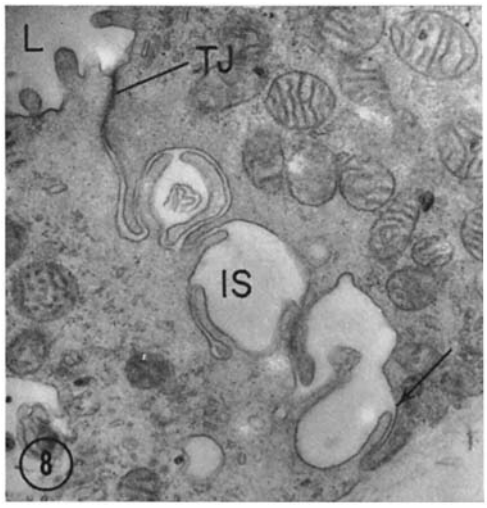

FIGURE 8 Intercellular space (*IS*) of vasopressin-treated tubule highly permeable to water (Period III); hypotonic infusion. The tight junction is unaltered (*TJ*). The intercellular space is dilated in its midportion but narrows abruptly at the base of the cells where the lateral membranes remain closely apposed (arrow). *L*, tubular lumen. × 20,000.

morphologically identical with those of tubules infused with solution during a state of low water permeability (Periods II and IV) (Figs. 11 and 12). There was no cellular swelling, vacuole formation, or enlargement of lateral intercellular spaces. A tubule in which the perfusion rate had been rapidly decreased to one-half of the original value prior to fixation was not appreciably different in appearance.

DISCUSSION

It is apparent, on the basis of these studies, that the simple view that the path traversed by water as it moves along an osmotic gradient out of the collecting tubule is exclusively intracellular, as had been originally proposed for toad bladder (10, 11), requires modification. The intercellular spaces observed during net flow of water induced by vasopressin in both collecting tubule and amphibian bladder (3, 4) are clear evidence of a potential extracellular (intercellular) route for water transfer. As a first approximation, it is assumed, though not proven, that water traverses both paths. This may occur simply as parallel flow through the cell and intercellular space. The more likely possibility is that water, unable to penetrate readily the junctional complex joining the cell apices, enters the cell cytoplasm from the urinary surface and then flows both into the intercellular spaces and out of the cell interior across the basal cell border. Formation of large intercellular spaces is observed when net water flow is induced by hormone only (in the presence of an osmotic gradient) and not by an osmotic gradient alone or by vasopressin under circumstances in which net flow of water is not accelerated.[1] Therefore, it is evident that the appearance of the spaces represents only the effect of bulk flow of solvent through the tissue, and is not a morphological expression of an "expansional" effect of the hormone.

Evidence that the primary changes in membrane structure and permeability to water caused by the hormone occur at the luminal or urinary surface of the cells was originally adduced from studies on membranes of amphibian tissues (2, 12). In these tissues, the blood surface of the cells is normally highly permeable to water, independent of the presence or absence of vasopressin, as indicated by the osmometric response to changes in tonicity of the solution bathing the blood border of the cells (12). It was demonstrated in toad bladder (2) that vasopressin increased the intracellular accumulation tritium-labeled water from the urinary side of the tissue, an effect interpreted to indicate that the hormone increased the permeability to water of the mucosal membrane. Similarly, in frog skin (12), when the solution bathing the external epidermal surface was hypotonic to that bathing the blood surface, the viable cell layer of the epidermis was observed directly and found to swell only following the addition of vasopressin to the blood surface of the cells. Since cell water is normally in osmotic equilibrium with the fluid bathing the blood border of the cells, swelling following treatment with vasopressin could have occurred only if the permeability to water of the mucosal membrane were increased.

In the rabbit collecting tubule, the site of increased permeability to water caused by vaso-

[1] Although in the absence of an osmotic gradient vasopressin has no effect on net water absorption, the hormone is effective under these conditions as attested to by the fact that it causes a marked increase in diffusional permeability as measured with isotopic water (6).

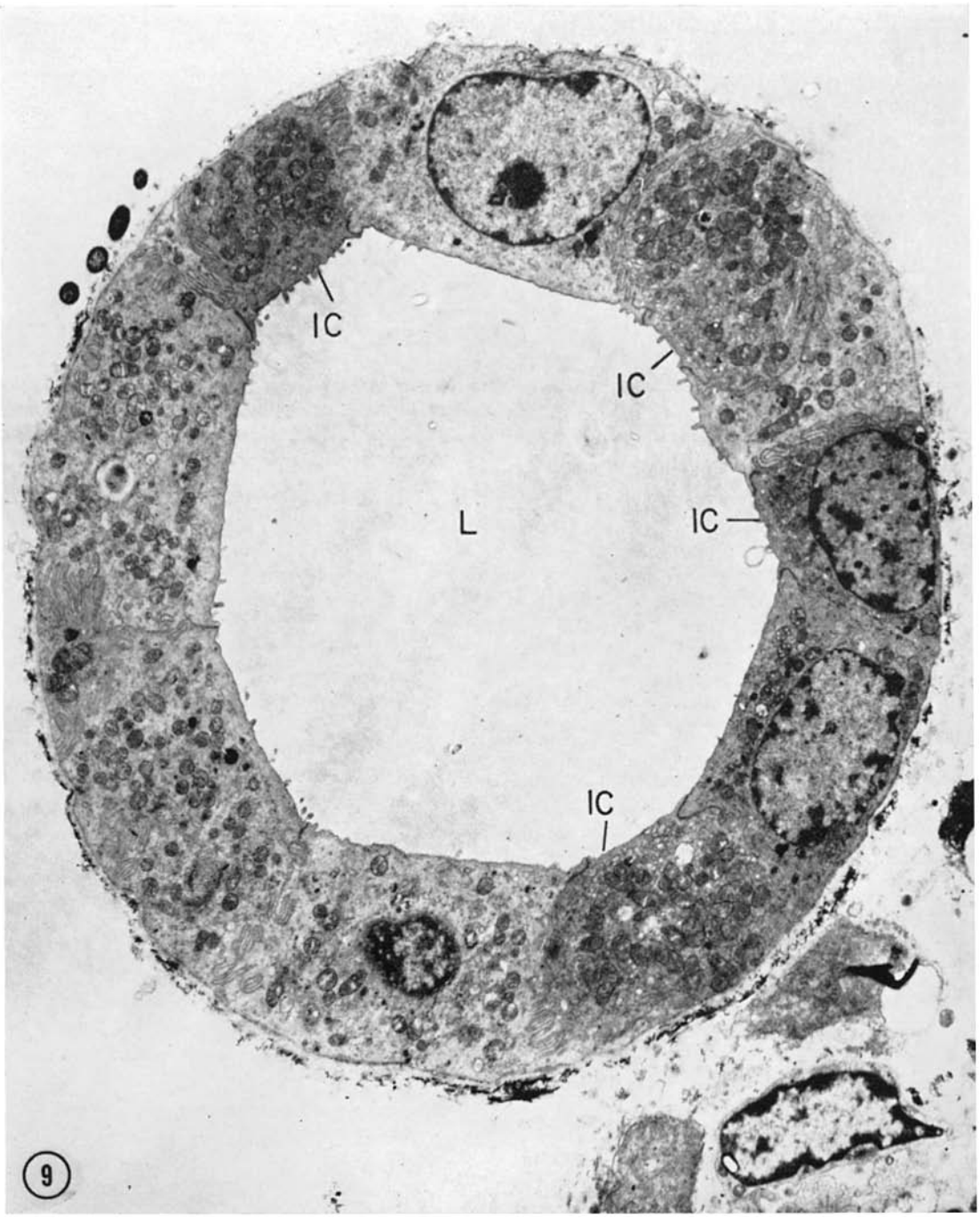

FIGURE 9 Low-power micrograph of a tubule following reversal of vasopressin effect (Period IV); hypotonic infusion. The tubular lumen (*L*) is nearly round. The cytoplasmic vacuoles are inconspicuous and the lateral cell membranes are closely apposed. This is the same tubule as in Fig. 2. *IC*, intercalated cells. × 4,100.

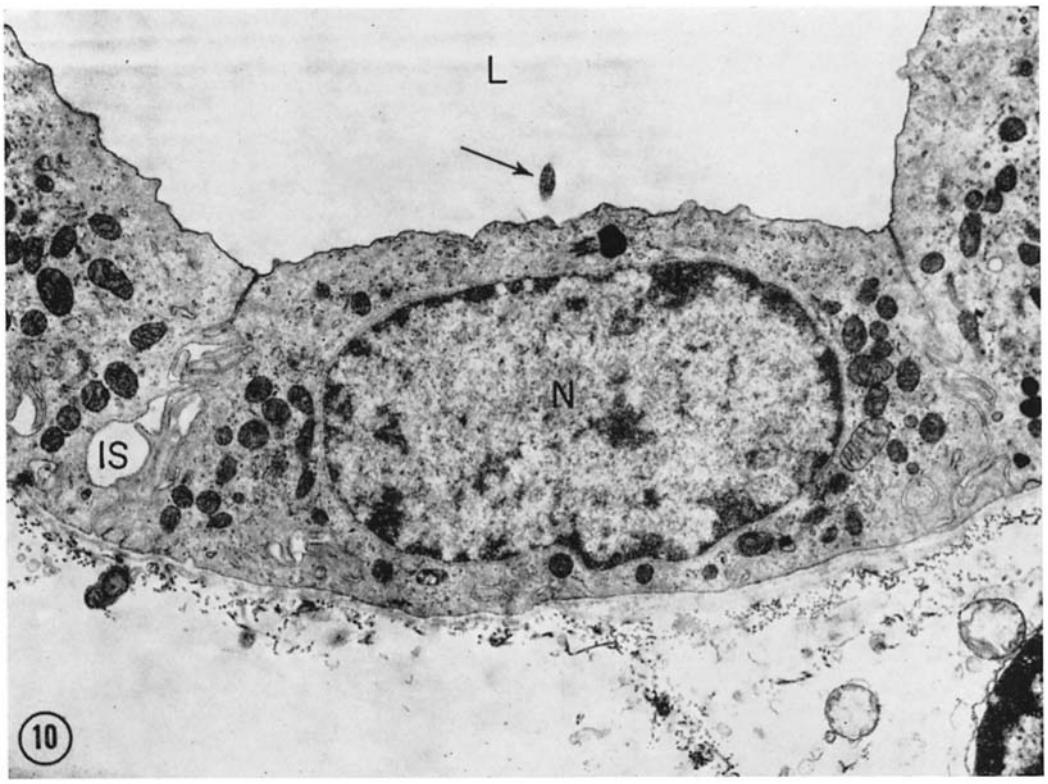

FIGURE 10 Lining cells of a freshly isolated tubule (Period I); hypotonic infusion. There is moderate dilatation of the intercellular space on the left (*IS*), but not on the right. The cell bulges slightly into the tubular lumen (*L*). A portion of a cilium is seen projecting into the lumen (arrow). *N*, nucleus. × 12,000.

pressin was determined by a somewhat similar approach. Decreasing the osmolality of the solution bathing the blood surface of the tubules caused swelling of the cells, independent of the presence or absence of vasopressin (unpublished observations). Thus, the peritubular or blood border of the cells is always highly permeable to water. In the absence of vasopressin, tubules perfused with a solution hypotonic to the outer medium have an appearance identical to that of tubules perfused with isotonic fluid. Only following treatment with vasopressin is significant swelling of the cells observed. Clearly, the luminal membrane in the absence of hormone is relatively impermeable to water, but when vasopressin is added to the blood border of the cells (6) the permeability to water of the luminal membrane increases and water enters the cells and intercellular spaces as it traverses the tissue.

At present, the precise localization of the site or sites of increased luminal membrane permeability to water is not possible. Although alteration in structure may occur at or near the luminal surface of the cells, no indication of this has been observed in electron micrographs. Indirect evidence interpreted to indicate that water enters the cell across the luminal cell membrane is based on the observation noted above that the cell layer swells following treatment with hormone. However, this conclusion must now be viewed with caution. Conceivably, similar alterations in thickness could be induced merely by an increase in volume of the intercellular space with subsequent distortion of the cells. Although we consider the latter explanation unlikely and favor the view that both spaces (cellular and intercellular) increase in volume during accelerated net water movement, the question has not been resolved satisfactorily. Until the point is clarified by volume measurements of individual cells, consideration must at least be given to the possibility that bulk flow occurs only between the

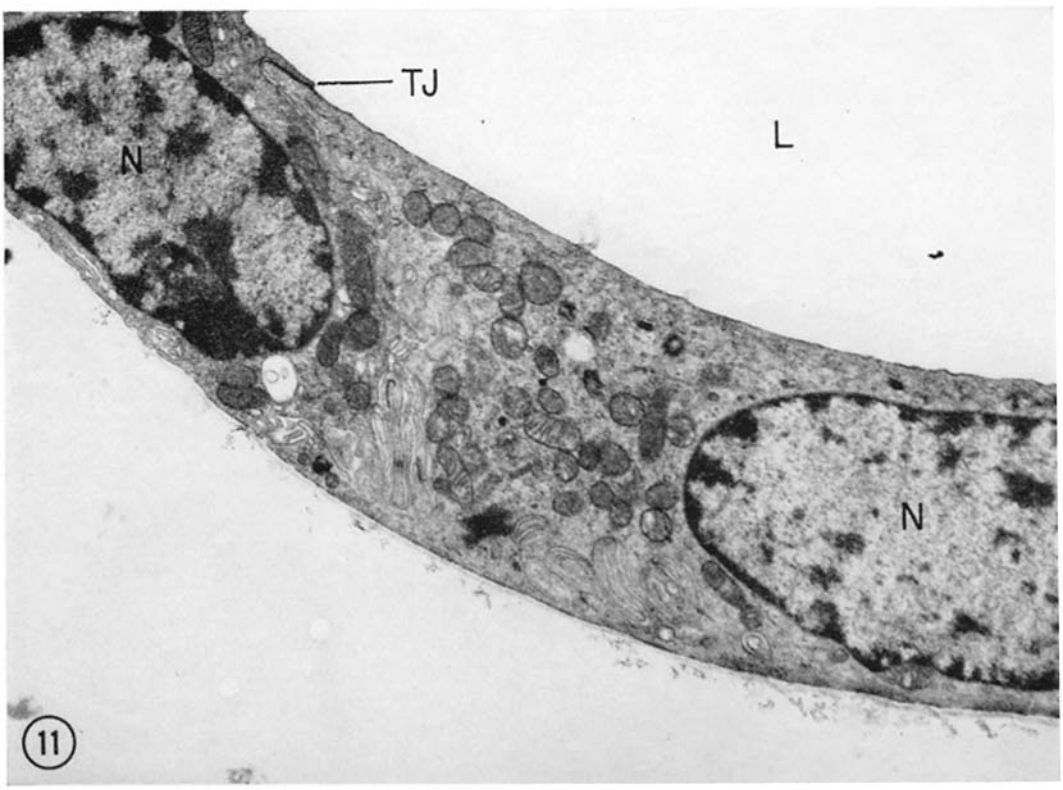

FIGURE 11 Vasopressin-treated tubule perfused with isotonic solution. There is no swelling of the cells nor dilatation of the lateral intercellular spaces. Intercalated cell on the left, lining cell on the right. *L*, tubule lumen. *TJ*, tight junction; *N*, nucleus. × 11,000.

cells in response to a hormone-sensitive alteration in the permeability characteristics of the junctional complex adjoining the apical surfaces of the cells.

The lateral intercellular space has been implicated as an important site of active transport in other tissues (13–15). In isolated rabbit gallbladder, net water absorption from lumen to bath occurs even against an established osmotic difference. At present, the mechanism of fluid transport is believed to involve the coupling of water with active sodium transport across the lateral intercellular, membranes. Fluid flow in the gallbladder, however, is not entirely analogous to that in collecting tubules since the collecting tubule, in contrast to gallbladder, transports minimal quantities of water coupled with sodium chloride transport (6) and shows no significant dilatation of the lateral intercellular spaces following treatment with vasopressin when no osmotic difference exists across its walls.

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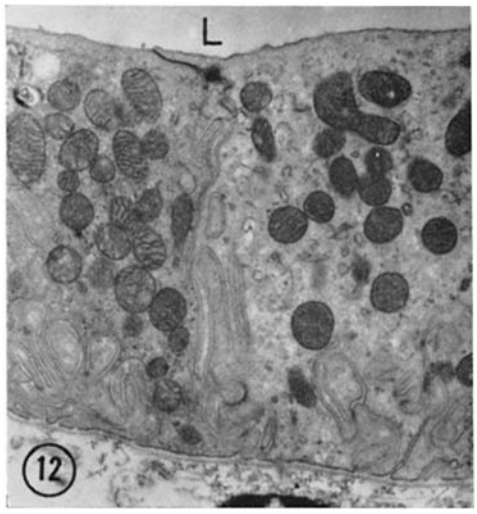

FIGURE 12 Vasopressin-treated tubule perfused with isotonic solution (corresponding to Period III). The lateral cell membranes are closely apposed. *L*, lumen × 17,000.

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